

**T cell subsets and the outcome of Haematopoietic Stem
Cell Transplantation**

Thesis presented by

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I, Adria Imelda Prieto Hinojosa, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Adria I. Prieto H

Abstract

Haematopoietic stem cell transplantation (HSCT) is an effective therapy for many malignant and non-malignant diseases. However, adverse events such as infections, Graft-versus-Host Disease (GvHD) and relapse limit the wider use of HSCT. The identification of factors that predict the outcome of the transplant can be a vital tool to offer patients better options for treatment. Since there is a significant immunological contribution to the outcome of the transplant, it is of interest to ask to what extent the immune status of the patient prior to transplant might influence their subsequent recovery.

After transplantation, the reconstitution of the T cell compartment in the patient relies on both expansion of existing T cells and the production of new ones via the thymus; in older patients this latter process is compromised by age-dependent thymic involution. Improvements in thymus function leading to improvement in immune reconstitution after an HSCT may provide significant benefits, potentially reducing mortality from both infections and GvHD.

In one study described in this thesis, an improvement in T cell reconstitution, in particular of the CD4 compartment, could be demonstrated after the administration of an LHRH agonist before transplantation from an allogeneic donor. Such an effect could not be demonstrated in an autologous setting. This may reflect a differential requirement for thymus function in the two transplant settings.

In additional studies, various immune parameters including T cell subsets and cytokine profiles from the patient, that could affect the transplant outcome were analysed. The findings indicate that high levels of regulatory T

cells (Tregs) as well as high levels of regulatory cytokines in patients pre-transplant are factors that predict relapse after transplantation. It is likely that these act by suppressing anti-tumour responses in the patient. These findings may provide a useful tool to stratify the patients into high and low risk categories prior to transplantation.

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Abbreviations

HSCT	=	Haematopoietic Stem Cell Transplantation
DLI	=	Donor Leukocyte Infusion
GvHD	=	Graft versus Host Disease
aGvHD	=	acute Graft versus Host Disease
cGvHD	=	chronic Graft versus Host Disease
UD	=	Unrelated Donor
UD-HSCT	=	Unrelated Donor Haematopoietic Stem Cell Transplantation
HLA	=	Human Leukocyte Antigen
CMV	=	Cytomegalovirus
GvL	=	Graft versus Leukaemia
RIC	=	Reduce Intensity Conditioning
NKT	=	Natural Killer T cells
NK	=	Natural Killer cells
PCR	=	Polymerase Chain Reaction
qRT-PCR	=	quantitative Real Time- Polymerase Chain Reaction
TRECs	=	T-cell Receptor Excision Circles
TCR	=	T Cell Receptor
TCR β	=	beta Chain of the TCR
Tregs	=	Naturally occurring CD4 ⁺ CD25 ^{hi} Regulatory T cells
CTLA-4	=	Cytotoxic T Lymphocyte Associated Antigen 4
GITR	=	Glucocorticoid Induced Tumour Necrosis Factor Receptor
PD1	=	Programmed Death 1
IL-4	=	Interleukin 4
IL-10	=	Interleukin 10
TGF β	=	Transforming Growth Factor β
FOXP3	=	forkhead box transcription factor 3
DC	=	Dendritic Cell
mDC	=	myeloid Dendritic Cells
pDC	=	plasmacytoid Dendritic Cells
TRM	=	Transplant related mortality
MHC	=	Major Histocompatibility Complex
INF- γ	=	Interferon-gamma
TNF- α	=	Tumour Necrosis Factor alpha
GvT	=	Graft versus Tumour
PTLD	=	Post-transplant Lymphoproliferative Disorder
EBV	=	Epstein Barr virus
pT α	=	Pre T-cell receptor
RAG1	=	Recombination Activating protein
APC	=	Antigen Presenting Cells
RSS	=	Recombination Signal sequence
TdT	=	Terminal deoxynucleotidyl Transferase enzyme
CDR3	=	Complementarity Determining Region 3
Tr	=	Regulatory T cells
Th3	=	Regulatory TGF- β producers
PBMC's	=	Peripheral blood Mononuclear cells

RMPI	=	Roswell Park Memorial Institute
DMSO	=	Dimethyl sulphoxide
cDNA	=	complementary DNA
M-MLV	=	Moloney Murine Leukemia Virus
PBS	=	Phosphate buffered saline
FCS	=	Foetal calf serum
mAb	=	monoclonal Antibodies
FITC	=	Fluorescein
PE	=	Phycoerythrin
PerCP	=	Peridinin-chlorophyll-protein Complex
APC	=	Allophycocyanin
FACS	=	Fluorescence Activated Cell Sorting
dNTP's	=	deoxynucleotide triphosphates
SDS	=	Sequencing detection system
LB	=	Lysogeny broth agar
TE	=	Tris-EDTA buffer
MgCl ₂	=	Magnesium chloride
NaCl	=	Sodium chloride
LiCl	=	Lithium chloride
NTC	=	Non template control
TfR	=	Transferrin receptor
OD	=	Optical density
CDR	=	Complementarity Determining Region
OS	=	Overall Survival
DFS	=	Disease Free Survival

Chapter 1

Introduction

Haematopoietic Stem Cell Transplantation (HSCT)

Haematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for a variety of life threatening diseases of the haematopoietic system, including malignancies, primary immune deficiencies, bone marrow failure syndromes and autoimmune diseases (van den Brink, et al., 2004). The principle of haematopoietic stem cell transplantation consists of the administration of high doses of chemotherapy and irradiation (conditioning regimen) to eliminate leukaemic or abnormal cells, and at the same time, to provide a space for the new healthy system to repopulate from transplanted stem cells. However, often not all the leukaemic cells are eliminated by the conditioning regimen, and those cells are responsible for relapse of the primary tumour. For some years, it has been demonstrated that the infusion of donor T cells, so-called donor lymphocyte infusion (DLI), induces remission in patients who had experienced relapse, providing a proof that donor cells can have an antileukaemic effect capable of inducing a sustaining remission.

Conditioning before HSCT

The conditioning regimen required prior to transplantation comprises both treatments, to eliminate the leukaemic cells from the recipient and also to prepare the recipient's immune system to tolerate and accept the new cells. This can be achieved with the use of high-dose chemotherapy with or without irradiation (myeloablative conditioning) or lower doses of chemotherapy and/or lower doses of irradiation (non-myeloablative conditioning) (Martin, et al., 2009).

Types of haematopoietic stem cell transplant

Haematopoietic stem cells can be collected from either the recipient's own stem cell population or can be obtained from sibling or unrelated donors. When cells are obtained from the recipient it is known as an autologous transplant. When cells are collected from other individual it is known as an allogeneic transplant, and in such cases, the recipients need to receive drugs to prevent either rejection of the transplanted haematopoietic stem cells or the development of graft versus host disease (GvHD). To avoid adverse reactions such as GvHD, T cells can be removed from the graft with depleting anti-T cell antibodies (T-cell depletion) or a stem cell population can be enriched (CD34 selection).

Haematopoietic Stem cell transplantation from Unrelated Donors

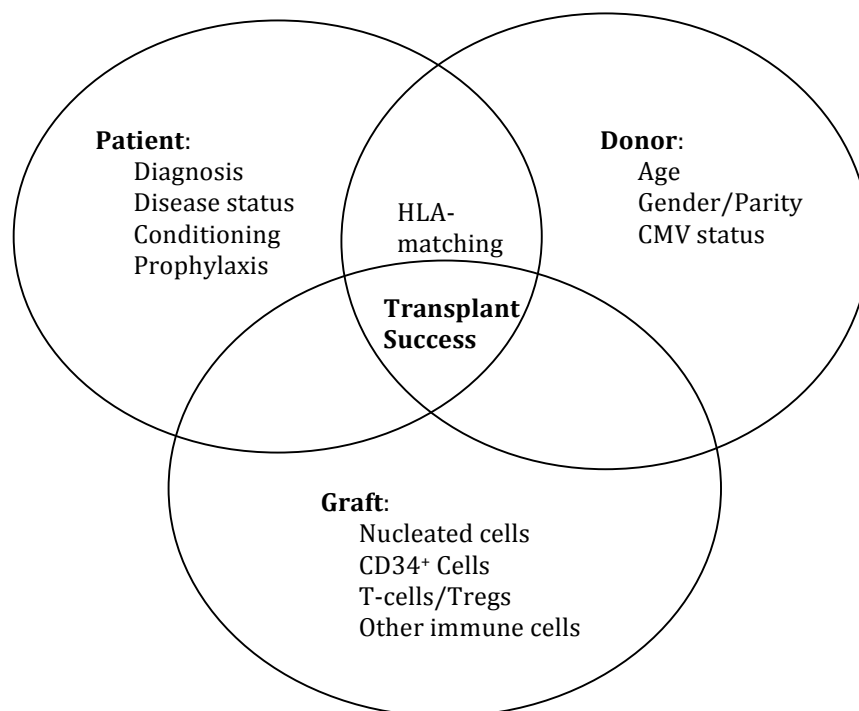
Although the curative effect of HSCT has been demonstrated, two-thirds of patients requiring an allogeneic HSCT do not have a matched sibling donor, thus HSCT from unrelated donors (UD) has considerably increased in recent years. As a result of novel approaches to HSCT that have led to more rapid engraftment and immune reconstitution, a reduction in morbidity and mortality post-transplant is seen, making the use of allogeneic HSCT from UD applicable to a wider range of patients. In Europe more than one third of allogeneic transplants performed employ haematopoietic stem cells collected from unrelated donors (Storb, et al., 2003). Nevertheless, despite its curative potential, HSCT remains associated with significant morbidity and mortality that may outweigh the beneficial effect in some cases.

Different studies comparing HSCT using siblings or unrelated donors have shown similar results with respect to transplant outcome, including the incidence of GvHD, incidence of relapse and overall survival. In part, that the results are comparable has been associated with an improvement in tissue-typing techniques (Remberger, et al., 2008). However, although the HLA-matching is essential for the success of HSCT, it does not prevent the major complications. Thus, there must be other non-HLA factors affecting the outcome of an HSCT. Some of those non-HLA factors are minor Histocompatibility antigens and polymorphic immune response genes.

Clinical factors affecting HSC transplant

A number of patient and donor factors need to be taken into account to select appropriate candidates for HSCT from unrelated donors. The major predictive factor for an UD HSCT is to have a suitable HLA-matched donor. Although, there are some “permissive” mismatches that can be analysed in specific clinical situations, the desirable situation and most important for transplant success is to have a 10/10 match. However, there are other factors that have been studied and are involved in UD-HSCT success, as shown schematically in Figure 1.1.

Figure 1.1. Patient and donor characteristics associated with haematopoietic transplant outcome.



Of these, the most important are:

- 1) Age, younger donors are preferred over older donors;
- 2) Gender, male donors are better than female donors;
- 3) Cytomegalovirus (CMV) status, matching CMV status is desirable;
and
- 4) Parity (in the case of female donors) (Armitage, 1994).

Although it is very important to consider donor characteristics in the context of an HSCT, there are also recipient factors that are needed to be taken into account to be able to define the most suitable donor. The recipient factors that need to be analysed are:

- 1) The type of haematological or genetic disorder,
- 2) The status of the disease at the time of the transplant;
- 3) The pre-transplant conditioning of the recipient;
- 4) The immunosuppressive prophylaxis; and
- 5) Whether or not the graft is T cell depleted (Singhal, et al., 2005).

Although the majority of allogeneic transplants from unrelated donors in the United Kingdom are performed with T cell depletion, other very important factors for successful transplant in the case of non-T cell depletion are the characteristics of the graft, particularly the dose of nucleated cells, the dose of CD34⁺ cells, the doses of other immunological cells such as NKT (specialised T cells that express the NK1.1 cell surface molecule and which carry a unique T cell receptor containing an invariant alpha chain; such cells recognise glycolipids presented by the CD1d non classical MHC molecule) and NK cell (natural killer cells), dendritic cells, and the dose of CD4⁺CD25⁺ and CD8⁺CD25⁺ T cells in the graft. All those cell subsets have been shown to influence the success of the transplant (Vela-Ojeda, et al., 2006) (Baron & Storb, 2005) therefore a detailed monitoring of the graft's composition is an important tool to predict the success of the transplant.

Even though all aforementioned factors play a pivotal role in the transplant outcome, the transplant outcomes are ultimately largely immune mediated events. Despite this, to date little is known about whether variations in immune parameters pre-transplantation can influence the overall transplant

outcome. Therefore precise identification of pre-transplant prognostic factors in both patients and donors, affecting the outcome after HSCT may help to identify patients who are more likely to benefit from additional approaches such as immunotherapy (Zorn, et al., 2005).

Immune factors affecting HSCT

Different groups have suggested several factors of the recipient's immune status pre-transplantation that have a predictive effect in HSCT outcome, such as:

- 1) T cell subsets, particularly the number of effector CD4⁺ T cells (Rosinski, et al., 2005);
- 2) Thymic function, analysed by recent thymic emigrants (Clave, et al., 2005);
- 3) Regulatory T cells (Zorn, 2006); and also
- 4) The pre-transplant levels of other immune cells (Cohen, et al., 2002) (Jones, et al, 2003) (Arpinati, et al., 2007) (Scott, et al., 2008).

Here I will analyse some of these factors and correlate their influence on the transplant outcome.

T cells subsets and Transplant outcome.

Tumour relapse remains the major cause of transplant failure due to the inability of the immune system to mount an effective and successful anti-tumour response after transplantation.

The role of immune effector cells in tumour biology is an active area of research and evidence has been accumulated that demonstrates an anti-tumour effect involving T cells in the allogeneic transplant setting. Pre-

transplant CD4⁺ T cells, in particular CD4⁺ memory T cells (CD4⁺CD45RA⁻CD62L⁻) in the recipient have been shown to correlate well with the outcome of HSCT independently of other known factors (Rosinski, et al., 2005). These reports suggest that the analysis of the CD4⁺ subset prior to transplantation might be used as a prognostic factor after transplant.

Another imperative issue for transplant success is the emergence of GvHD. Certain T cell subsets, particularly those expressing the activation marker CD25 correlate with the degree and incidence of GvHD. Stanzani *et al* (2004) reported that higher frequencies of both CD4⁺CD25⁺ and CD8⁺CD25⁺ T cells in the graft were seen in patients with acute or chronic GvHD when compared with those who did not experience GvHD. However, they could not define whether these CD25⁺ cells were regulatory or activated T cells, and they suggested that more work to identify regulatory T cells was needed (Stanzani, et al., 2004), to clarify their influence in HSCT outcome.

Therefore, there is evidence to support that the recipient's T cell profile prior to transplantation might influence the transplant outcome, and its analysis therefore could be used as a predictive factor.

Thymic function and transplant outcome.

Another major factor influencing HSCT outcome is thymic function (Zorn, 2006). The thymus is the primary anatomic site of T cell development and the regeneration of an adequate T cell number and repertoire diversity are key elements in the recovery of immune competence (Stanzani, et al., 2004).

Thymic function and the production of recent thymic emigrants can be evaluated through the quantitation of T-cell receptor excision circles (TRECs) by Real-Time Polymerase Chain Reaction (qRT-PCR). TRECs arise from the rearrangement event that deletes the TCR delta locus during commitment of a T cell to the alpha/beta lineage; they are stable, and neither replicate nor degrade during cell division but partition into one daughter cell, and they decrease with age following thymic atrophy since they will be lost as a result of normal cell turnover and not replaced (Douek, et al., 1998) (Talvensaari, et al., 2002). After transplantation, TREC production is the best indicator to date of thymic function and TREC levels correlate well with naïve T cell levels (Baron & Sandmaier, 2005) (Stanzani, et al., 2004).

A number of studies have shown that patients with better thymic function before transplantation have better early recovery of TREC number and TCR β repertoire diversity than patients with lower thymic function. Therefore, TREC quantification may be useful in assessing risk prior to transplantation (Zorn, 2006) (Zhang, et al., 1999) (Douek, et al., 1998).

Regulatory T cells and transplant outcome.

A population of lymphocytes with immune suppressive capacity has been reported, termed regulatory T cells (Tregs), representing 5 to 10% of the normal T cell compartment in mice and humans (Sakaguchi, et al., 1985). A precise definition of these cells is not conclusive as yet, however, they are naturally occurring, predominantly confined to the CD25^{hi} subset of CD4⁺ T

cells, they appear to preferentially express the transcription factor FOXP3 along with varying levels of cytotoxic T lymphocyte associated antigen 4 (CTLA-4), glucocorticoid induced tumour necrosis factor receptor (GITR), and programmed death 1 (PD-1). Upon stimulation, they produce regulatory cytokines such as IL-4, IL-10 and transforming growth factor β (TGF- β).

Tregs have an important role in controlling autoreactive T cells and maintaining peripheral tolerance to self-antigens, however their role in transplant outcome remains controversial (Sakaguchi, et al., 2001).

A potential clinical benefit of Tregs in controlling GvHD and reducing the complications of allogeneic HSCT has been suggested. The recent understanding of the function of Tregs may provide a tool to improve the outcome of HSCT by creating a balance between GvHD and GvL effects. In mice, it has been reported that a high number of CD4⁺CD25⁺ T cells correlates with a delay in the development of GvHD (Yagi, et al., 2004). Also, in mice, it has also been reported that the infusion of CD4⁺CD25⁺ T cells can control even established GvHD (Sakaguchi, et al., 1985).

However, the role in humans is more controversial, with different results being obtained by different groups.

Clark *et al* (2004) found an increase in the absolute number of CD4⁺CD25^{hi} T cells in adult patients who underwent an HSCT from sibling donors and developed GvHD (Clark, et al., 2004). Sanchez *et al* (2004) also reported that the ratio of activated CD4⁺ effector cells/CD4⁺CD25⁺ cells was higher in patients who developed chronic GvHD in comparison with patients who did not (Sanchez, et al., 2004).

In contrast, Zorn *et al* (2006) found a decrease in the frequency of CD4⁺CD25⁺ T cells in patients with chronic GvHD and also showed a correlation between a decrease of FoxP3 expression in peripheral blood lymphocytes (PBL), measured by quantitative PCR, in patients with chronic GvHD (Zorn, 2006). Miura *et al* (2004) also showed a decrease in the expression in FoxP3 in PBMC, measured by quantitative PCR, in patients with acute and chronic GvHD (from patients who underwent HSCT from sibling donors or auto HSCT), and also showed a correlation between the expression of FoxP3 and the severity of GvHD (Miura, et al., 2004).

However in contrast to both of the above, Mening *et al* (2005) reported that comparable numbers of CD4⁺CD25^{hi} T cells and FoxP3 levels were found in patients who underwent HSCT from HLA identical siblings, with or without chronic GvHD (Meignin, et al., 2005). Clearly, more information is required to elucidate the role of Treg in humans.

Other Immunological Cells and HSCT outcome

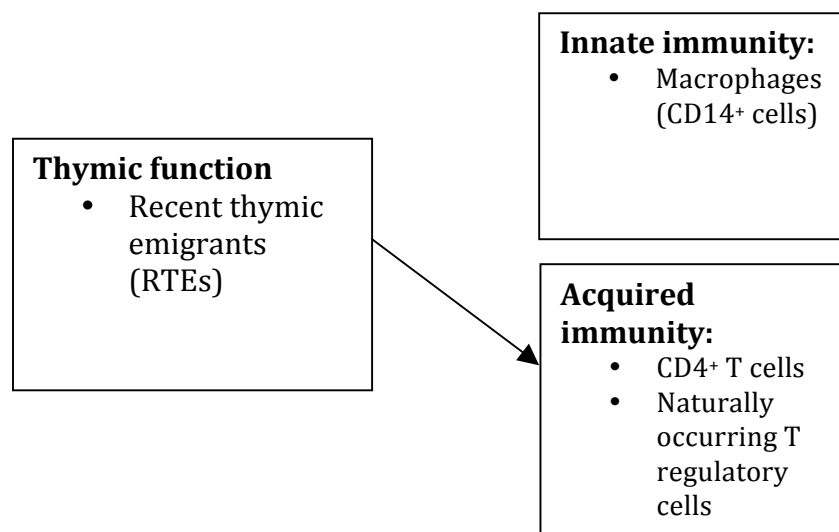
Recently the impact of the pre-transplant levels of other immunological cells on HSCT outcome has been investigated. In particular, the levels of both major types of dendritic cells, myeloid (mDC) and plasmacytoid (pDC) dendritic cells along with monocytes (CD14⁺ cells) have been studied. Dendritic cells and monocytes were measured prior to the initiation of the conditioning regimen in both related and unrelated transplant. The results suggested that higher levels of CD14⁺ cells prior to transplantation were predictive for the onset of aGvHD in patients receiving a transplant from a related donor but no effect was seen with patients receiving transplant from an unrelated donor. On the other hand, the levels of dendritic cells did not

affect the transplant outcome. Therefore, this study suggests that evaluation of other non T cell immunological factors and immune cells, could be important in predicting the success of the transplant (Arpinati, et al., 2007).

Another population of immune cells associated with the success of the transplant are neutrophils; an association between neutropenia pre-transplant and an increase in the infection rate has been found, in particular of infections produced by Gram-positive bacteria. In general, the overall mortality is increased in patients who present with neutropenia pre-transplant (Scott, et al., 2008).

In summary, shown schematically in Figure 1.2, all of the observations about the role of immune, T cells subsets, including Tregs, and the levels of TREC pre-transplant, suggest the possibility of the identification of those patients at higher risk of relapse, infections or cGvHD based on their immune status prior to transplantation.

Figure 1.2. Patient pre-transplant immune factors affecting transplant outcome



Major complications in HSCT

There are complications associated with the transplant procedure and those not associated with the underlying disease are denominated as transplant related mortality (TRM), which can occur early post-transplant. The TRM complication can be classified as early (occurring within the first 90 day after transplant) or late, occurring beyond 90 days post-transplantation. These complications are often related more to the conditioning regimen used.

Despite the fact that recent advances have improved the prognosis of HSCT, there are major complications such as graft versus host disease (GvHD), infections and relapse of the primary malignancy that are still major causes of death after transplantation and are a limitation on the wider use of unrelated donors, and the wider application of Haematopoietic stem cell transplantation for non-malignant diseases.

Infections

Although better diagnostic tools and better treatments have been developed over time, infectious complications after HSCT remain a major cause of death after HSCT (Gratwohl, et al., 2005). The major factor involved in the development of infections is the profound immune deficiency following HSCT with full immune reconstitution taking up to two years (Martin, et al, 2009). At least 10% of all deaths after HSCT are due to infections. The causes of lethal infections are divided into bacterial, viral, fungal or parasitic infections. Other pre-transplant factors along with immune reconstitution that are associated with infectious complications are age and T-cell

depletion, though these also affect the rate of immune reconstitution. The impaired T cell reconstitution after HSCT has also been associated with other HSCT complications, in particular with the incidence of GvHD and relapse and is therefore a major cause of morbidity and mortality (Giralt, et al., 1997) (Kassim, et al., 2005).

Graft Versus Host Disease (GvHD) and Transplant failure.

Graft versus host disease (GvHD) is the major complication after allo-HSCT. Acute GvHD manifests within 100 days post-transplant and is induced by donor T-cells responding to mismatched host polymorphic histocompatibility antigens, while chronic GvHD manifests later (more than 100 days post-transplant) and has some features of autoimmune diseases (Toubai, et al., 2008). Chronic GvHD can arise *de novo* or as an extension of aGvHD, and it is thought to be induced also by donor T-cells. However, the exact mechanism of cGvHD remains unclear (Toubai, et al., 2008).

The physiopathology of GvHD is not completely understood; however it is thought to be initiated by alloreactive donor T cells that recognise MHC class I and class II molecules on the surface of host cells (direct allorecognition), as well as host peptides presented by donor dendritic cells (indirect allorecognition) (Ferrara, 2000). The infiltration of target organs by donor leukocytes is one of the key processes in GvHD (Gratwohl, et al., 2005); the activation and expansion of these donor T cells leads to a deregulation in production of pro-inflammatory cytokines (e.g. INF- γ and TNF- α) in both T cell and other immune cells, and the recruitment of additional inflammatory

effector cells that further damage affected tissues (Remberger, et al., 2008) (Madrigal, et al., 1999) (Madrigal, et al., 1997).

To date, the most effective way to avoid GvHD in unrelated donor haematopoietic stem cell transplantation (UD-HSCT) is T cell depletion of the graft (Przepiorka, et al., 1999) (Przepiorka, et al., 2001). However, this method results in a greater incidence of graft failure; higher risk of opportunistic infections; tumour relapse; secondary lymphoproliferative diseases, and delay in immune reconstitution (Urbano-Ispizua, et al., 2002) (Vela-Ojeda, et al., 2006) (Baron, et al., 2005).

On the other hand, there is an association between GvHD and relapse, as mentioned before, patients who receive infusions of grafts containing T cells show less recurrence of their disease, demonstrating that in addition to the conditioning regimen aimed to eliminate the leukaemic cells, an immune reaction occurs mediated by donor T cells that is important for the elimination of residual tumour cells. This is the so-called Graft versus Tumour or Leukaemia (GvT or GvL) effect. Therefore, it is clear that donor T cells have an impact improving engraftment and immune reconstitution after HSCT (Baron & Storb, 2005). For several decades then, the main challenge of allogeneic HSCT has been the balancing of these two reactions (GvT versus GvHD) that has not yet been achieved (Baron & Sandmaier, 2005).

Graft failure

Finally, transplant failure occurs when the new haematopoietic system fails to grow successfully in the marrow of the recipient. This may result from

insufficient stem cells being transplanted or from recipient immune responses directed against the donor haematopoietic cells.

Relapse of the primary tumour

Relapse or disease recurrence is defined as the presence of cytological molecular or genetic markers of the underlying disease. Relapse of the primary tumour remains the major cause of treatment failure, and can occur anytime during therapy or after HSCT. In fact, 20 to 50% of patients with acute leukaemia relapse after an HSCT (Lee, et al., 2000). One factor that has been associated with an increase incidence of relapse is the stage of the disease at the time of transplant. A second, very important factor is whether or not the graft was T cell depleted, which relates to the presence or absence of the graft versus leukaemia effect (GvL) (Lee, et al., 2000). It has been demonstrated, that after allogeneic HSCT from a fully HLA matched donor, T cells from the donor can mount an alloresponse against minor histocompatibility antigens of the recipients. Those allogeneic responses can be restricted to host haematopoietic cells and can create an effective anti-tumour response. Such GvT response is also known as the Graft versus Leukaemia effect (GvL) and prevents the patient' suffering relapse of the primary tumour (Horowitz, et al., 1990).

More recently other genetic factors have been associated with increase relapse rate, such as DP matching and the presence of polymorphisms in the NOD2/CAR15 gene, particularly in T cell depleted transplant settings (Mayor, et al., 2007).

Post-transplant lymphoproliferative disorder (PTLD)

Another important complication after HSCT is a group of malignancies that are associated with Epstein-Barr virus (EBV) infection. There are several factors associated with the induction of PTLD, EBV mismatch between donor and recipient, the use of T-cell depletion therapy and genetic predisposition of the recipient. The overall incidence of PTLD is 2.5% in the first year post-transplant.

Transplant success

The successful hematopoietic stem cell transplantation (HSCT), in both autologous and allogeneic settings, requires a rapid and durable engraftment, considered successful when the patient achieves neutrophil counts $> 500/\mu\text{l}$ and platelet counts $> 20,000/\mu\text{l}$ (Gonçalves, et al., 2009). Engraftment can be achieved with a combination of the high dose chemo/radiotherapy, transplant conditioning and the anti-leukaemic effect of the allogeneic T cells in the graft, and it can be analysed by monitoring engraftment and reconstitution of the immune system by flow cytometry and TREC quantification. Also it is in this context important to study the diversity of the T cell population. The period following HSCT is a period of profound immunodeficiency associated with complications such as infections and relapse. And thus, mechanisms to improve immune reconstitution have become an area of great interest. New drugs that enhance the immune reconstitution, in particular the T cell lineage have been studied to improve the outcome after transplantation.

Immune reconstitution following HSCT

Better outcome following HSCT depends on the complete reconstitution of the host's immune system. The recovery of the complete immune system after an HSCT follows different paths for the different cellular components. There is a relative fast recovery of cells of the innate immunity, and the first cells to recover are monocytes and NK cells. Although the levels of B cells also recover rapidly, the levels of immunoglobulins remain low. The T cell lineage remains low up to one year after transplantation, mainly due to a low level of CD4⁺ cells, which remain low up to 2 years post-transplant. Consequently, there is an inversion on the CD4/CD8 ratio. That observation has been supported by TREC quantification, where a low TREC count can be found even a year after transplant, and the low TREC level is mainly in the CD4⁺ T cell compartment (Storek, et al., 2008). Also the diversity of the TCR diversity remains restricted up to two years after transplant, which may imply a limited response against antigens and an increased risk of infections.

T cells reconstitution after HSCT

T cells can reconstitute through two different mechanisms after transplantation, the progenitor haematopoietic cells can reconstitute the T lymphocyte population using the thymic dependent pathway or pre-existing T lymphocytes can reconstitute normal levels using the thymic independent homeostatic pathway.

The thymic independent pathway involves an expansion of pre-existing naïve and memory T cells derived from mature T cells present in the donor

graft, the limited numbers of these producing an oligoclonal expansion and a narrow T cell repertoire (Bomberger, et al., 1998) (de Gast, et al., 1985).

The thymic dependent pathway involves the development *de novo* of T cells in the recipient from the transplanted donor stem cells. During, this process T cell reconstitution recapitulates the T cell ontogeny, in which T cell progenitors undergo a process of expansion, maturation and differentiation in the thymus. Clearly, therefore, a functional thymus is required (Mackall, et al., 1993). The thymic dependent pathway has the advantage of generating long-term naïve T cells and a broader TCR repertoire in contrast with the thymic independent pathway. However, the thymus dependent pathway is age-dependent as a consequence of thymic involution with increasing age (Bomberger, et al., 1998) (Weinberg, et al., 1995) (Mackall, et al., 1995) (Storek, et al., 1995). The reconstitution by the thymic dependent pathway can be analysed by flow cytometry. Traditionally, the naïve T cells can be studied by the expression of the surface marker CD45RA (Johannisson & Festin, 1995). However, it has been demonstrated that this marker is not accurate (Arlettaz, et al., 1999). The best way at present to analyse the reconstitution of newly generate T cells is by quantification of the T cell excision circles (TRECs) by real-time PCR (Fallen et al, 2003).

T cell ontogeny

Lymphoid progenitors from haematopoietic cell precursors migrate from the bone marrow to the thymus where they begin a period of differentiation and complete their maturation. The thymic microenvironment directs the

differentiation (Haynes & Heinly, 1995). In the thymus, T cells also acquire their specific markers: TCR α and β chains, CD3, CD4 and CD8, and they go through a period of thymic education, involving processes of the positive and negative selection (Starr, et al., 2003).

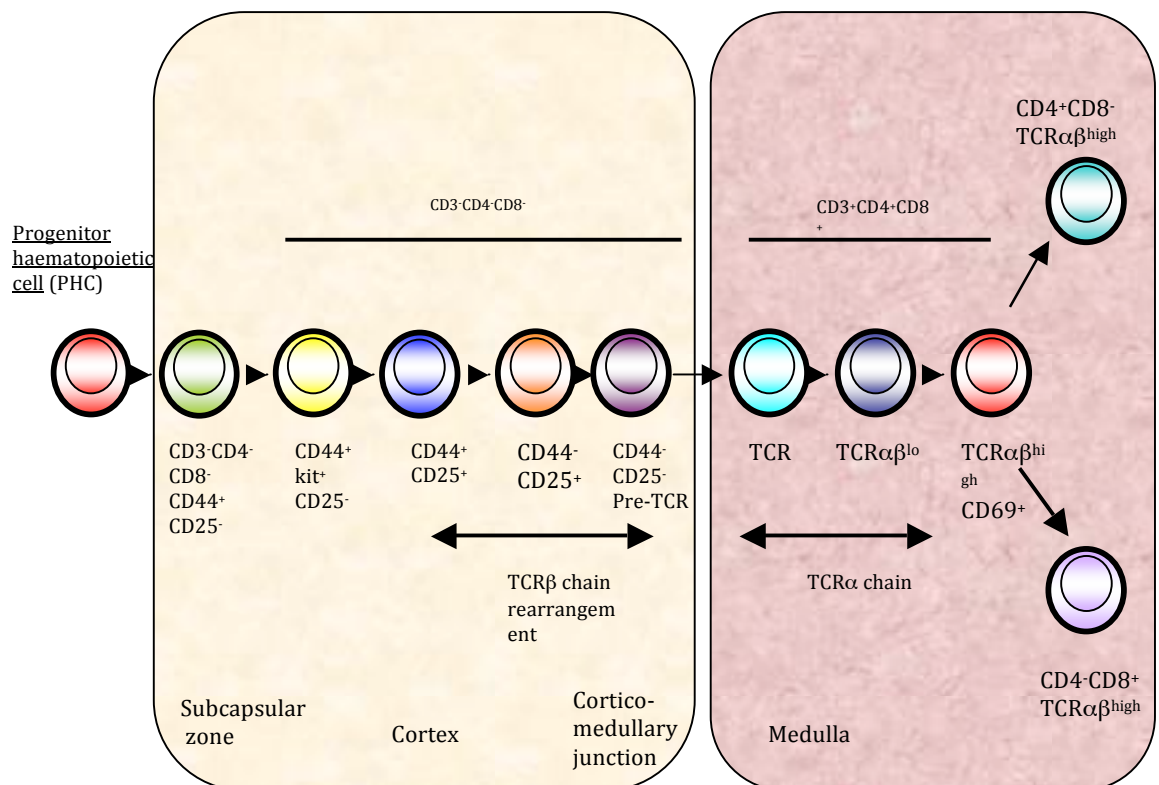
The thymus is a multi-lobed organ with cortical and medullary areas surrounded by a capsule. Shown schematically in Figure 1.3, the lymphoid progenitors enter the subcapsular cortical area in which cells interact immediately with the thymic stroma; during that period cells undergo a period of extensive proliferation. As cells continue their differentiation they move from the cortex to the medulla, into a different microenvironment that promotes T cell maturation.

T cell maturation from lymphoid progenitors can be analysed through the expression of different surface markers. The first marker expressed is CD2, when the cells have not rearranged their TCR genes (CD2⁺CD3⁻) and they are also negative for the CD4 and CD8 markers (so-called double negative period CD4⁻CD8⁻). Then T cell precursors will express the adhesion molecule CD44, followed by the expression of the α chain of the IL-2 receptor (CD25) (Haynes, et al., 1988).

As T cell precursors become CD44^{low} CD25⁺ double negative cells, they rearrange their T cell receptor loci. From the double negative phase about 10-20% of the cells will rearrange the $\gamma\delta$ TCR, to complete their differentiation as a $\gamma\delta$ T cells, while the majority of the cells, about 80-90% will commit to rearranging their $\alpha\beta$ TCR, becoming $\alpha\beta$ T cells. In this process they rearrange the TCR β chain of one chromosome and, if that rearrangement fails then a rearrangement of the second chromosome

occurs. The rearrangement of a productive β chain is followed by its expression on the cell surface along with CD3 marker and a surrogate α chain, pT α (pre T cell receptor α), which provides a signal that causes the cell to stop rearranging the β chain, followed again for a period of proliferation in which cells start to express both the CD4 and the CD8 markers, and become double positive cells (CD4⁺CD8⁺ T cells). At this point they stop expressing the CD25 marker. Double positive cells then re-express RAG-1 and RAG-2 enzymes to rearrange their α chain genes. This can happen on both chromosomes at the same time. The double positive $\alpha\beta$ T cells move then towards the cortico-medullary junction where further processes of negative selection eliminate self-reactive T cells. Only after this mature T cells leave the thymus (Haynes, et al., 1988).

Figure 1.3. T cell ontogeny



Positive selection

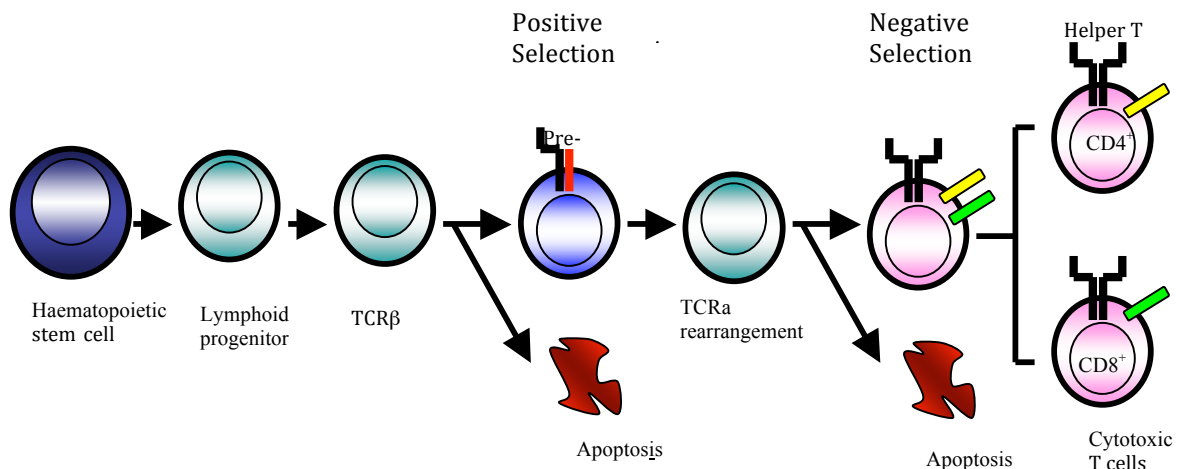
The process of selection of the T cell specificity takes place in two discrete stages during T cell development, as shown in Figure 1.4. Positive selection occurs when double positive T cells bind cortical thymic epithelial cells expressing either Class I or Class II MHC molecules/peptide complexes with high affinity to get a survival signal, allowing them to continue their maturation process (Owen & Moore, 1995). Therefore, positive selection appears to be based on low reactivity with self-peptides presented by MHC molecules of thymic epithelium (Hogquist et al., 1994) (Hogquist, et al., 1997).

Negative selection

Negative selection occurs when double positive T cells bind to self derived APC (macrophages and dendritic cells) expressing either Class I or Class II MHC molecules/self peptide complexes with a high enough affinity to receive an apoptosis signal and therefore, clonal deletion (Viret & Janeway, 1999).

The recognition of peptides bound to the MHC molecules through negative and positive selection have an impact in shaping the T cell repertoire.

Figure 1.4. Positive and Negative Selection of T cells



T cell receptor (TCR) and T cell receptor rearrangement

T cell receptor (TCR) is a membrane bound heterodimer composed of two polypeptide chains ($\alpha\beta$ or $\gamma\delta$) linked by a disulphide bond and associated with a non-polymorphic membrane bound complex of proteins known collectively as CD3 (Clevers, et al., 1988) (Rudd, et al., 1994) (Weiss, et al., 1994).

95% of T cells express the $\alpha\beta$ T cell receptor (TCR $\alpha\beta$) and only 5% of T cells express the alternative $\gamma\delta$ T cell receptor (TCR $\gamma\delta$).

The extracellular domains of the TCR contain a constant (C) domain and a variable (V) domain. The V domain interacts with the antigen and with the major histocompatibility molecules and confers specificity to the T cell.

The TCR loci

The TCR genes are located on chromosomes 7 and 14. The genes encoding the TCR δ (TCRD) chain are located within the TCR α (TCRA) loci on chromosome 14q11-12, whereas the TCR β (TCRB) and TCR γ (TCRG) genes are located on chromosome 7, positions q31-35 and p15, respectively (Caccia, et al., 1984) (Morton, et al., 1985) (Isobe, et al., 1985) (Wilson, et al., 1988) (Rabbitts, et al., 1985) (Le Franc, 2000). Each of the four genes must be assembled together during T cell ontogeny by somatic recombination and each of them is composed of a variable (V) amino-terminal region and a constant (C) region. The variable region of the alpha locus (TCR α) contains V and J gene segments (V α and J α) while the beta locus (TCR β) contains D β gene segments in addition to the V β and J β gene segments.

The gamma (γ) and delta (δ) chains also contain the variable (V) and the constant (C) regions, and like the $\alpha\beta$ TCR they have the V and J gene segments. The delta locus also contains the D segment.

The multiple non-contiguous V, D and J segments undergo rearrangement during T cell development to form the complete V domain, which is responsible for antigen recognition.

The TCRA gene consists of 70 V gene segments, divided into 41 families, and at least 61 J segments consisting of 50 functional J genes and with a single C gene (Roman-Roman, et al., 1991) (Yoshikai, et al., 1985) (Kimura, et al., 1987) (Klein, et al., 1987).

The TCRB locus, shown in Figure 1.5, contains approximately 67 V genes of which 47 are functional and 19 are pseudogenes (Rowen, et al., 1996). The functional genes are grouped into 23 families based upon greater than 75%

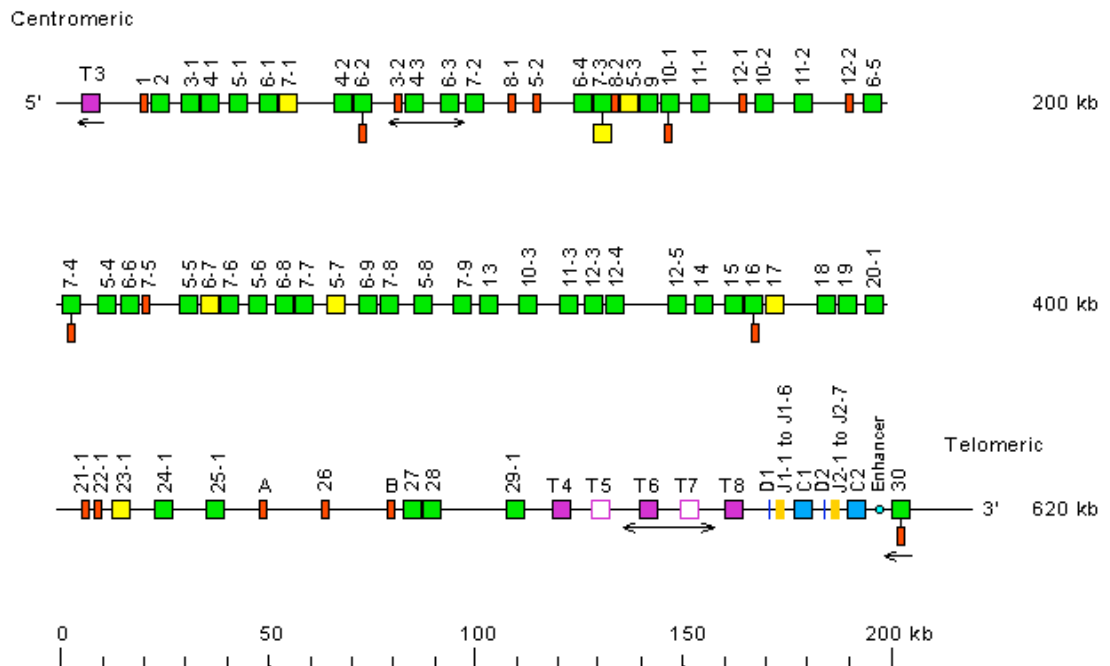
nucleotide similarity (Toyonaga, et al., 1985). There are also 2 D genes segments, 13 J segments, and 2 C genes. The D, J and C genes form two D-J-C clusters.

The TCRG consist of 14 V gene segments and a duplicate J-C cluster, one cluster containing three J genes and one C and the other one containing two J genes and one C gene.

The TCRD locus is located within the TCRA locus between the AV and AJ gene segments (Le Franc, 2000). It is flanked by TCRD deleting elements (Ψ JA and δ REC), and during the commitment of the T cell to express $\alpha\beta$ T cell receptor (TCR) the delta locus is eliminated by rearrangement between these deleting segments. The germline encoded TRCD locus contains eight V segments, three D segments and four J segments.

Figure 1.5. Locus representation of human TCRB locus on chromosome 7 (7q39).

Lefranc, M.-P. and Lefranc, G., *The T cell receptor FactsBook*, Academic Press, London, 398 pages (2001)



TCR Diversity

T cells have a unique ability to recognise a wide variety of non-self antigens, which is necessary for their immunological function. Therefore, the generation of a broad TCR diversity is very important to allow immune responses to many different infectious agents. The T cell receptor diversity is generated during T cell development in the thymus as a consequence of different mechanisms:

a) Stochastic TCR gene rearrangement: different V, D, J gene segments are brought together in each T cell progenitor into a continuous V-D-J coding block through the recombination activating genes, RAG1 and RAG 2 (Talvensaari, et al., 2002) (Zhang, et al., 1999) and the terminal deoxynucleotidyl transferase enzyme (TdT) (Landau, et al., 1987). The TCR gene segments are flanked by recombination signal sequences (RSS), which are the sites for the VDJ recombinase and mark the points where the somatic recombination occurs.

b) Nucleotide insertion and deletion at junctional sites: incorporation of template dependent palindromic “P” nucleotides and template independent “N” nucleotides at the junctional sites increases the diversity of the TCR (Lewis, 1994) (Nadel & Feeney, 1997) (Alt & Baltimore, 1982) (Gilfillan, et al., 1993). The junctional region (V(D)J) encodes the hypervariable CDR3 (Jones, et al., 2003). The CDRs (CDR1, CDR2 within the V genes and CDR at the V(D)J junction) are the regions of greatest variability and are the binding sites for antigen, the CDR3 is positioned at the centre and it is the one in direct contact with the MCH/peptide complex).

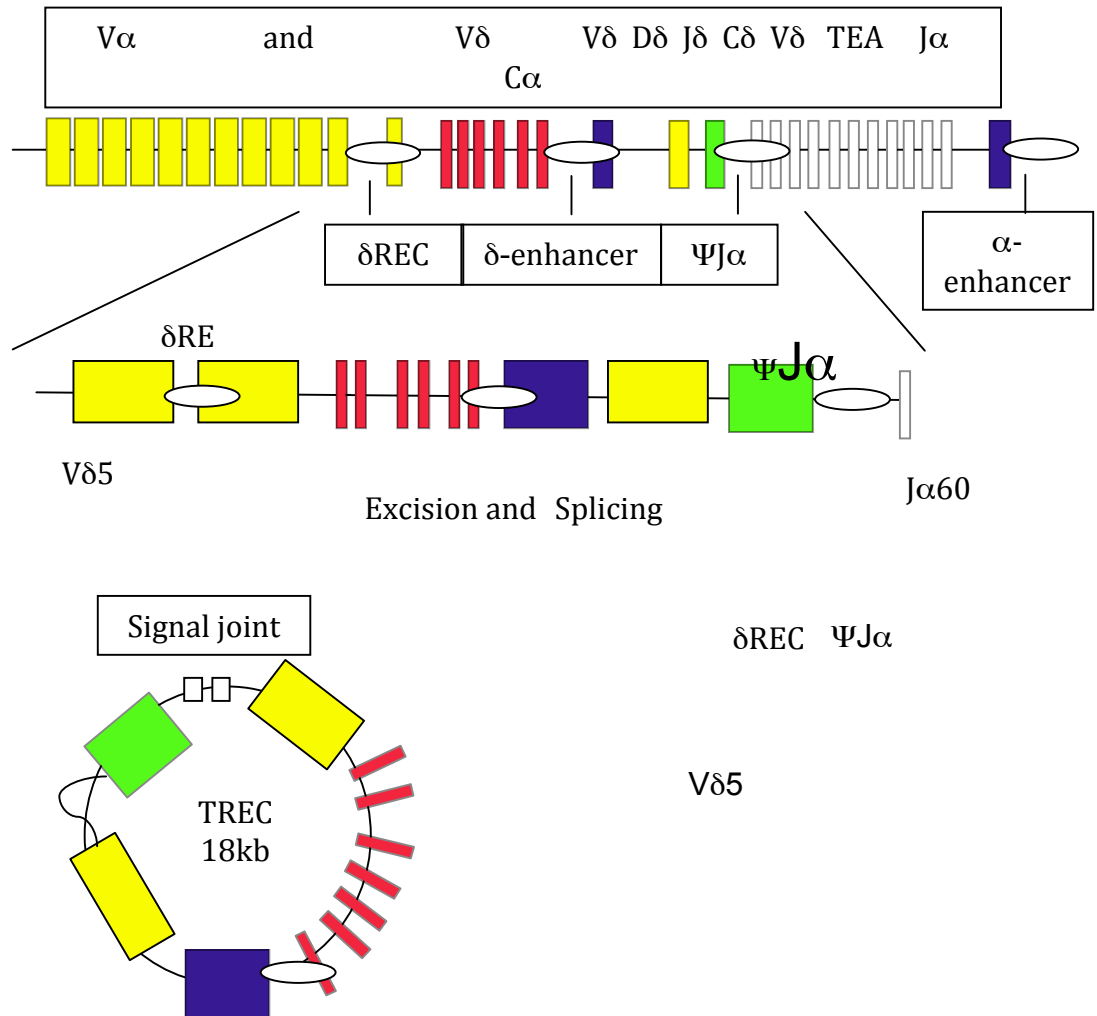
c) Combinatorial recombination: the different combinations between individual TCRA and TCRB chains or TCRG and TCRD also increase the diversity of the TCR repertoire.

The estimated repertoire of unique TCR has been calculated at around 10^{15} (Davis & Bjorkman, 1988).

T cell receptor excision circles (TRECs)

The generation of TCR diversity occurs in the thymus through the recombination of gene segments encoding the variable region of the α and β chains (see Figure 1.6). During the recombination of the TCRA locus a by-product is generated by the deletion of the TCRD locus, this by-product is known as the TCR excision circle (TREC). This deletion step is important during T cell differentiation and is the sign of the definite commitment of thymocytes to the $\alpha\beta$ T lineage. This molecule is an extra-chromosomal DNA circle that is stable, is not replicated during mitosis and consequently is diluted-out in each cell division. The quantification of these molecules is considered a valuable tool to estimate thymic function and to evaluate immune reconstitution after HSCT, chemotherapy or therapy for immunodeficiency. The quantification can be made from both the CD4⁺ and the CD8⁺ population. Different techniques to measure TRECs are used such as real-time PCR, quantitative competitive PCR and PCR-ELISA. The most frequently and widely used to date is the real-time PCR.

Figure 1.6. The formation of T cell receptor excision circle (TREC) during TCR α rearrangement.



T cell subsets

The advent of the monoclonal antibodies and flow cytometry made possible the analysis of functional T cell subpopulations. The most representative T cell subsets are CD4⁺ and CD8⁺ T cells, better known by their functionality, thus CD4⁺ T cells or helper T cells and CD8⁺ T cells or cytotoxic cells. However, CD4⁺ and CD8⁺ T cells can also be identified by their maturation stage using another set of surface markers such as: CD45RO/CD45RA, CD27, CD28 and CCR7. The first studies conducted analysing the CD3⁺/CD4⁺/CD8⁺ T cell subsets recovery after transplantation showed similar results where substantial impairment of the CD4⁺ subpopulation was found independently of other clinical factors while a fast recovery to normal values was found in the CD8⁺ subpopulation (Atkinson, et al., 1982) (Janossy, et al., 1986). Even more, some groups demonstrated that the recovery of the CD8⁺ T cell subsets was mainly due to a clonal proliferation of mature T cells present in the graft, as was shown in patients positive for CMV infection (Peggs, et al., 2003).

Eventually, other markers such as CD45RA, CD45RO and CD27 together with CD4 and CD8 were shown to be better markers to define the maturation status of T cells. The CD45 molecules are known to appear in various isoforms generated by alternative splicing of variable exons 4, 5 and 6 of the extracellular domain in various thymocyte subsets and changes in CD45 isoform expression has been correlated with positive selection and thymocyte development (Fukuhara, et al., 2002). The expression of the CD45RA isoform represents naïve, recent thymic emigrant T cells; they represent the 95-99% of the lymphocytes at birth while an increase of the

cells expressing the CD45RO isoform is observed during the first two decades of life, and this conversion on the phenotype is associated with an increase in memory cells (Cossarizza, et al., 1996). Therefore, the analysis of the CD45RA/RO expression might be helpful to define the origin of the new cells after transplantation and the reconstitution pathway followed after transplantation. However, it has become clear that this alone is insufficient, since CD45RO⁺ cells can re-express CD45RA as they mature further.

Phenotypic analysis using CD45RA/RO monoclonal antibodies performed in post-transplanted patients showed a disparity between the phenotype of neonatal and post-transplantation reconstitution. As mentioned previously, in neonates the phenotype of CD4⁺ T cell is associated principally with CD45RA expression, which is associated with naïve T cells and the naïve T cell phenotype is inversely correlated with age. While in post-transplanted patients there is an inversion and CD4⁺ T cells are associated with the expression of CD45RO, which represents a memory marker (Storek, et al., 1995). Indicating that T cells expressing the CD45RA marker are generated through the thymic dependent pathway and are better known as recent thymic emigrant (RTEs), while cells expressing the CD45RO marker are generated through peripheral expansion. However, a regression of the CD45RO⁺ phenotype to the CD45RA⁺ has been recently observed, which complicates the analysis of the recent thymic emigrant through the expression of their surface markers, making evident the need for better ways to define the recently formed T cells (Haynes, et al., 2000).

Using other surface markers the naïve T cells are defined as CD45RA⁺/CD27⁺/CCR7⁺/CD28⁺, the central memory cell are defined as

CD45RA-/CD27+/CCR7+/CD28+ and finally the effector memory T cells are defined as CD45RA-/CD27+/CCR7-/CD28+ (Okada, et al., 2008).

However, apart of the CD4 and CD8 functional and maturation stage analysis through the analysis of their surface markers, different CD 4 subsets can be analysed through their cytokine production profile. Therefore, CD4⁺ cells have two major functional subsets: Th1 and Th2. However, recently other CD4 subsets have been described: Th17 and regulatory cells. The CD4⁺ Th1 T cells are characterised by the production of INF- γ and IL-12 cytokines, while the Th2 T cells subsets is characterised by the production of IL-4, IL-5 and IL-13. The Th17 subset has been recently described, which produces IL-17, and have been implicated in autoimmune disease (Harrington, et al., 2005) (Park, et al., 2005).

Figure 1.7. T cell subsets.

Phenotype definition of naïve, memory, effector and terminal effector cells depending on the expression of CD45RO and CD27 and phenotype definition of naïve, central memory, peripheral memory and effector cells depending on the expression of CD45RA and CCR7.

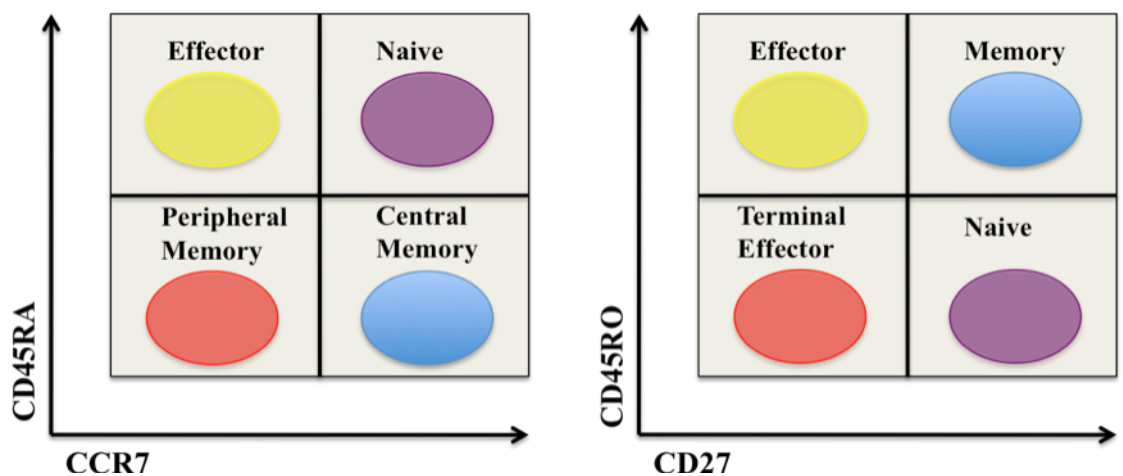


Table 1.1. Definition of T cell subsets by the expression of different markers
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	CD45 RA	CD27	CCR7	CD28
Naïve	+	+	+	+
Central Memory	-	+	+	+
Effector	-	+	-	+
Terminal effector	-	-	-	-

T cell subsets with regulatory function

Another important subset of T cells that have been correlated with the HSCT outcome are the regulatory T cells. Different regulatory T cells have been defined up to now: a) Naturally occurring CD4⁺CD25^{hi} T cells (Tregs); b) Regulatory T cells 1(Tr1); and finally c) Regulatory T cells TGF-β producers (Th3).

Definition of naturally occurring CD4⁺CD25^{hi} T cells: Tregs

Tregs are naturally occurring T cells and represent a distinct and functional T cell subset developed in the thymus expressing the surface markers CD4 and CD25. They represent the 5-10% of peripheral CD4⁺ T cells. They exert suppressive functions over other T cells and over other non T-cells such as

B-cells, NK cells and dendritic cells, in a cell-cell contact dependent manner. They play a central role in controlling autoimmunity, and contribute to the maintenance of self-tolerance, also an important role in controlling tumour immunity and transplantation tolerance has been observed (Sakaguchi, et al., 2001) (Shevach, 2002). The forkhead transcription factor FOXP3 is preferentially expressed by Tregs and it has been used as specific marker to identify them.

Regulatory T cells 1 (Tr1)

Another type of T cell that exhibits an immunomodulatory function through the secretion of high levels of IL-10 are known as Tr1. These are generated through chronic activation in the presence of interleukin-10 (IL-10). Tr1 cells can suppress antigen-specific responses of naïve and memory T cells through the release of IL-10 and by inhibiting the secretion of TNF α and INF γ (Sundstedt, et al., 1997) (Groux, et al., 1997) (Roncarolo, et al., 2001).

Th3 cells

Th3 T cells are another subset with regulatory/suppressive functions over Th1 and Th2 cells. Their suppression activity is exerted through the production of Transforming Growth Factor beta (TGF β) (Weiner, 2001). Recently it has been demonstrated that TGF β can induce the expression of the transcription factor FOXP3 in the periphery, which induces the generation of new Tregs in the periphery. The peripheral Tregs seem to be different from the thymic derived Tregs, however, a close interaction

between the different types of suppressive cells has been documented (Carrier, et al., 2007) (Carrier, et al., 2007).

The role of the various T cell subsets present in patients prior to transplantation in determining the outcome of the transplant forms the subject of this thesis.

Chapter 2

Material and Methods

Isolation and cryopreservation of peripheral blood cells

Reagents used for isolation and cryopreservation

Heparin
Lympholyte
RPMI 1640
Trypan blue
FCS
DMSO

Isolation of peripheral blood mononuclear cells (PBMC's)

Peripheral blood samples of a volume of 40ml were transferred to 50ml Falcon tubes containing 50 IU Heparin (CP Pharmaceuticals, Wrexham, UK). Peripheral blood mononuclear cells (PBMC's) were separated from whole blood by density-gradient centrifugation as follows: 10ml of Lympholyte (Cedarlane Laboratories Limited, USA) was added to a 20ml Universal tube. The Ficoll contained in Lympholyte is a neutral high mass hydrophilic polysaccharide. 10ml of whole blood was layered carefully on top of the Lympholyte solution allowing formation of a defined interface between the blood and Lympholyte. The samples were then spun at 2200 rpm for 20 min in a Heraeus 2.0 centrifuge (Heraeus, Brentwood, UK) with no braking to allow the formation of the buffy coat layer. Centrifugation of samples with this reagent results in the separation of different layers containing the plasma on the top, then the PBMC's followed by Ficoll and finally on the

bottom the erythrocytes and granulocytes. Using a plastic sterile Pasteur pipette, the buffy coat layer containing PBMC's was removed from the Lympholyte/plasma interface and transferred to a 50ml Falcon tube. The Falcon tubes were then filled to the top with RPMI 1640 (Biowhittaker, Wokingham, UK) and samples were centrifuged at 1800 rpm for 10 min. The supernatant was then decanted, the cell pellet resuspended in a further 50ml of RPMI 1640 and the samples spun at 1600 rpm for 10 min. Before freezing the PBMC's cell were counted.

Cell Counting and Viability

The cell pellet was resuspended in 10ml of RPMI 1640 and cells counted by the dye exclusion method. 10 μ l of the resuspended cells were stained with an equal amount of sterile filtered PBS solution containing 0.4% (w/v) Trypan blue (BDH) and counted using an haemocytometer (Neubaur Chamber, Weber). Following the isolation and counting of PBMC's, cells were cryopreserved in liquid nitrogen until further use.

Storage and Cryopreservation

PBMC's were isolated as described previously. The cells were then spun at 1600 rpm for 5 min and the cell pellet resuspended at a concentration of 5-10 \times 10⁶ cell/ml in freeze mix (heat inactivated foetal calf serum, FCS and

10% dimethyl sulphoxide (DMSO) (BDH)). The cell suspension was then transferred to freezing vials (Nunc, Nalgene, USA) in 1ml aliquots containing a maximum of 1×10^{10} cells. The freezing vials were placed inside polystyrene boxes, and the boxes were stored at -70°C and after a minimum of 24 hours cells were transferred to liquid nitrogen until use.

Cryopreserved samples were put back in culture by rapid warming at 37°C , followed by at least two washing steps in 50 ml RPMI 1640 (Bio Whittaker) using centrifugation at 1600 rpm for 10 minutes and incubation in complete medium for 12 to 24 hours prior to further use to allow for recovery of cell surface molecules and cellular function.

Extraction of nucleic acids

RNA Extractions and cDNA synthesis

Total RNA was extracted from 5×10^5 PBMC's with RNeasy Mini Kit (QIAGEN Inc, Valencia, California) according to manufacturer's protocol.

Samples were lysed and homogenised using guanidine-isothiocyanate lysis. 75 μl of buffer RLT (guanidine thiocyanate containing buffer provided with the kit) containing 1% (v/v) β -mercaptoethanol (Invitrogen) was added to the cell pellet, and the sample was mixed by vortexing for 1 minute. After addition of 5 μl of a 4 ng/ μl poly-A RNA solution as a carrier to the lysate, homogenisation was performed by resuspension using a fine pipette tip. This

resulted in disruption of plasma membranes, release of RNA, shearing of high molecular weight genomic DNA and reduction of viscosity of the cell lysate. 75 μ l of 70% sterile, American Chemical Society (ACS) grade ethanol (Sigma Aldrich) was added to provide ideal binding conditions. The well-mixed lysate was then loaded onto a column in a collection tube resulting in binding of RNA to the silica-gel-membrane in the column. This procedure isolates RNA molecules longer than 200 nucleotides thereby enriching for mRNA. Tubes were spun at 13,000 rpm for 15 seconds and the flow-through discarded. Samples were washed by addition of 350 μ l buffer RW1 (supplied with the kit), spun at 13,000 rpm for 15 seconds and the flow-through discarded. Traces of DNA that may co-purify were removed by deoxyribonuclease (DNase) treatment on the column using digestion with RNase-free DNase. Therefore DNase I (2.72 units/ μ l) was diluted 1:8 in buffer RDD (supplied with the kit) and 80 μ l were pipetted directly onto the membrane for incubation at room temperature for 15 minutes. DNase and contaminants were washed off by addition of 350 μ l buffer RLT and centrifugation at 13,000 rpm for 15 seconds. The flow through was discarded and the column transferred to a new collection tube for a further wash with 500 μ l buffer RPE (supplied with the kit). After centrifugation and discarding of the collection tube, 500 μ l of 80% sterile, ACS grade ethanol (Sigma Aldrich) were added and the membrane dried by centrifugation at 13,000 rpm for 2 minutes. The spin was repeated in a new collection tube at 13,000 rpm for a further 5 minutes. Purified RNA was captured in a new collection tube by elution with 40 μ l RNase-free water and centrifugation at 13,000 rpm for 1 minute resulting in a final volume of approximately 35 μ l

RNA. It was either used for RT-PCR immediately or stored at -70°C .

Reverse transcription of the RNA extracted was performed using $1\mu\text{g}$ RNA solution in a volume of $10\mu\text{l}$ with $1\mu\text{l}$ of $500\text{ ng}/\mu\text{l}$ random primers (Promega) and $1\mu\text{l}$ of 10 mM dNTPs (Bioline) for 5 minutes at 65°C and subsequent chilling on ice. The resulting $12\mu\text{l}$ template was reverse transcribed in a $20\mu\text{l}$ total reaction volume with 400 units Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Promega), 10 mM DDT and 80 units RNasin RNase inhibitor (Promega) in M-MLV RT buffer (Promega) by incubation for 50 minutes at 37°C . Afterwards, the reaction was terminated by incubation at 70°C for 15 minutes and the resulting cDNA was stored at -20°C .

The quality of the cDNA was confirmed by β -actin amplification as follows: $0.5\mu\text{l}$ cDNA was amplified in a $25\mu\text{l}$ reaction volume using 0.5 units Taq (Bioline), 2 mM MgCl_2 (Bioline), 0.4 mM dNTPs (Bioline), 0.4 mM 5' β -actin primer (5'-TCA TGA AGT GTG ACG TTG ACA TCC GT-3') (Promega) and 0.4 mM 3' β -actin primer (5'-CTT AGA AGC ATT TGC GGT GCA CGA-3') (Promega) in PCR buffer (Bioline). PCR conditions were 94°C for 2 minutes followed by 94°C for 30 seconds, 65°C for 1 minute and 68°C for 2 minutes for 40 cycles and final polymerisation at 68°C for 7 minutes. $5\mu\text{l}$ of PCR products were subjected to gel electrophoresis in a 2% agarose gel to test amplification of the constitutive gene β -actin (amplicon of 285pb).

Preparation of cell lysates

Reagents

- Proteinase K 100mg/ml

Amplification of TREC molecules was carried out directly from a cell lysates. Cell lysates were prepared from PBMC's. Cells were counted and transferred (5×10^5) to a conical 1.5ml microfuge tubes (Eppendorf, Germany). The samples were centrifuged at 13000 rpm for 1 min in a Heraeus Sepatech Biofuge 15 (Heraeus Brentwood, UK). The supernatant were aspirated by vacuum suction and the cell pellet lysed immediately. For every 1×10^5 cells in the cell pellet, 10ml of 100mg/ml proteinase K solution (Sigma, Saint Louis, MO, USA) was layered on the top of the cell pellet taking care of not touch the cell pellet. The sample was then vortexed to detach the cell pellet from the bottom of the tube and the sample flicked, letting the pellet and the proteinase K solution be together at the bottom of the tube. The samples were then incubated for 2 hours at 56°C in a heating block. After 2 hours the samples were vortexed and spun down briefly (10 sec) in a Heraeus Sepatech Biofuge 15 and then incubated at 95°C for 15 min to inactivate the proteinase K. Finally samples were vortexed and spun down at 13000 rpm for 1 min in a Heraeus Sepatech Biofuge 15, and stored at -70°C until use. For the amplification 5µl of the cell lysate were added to each PCR reaction.

Flow Cytometric Analysis

Reagents

- 1% Phosphate buffered saline (PBS)
- Foetal calf serum (FCS)
- Monoclonal antibodies (see the table below)
- 1% Paraformaldehyde
- Fixation/permeabilization buffer

Immunofluorescence staining

Many immunologically important cells can be defined based on what molecules are present on their surface. Numbered human clusters of differentiation (CD) are used to classify many epitopes on the cell surface of leukocytes. A combination of several surface markers is often used to associate cells with certain immune functions or properties.

Antibodies directed against different CDs were purchased from different companies. These had fluorochromes FITC, PE, PerCP or APC directly conjugated and were used as described above.

PBMC's were washed in 1% Phosphate buffered saline (PBS) (BioWhittaker, Walkersville, MD, USA) and 2.5% Foetal Calf Serum (FCS) (BioWhittaker, Walkersville, MD, USA), the solution of PBS and FCS will be called from here, FACS buffer. A total of 300,000 PBMC's were incubated for 25 min at 4°C with a series of fluorescent labelled mono-clonal Antibodies (mAbs) specific for: CD3, CD4 CD8, CD27, CD45RO, CCR7, CD45RA and CD25 conjugated to fluorescein, phycoerythrin, PerCP or APC (BD Bioscience, San Jose, California, USA). Antibodies were used at 1:100 dilution and cells were washed twice in

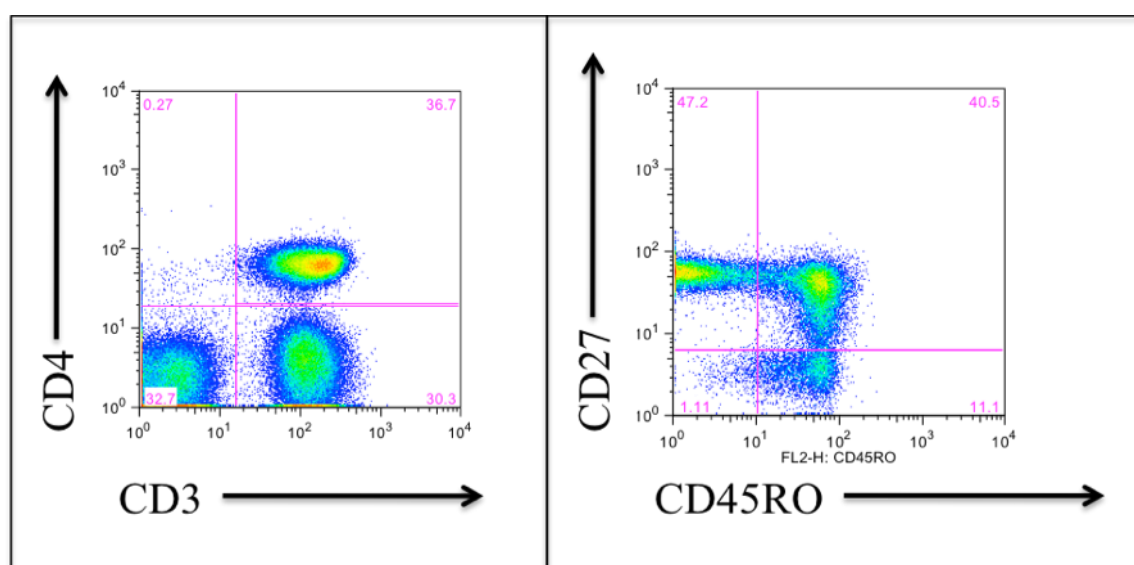
FACS buffer followed by fixation with 1% paraformaldehyde until acquisition. Immunophenotypic analysis of the stained and fixed cells was performed using FACSCalibur flow cytometer and CellQuest software (BD Bioscience, San Jose, California, USA). The frequency of each cell subset was calculated as a percentage of positive cells in the total lymphocyte gate. Figure 2.1 shows the phenotypic analysis of CD4 T cell subsets.

Table 2.1. Antibody dilutions used for flow cytometry staining

Antibody	Label	Company	Dilution	Clone
CD3	APC	BD Bioscience	1:125	HIT3a
CD4	PerCP	BD Bioscience	1:25	SK3
CD8	PerCP	BD Bioscience	1:25	SK1
CD27	FITC	BD Bioscience	1:20	M-T271
CD45RO	PE	BD Bioscience	1:20	UCHL1
CCR7	PE	R&D	1:10	150503
CD45RA	FITC	BD Bioscience	1:20	L48
CD25	PE	eBioscience	1:25	2A3
FOXP3	FITC	eBioscience	1:25	PCH101
CTLA-4	PE	eBioscience	1:20	eBio20A (20A, A3)

Figure 2.1. Phenotypic analysis of CD4⁺ T cells (CD3⁺CD4⁺).

The left panel of the figure shows the gating of lymphocytes to define the CD3⁺CD4⁺ population; on the right side of the figure are shown the different T cell maturation stages, naïve (CD27⁺CD45RO⁻), memory (CD27⁺CD45RO⁺), effector (CD27⁻CD45RO⁺) and terminal effector (CD27⁻CD45RO⁻) as defined by flow cytometry.



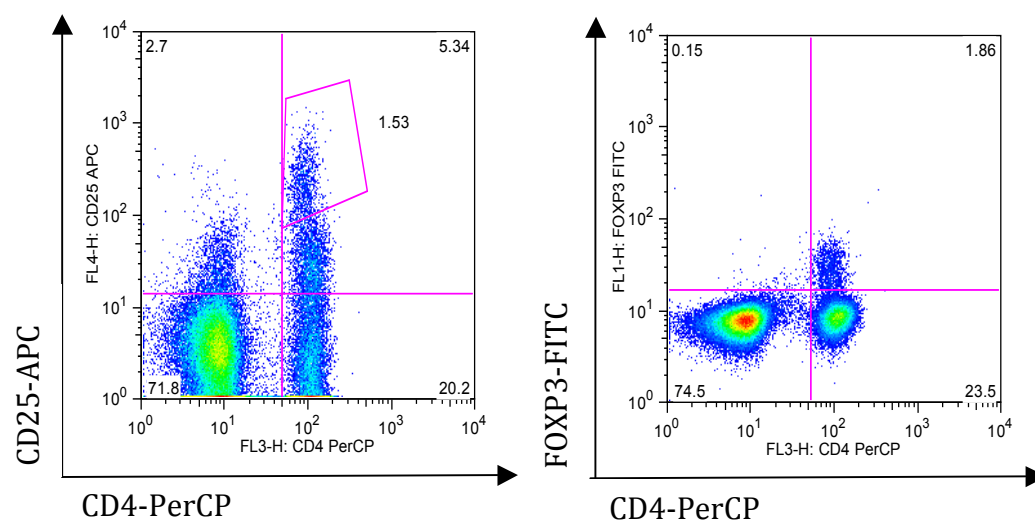
Intracellular staining

For FOXP3 intracellular staining a kit for fixations and permeabilization from eBioscience, Inc (San Diego, California, USA) was used. 100ml of previously prepared cells were used per sample, staining normally for surface markers for CD3, CD4 and CD25 (BD Bioscience), after washing, cells were

resuspended by vortex and 1ml of freshly prepared Fixation/Permeabilization solution was added and vortex again, and incubated for 1 hour at 4°C. After incubation samples were washed two times by adding 2ml of 1x Permeabilization buffer and centrifuged and supernatant was decanted. 5ml anti-human FOXP3 antibody was added per sample with 45ml of Permeabilization buffer, incubated for 30 minutes at 4°C in the dark. After incubation cells were washed again two times followed by fixation with 1% paraformaldehyde until acquisition. Intracellular staining with FOXP3 is presented in Figure 2.2.

Figure 2.2. Flow cytometry analysis of naturally occurring Tregs.

Panel A shows Tregs by the expression of CD4 and CD25^{hi}; while in panel B shows Tregs by the expression of CD4 and FOXP3. The frequencies of Tregs showed by the different staining strategies are not different.



Flow Cytometry

Cells stained with fluorochrome-conjugated antibodies were visualised using flow cytometry using FACSCalibur flow cytometer and CellQuest software (BD Bioscience, San Jose, California, USA) as it was presented on Figures 2.1 and 2.2. This technique is based on the principle that a beam of a single wavelength light is directed onto a stream of fluid. Detectors are aimed at the point where the stream passes through the light beam. The FACSCalibur has one detector positioned in line with the light beam, called Forwards scatter (FSC), and five detectors positioned perpendicular to it. The latter are the Side Scatter (SSC) and four fluorescent detectors. These detect green FITC emission (maximum 530 nm), orange PE emission (maximum 585 nm) or red PerCP emission (maximum 650 nm), all excited by a blue argon laser (488 nm); or long red-blue APC emission (maximum 670 nm) excited by a red diode laser (635 nm). Each cell within the acquired liquid that passes through the beam scatters light. Light scattered in forward direction is directly proportional to the size of the cell (FSC) whereas light scatter at larger angles to the side (SSC) is caused by granularity and structural complexity inside the cell. In addition fluorochromes on that cell are excited into emitting light. Light scattered to the sides is focused through a lens system and is collected by the SSC detector located at around 90 degrees from the laser's path. Fluorescent light travels along the same path as the side scatter signal and is detected through a series of filters and mirrors so that particular wavelengths information is delivered to the appropriate detectors. The combination of scattered and fluorescent light that is picked

up by the detectors is eventually converted to electronic signals.

Filters are used in order to separate fluorescence emission from the excitation light source and to resolve different colour. Detection channels (FL) therefore pass light in a specified range of wavelength (band-pass). Fluorochromes, however, emit light over a range of wavelengths and a signal from one fluorochrome may therefore overlap in a detector used for another fluorochrome. The spectral overlap was manually compensated using control samples stained with each single colour, and the portion of one detector's signal was subtracted from the other, leaving only the desired signal.

Selection of CD4 and CD8 subsets from PBMC's

Isolation of CD4⁺ and CD8⁺ T-cells was carried out using EasySep positive magnetic separation (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions and positive selection MS⁺/RS⁺ columns with a maximum capacity of 1x10⁷ labelled cells.

Molecular Biology

Real Time Quantitative PCR

Reagents

- ROX dye
- Distilled water
- Platinum Taq (Buffers and Magnesium)

- dNTPs
- Primers and probes

Real-Time PCR was carried out using an ABI/PRISM 7500 sequence detection system (Taqman[®]) and SDS software (Applied Biosystems, Foster City, CA) to quantify TRECs in cell lysates from donor and patient samples and to quantify FOXP3 and CD71 in diluted complementary DNA (cDNA) from each patient sample.

The PCR reactions were set up in 96-well optical reaction plates (Applied Biosystems, Foster City, CA, USA); sealed using Optical Adhesive Covers (Applied Biosystems, Foster City, CA, USA) and transferred to the ABI/PRISM 7500 Sequence Detector for analysis.

The PCR was carried out using Platinum Taq (Invitrogen Ltd., USA) and ROX dye (Invitrogen Ltd, USA) was employed in each reaction as a background reference.

Each 25 μ l reaction contained 5 μ l of cell lysates or 5 μ l of cDNA and 5 μ l of the specific standard (TREC, FOXP3 or CD71 standard), and the final concentration of 1U Platinum Taq (Invitrogen Ltd., USA), 3.5mM MgCl₂, 0.25mM dNTPs, 12.5mM of each primer (Table 2.2), 3.75mM fluorescent labelled probe (Table 2.2), 2.5 μ l Platinum buffer (Invitrogen Ltd., USA) and 0.5 μ l of ROX reference dye. One cycle of denaturation (95° for 5 minutes) was performed, followed by 40 cycles of amplification (95°C for 30 sec, 60°C for 1 minute).

Cloning and Sequencing of TREC and FOXP3

Amplification of TREC junctional sequences and FOXP3 was performed using

the primers from Table 2.1 in 50 μ l reactions using 2.5U High Fidelity Platinum Taq (Invitrogen) to circumvent the occurrence of mutations. Other reagents included 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (Bioline), 2 mM MgSO₄ (Invitrogen) and 0.2 μ M of both primers in 1x High Fidelity buffer (Invitrogen). The PCR amplification programme involved 95°C for 5 minutes followed by 40 cycles of 30 seconds at 95°C, 60 seconds at 60°C.

PCR products were subjected to electrophoresis on a 2.0% TBE gel.

Bands corresponding to a size of 100 bp were recovered from the gel using GFX gel band purification kit (GE Healthcare) and ligated into pCR2.1 vector using the pCR2.1 TOPO-TA cloning kit (Invitrogen) as it is presented in Figure 2.3.

The pCR2.1 TOPO-TA vector contains single 3'-thymidine (T) overhangs for TA cloning and the enzyme Topoisomerase I covalently bound to its DNA via 3' phosphate.

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds single deoxyadenosine (A) to the 3' ends of PCR products. The overhanging T residues of the vector allow for efficient ligation of PCR inserts into the vector. High Fidelity Platinum Taq (Invitrogen) likely resulted in less efficient cloning due to partial removing of A' overhang but was used as its advantages of proofreading may outweigh this disadvantage.

The enzyme topoisomerase I from Vaccinia virus specifically recognises the pentameric sequence (C/T)CCTT and cleaves the phosphodiester backbone of duplex DNA after that sequence in one strand (Shuman, 1991) enabling the DNA to unwind. The energy resulting from this cleavage is conserved by

formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-275) of the enzyme. This bond can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, thus reversing the reaction and releasing the enzyme (Shuman, 1994). Therefore topoisomerase I functions both as a restriction enzyme and ligase, can therefore cleave and re-join DNA during replication and enables faster ligation of PCR products than conventional methods. Finally a stop solution prevents free topoisomerase from re-binding and nicking the plasmid.

Briefly, 1µl or 4 µl of PCR product diluted in sterile water were incubated with 1µl vector solution and 1 µl salt solution in a total volume of 5 µl at room temperature each for either 5 or 25 minutes before storage at -20°C. No difference was observed between the four experimental variations.

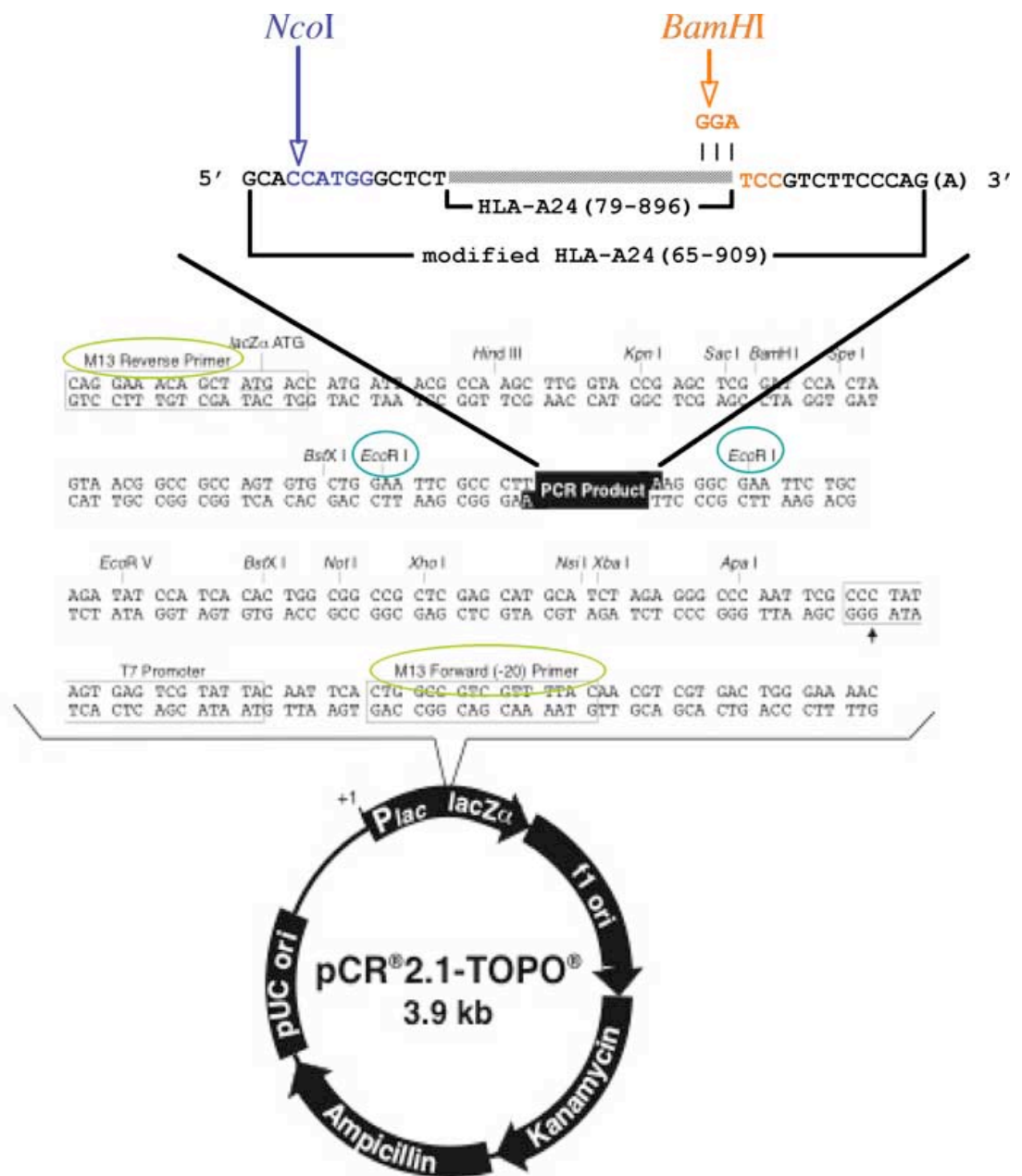
TOP10F' cells (Invitrogen) were transformed with pCR2.1 containing either TREC or FOXP3 by calcium chloride (CaCl₂) membrane permeabilisation. Therefore cells were chilled at 4°C for 30 minutes in the presence of plasmid DNA and divalent Calcium cations (present in the cell suspension) that prepare the cell walls to become permeable and were then briefly heat shocked (at 42°C for 3 minutes) causing the DNA to enter the cells. Transformed cells were grown overnight on LB plates (10g Tryptone, 5g Yeast Extract and 10g NaCl per L, pH 7) containing Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Alexis Biochemicals), 5-Bromo-4-Chloro-3-indolyl-β-D-galactoside (X-gal) (Sigma) and 80µg/ml Kanamycin. Single white colonies were selected and grown overnight or 16 hours in LB medium containing 50 µg/ml Kanamycin.

Plasmid DNA was extracted by midiprep and tested for the existence of an

insert by PCR amplification and checked for 100pb products on an agarose gel electrophoresis.

Figure 2.3. Illustration of ligation of *Nco* I/*Bam*H I modified HLA-A24 insert into pCR2.1 vector.

The figure shows the site of ligation of the *Nco* I/*Bam*H I modified HLA-A24 PCR product into pCR2.1 vector (modified from a pCR2.1 TOPO map provided at <http://www.invitrogen.com>). *Eco*RI restriction sites and sites for sequencing with M13 primers that were used in further experiments are highlighted in circles.



Plasmid DNA midiprep

This method was modified from the originally alkaline lysis described by Birnboim and Doly in 1979 (Birnboim & Doly 1979). 50 ml overnight cultures were spun at 3000 rpm for 20 minutes and cell pellets resuspended in 1 ml of a solution containing 25 mM trishydroxymethylaminomethane (2-amino-2-hydroxymethyl-1, 3-propanediol; abbreviated: Tris) and 10 mM ethylenediaminetetraacetic acid (EDTA) at pH 8. Then 2 ml of freshly prepared solution containing 200 mM sodium hydroxide (NaOH) and 1% (v/v) sodium dodecyl sulphate (SDS) was added and incubated on ice for 5 minutes. The lysis of bacteria under alkaline conditions results in precipitation of DNA and proteins. 3 M sodium acetate (acetic acid) at pH 4.8 was added and incubated on ice for 45 minutes. The acetate-containing buffer neutralizes NaOH in the previous lysis step. It results in large and less supercoiled chromosomal DNA (and proteins) forming an insoluble white, rubbery precipitate with SDS while small bacterial DNA plasmids can renature and stay in solution. After centrifugation at 4000 rpm for 20 minutes, supernatant was added to tubes containing 8 ml of absolute ethanol, gently mixed by inversion and spun at 4000 rpm at 4°C for 10 minutes. 400µl of solution containing 10 mM Tris and 1 mM EDTA at pH 8 was added to DNA pellets for incubation at 65°C for 10 minutes. After addition of 500µl Lithium chloride (LiCl) the resulting solution was cooled to -20°C for at least 10 minutes. It was then spun at 10000 rpm at 4°C for 10 minutes and 1 ml of absolute ethanol was subsequently added to supernatant resulting in precipitation of DNA. Further centrifugation at

10000 rpm at 4°C for 10 minutes was performed to enable aspiration of supernatant. The resulting DNA pellet was dried in a 37°C incubator for 10 to 20 minutes before resuspension in 50µl water and incubation in a water bath at 65°C for 10 minutes.

Plasmid DNA miniprep

The midiprep method described above was the preferred method of plasmid DNA isolation because of high DNA yields with good purity. Occasionally a miniprep method was used when a short processing time was prioritised to high DNA yields.

Therefore DNA was extracted by StrataPrep plasmid miniprep kit (Stratagene) according to the manufacturer. This method employs a modification of the alkaline method of cell lysis (Birnboim & Doly, 1979) and binding of DNA on a silica-based fibre matrix.

Briefly 1.5 ml of a 10 ml overnight culture were spun at 13,000 rpm or 1 minute and the supernatant discarded. The pellet was resuspended in 100µl of a ribonuclease-containing solution [50 mM Tris HCl (pH 7.5), 10 mM EDTA, 50 µg/ml RNase A] and gently mixed with 100µl of a cell lysis solution [0.2 M NaOH, 1% (w/v) SDS], and 125µl of a DNA-binding salt solution (containing an undisclosed chaotropic salt). As described above the lysis of bacteria under alkaline conditions resulted in precipitation of DNA and proteins. A salt neutralising buffer results in large and less supercoiled chromosomal DNA forming an insoluble white, rubbery precipitate with SDS

while small bacterial DNA plasmids can renature and stay in solution. The sample was then spun at 13,000 rpm for 5 minutes. The DNA containing supernatant was then transferred to a cup with fibre matrix, to which the DNA binds. The cup was seated inside a receptacle tube and contaminants were subsequently washed from the cup with wash buffer. Therefore the tube was spun at 13,000 rpm for 30 seconds. After removal of liquid from the tube, 750 μ l of wash buffer [5 mM Tris HCl (pH 7.5), 50 mM NaCl, 1.25 mM EDTA and 50% (v/v) ethanol] were added to the cup and spun inside the tube at 13,000 rpm for a further 30 seconds. After removal of filtered liquid, the tube was spun for an additional 30 seconds and the cup was then transferred to a new tube. 50 μ l sterile deionised water was placed on top of the fibre matrix and incubated at room temperature for 5 minutes for elution of the purified plasmid DNA, which was captured in the tube by centrifugation at 13,000 rpm for 30 seconds.

Absorbance measurement of DNA and RNA

DNA and RNA absorb ultraviolet light with an absorption peak at 260 nm wavelength. The amount of light absorbed can be related to the concentration of the absorbing molecule. At a wavelength of 260 nm, the extinction coefficient for double-stranded DNA is 20 (mg/ml)⁻¹ cm⁻¹; for single-stranded DNA and RNA it is 25 (mg/ml)⁻¹ cm⁻¹. Thus, an optical density (OD) of 1 corresponds to 50 μ g/ml for double-stranded DNA, 40 μ g/ml for single-stranded DNA and RNA.

Nucleic acid concentration was measured at 260 nm and protein contamination was assessed by the ratio of absorbance at 260 nm: 280 nm (which corresponds to 2 in pure nucleic acid solutions) using a Fenway 6405 spectrophotometer.

Control plasmid quantification

The concentration of the plasmid DNA was quantified by spectrophotometry and the result expressed as ng/ μ l. The calculation of the number of molecules was then carried out as follows:

- The average molecular weight of a nucleotide is 330 Daltons.
- A base pair is formed of two nucleotides; the average molecular weight of a base pair is 660 Daltons.
- The 3.9Kb plasmid used plus the fragment size inserted, has 3965 base pairs and thus weighs $(3965 \times 660) = 2616900$ gram/mole
- One mole has 6×10^{23} molecules. Therefore 1 molecule weights $2616900 / 6 \times 10^{23} = 4.36 \times 10^{-18}$ gm
- If the stock plasmid has an OD reading at 260 of 0.872, then the DNA concentration is $0.872 \times 50 \text{ ng}/\mu\text{l} = 43.6 \text{ ng}/\mu\text{l}$
- $43.6 \text{ ng} = 43.6 \times 10^{-9} \text{ gm}$, so
- $(43.6 \times 10^{-9} \text{ gm}) / (4.36 \times 10^{-18} \text{ gm}) = 1.0 \times 10^{10}$ molecules/ μ l

The number of molecules was calculated per μ l of plasmid solution. The standard plasmid was then diluted in dH₂O to give a solution with a calculated concentration of 1×10^8 molecules. Following this, a series of 10

fold dilutions was set up to give a set of standards with a calculated concentration of 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 molecules and stored frozen at -20°C until use.

Detection and Quantification of TREC

To quantify TREC a series of standard dilutions of plasmid containing the signal-joint breakpoint was used following Douek and cols protocol (Douek, 2000). Each sample was run in triplicate along with the dilution series of TREC plasmid and not template control (NTC). Data was normalised based on the percentage of CD3^+ T cells in total PBMCs and calculated as number of TREC per 1.5×10^5 CD3^+ cells. TREC amplification is shown in Figure 2.4.

Quantification of FOXP3

Total RNA was purified from PBMC's with RNeasy Mini Kit following manufacturer's protocol (QIAGEN Inc, Valencia, California). Complementary DNA (cDNA) was synthesised using M-MLV Reverse Transcriptase and Random Hexamers (Promega Corporation, Madison Wisconsin, USA) according to manufacture's instructions as described in section 2.2.1. 1:10 dilution of cDNA was used to perform quantitative PCR.

To allow quantification of FOXP3 molecules by Real-Time PCR, it was essential to prepare different set of standards, as described previously.

PCR was performed using FOXP3 primers and TaqMan® probes designed by Emanuel Zorn (verbal communication), the sequences of which are shown in

table 2.6. For internal control, Human Tfr (CD71) assays-on-demand from TaqMan® (Applied Biosystems, USA) was used.

To quantify the expression of FOXP3 and CD71 series of standard dilutions of plasmid containing FOXP3 and CD71 were used. Each sample was run in triplicate along with the dilution series of the plasmids and not template control (NTC).

Normalisation of FOXP3 expression was made by dividing the total expression of FOXP3 by the total expression of CD71 and the multiply by 100.

$$\text{Normalisation of FOXP3} = \frac{(\text{Total expression of FOXP3}) \times 100}{\text{Total expression of CD71}}$$

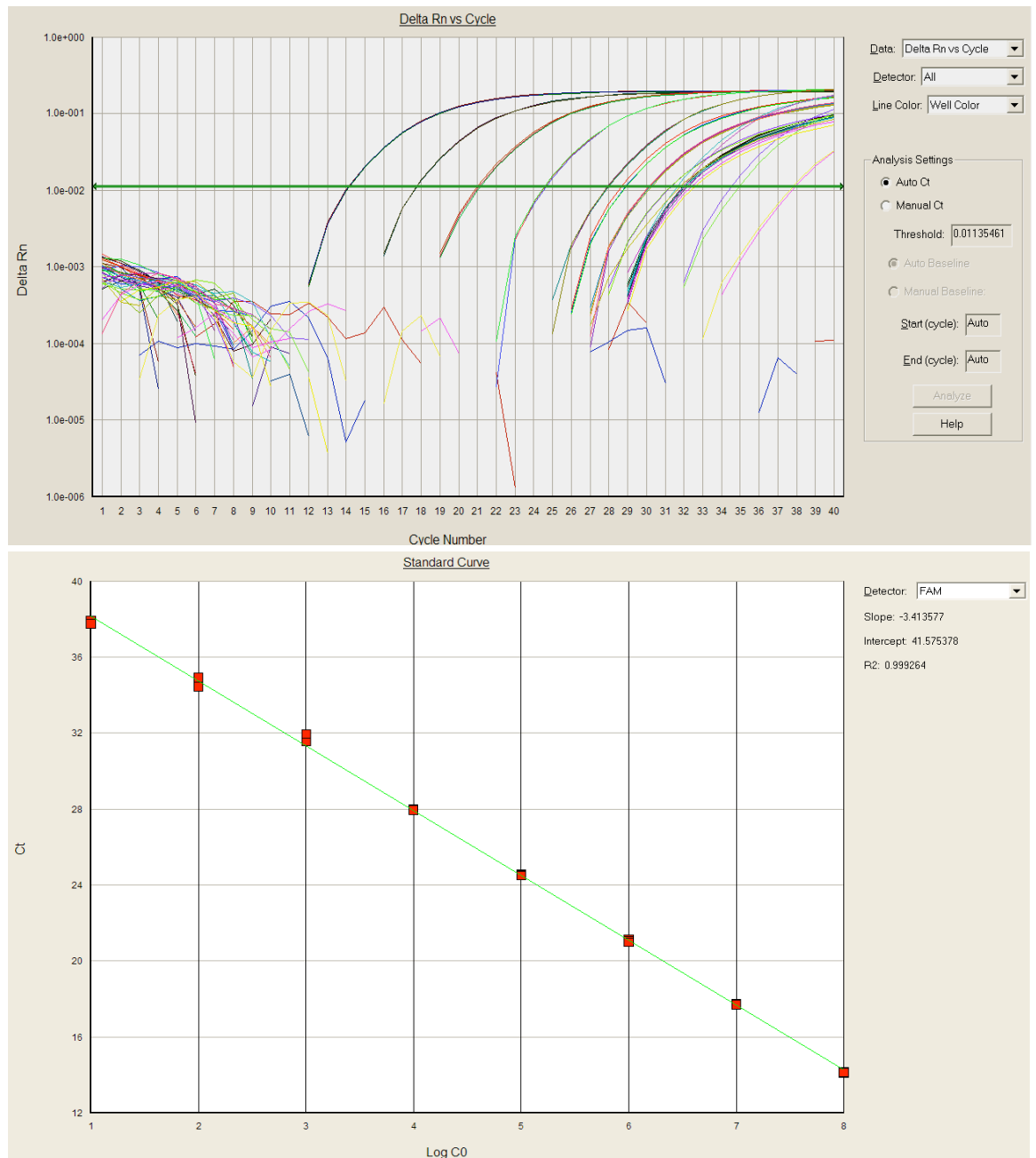
Table 2.2. Primers and probes used for qPCR.

Probe for TREC quantification was labelled with FAM (reporter dye) and TAMRA (quencher dye). The FOXP3 probe was labelled with VIC (reporter dye) and TAMRA (quencher dye)

Gene	5' primer	3' primer	Probe
TREC	cacatcccttcaacctgct	Gccagctgcagggttagg	FAM-acacctctggttttgtaaagggtcccact-TAMRA
FOXP3	gcctcctcttctccttgaa	Gtgaggctgatcatggct	VIC-ccatcgcagctgcagctctcaa-TAMRA

Figure 2.4. The quantification of TREC was by real time PCR using an ABI 7500 machine.

The upper figure shows the exponential amplification of samples. For the absolute quantification it was required to have a standard curve prepared from the cloned TREC and FOXP3, which is shown on the lower figure in a log scale, showing an r^2 of 0.9999.



Blue/white colony selection

The PCR2.1 vector (Invitrogen) enables screening of successful cloning reactions through the colour of the bacterial colonies. The molecular mechanism for blue white screening is based on the Lac operon and is briefly explained here. PCR2.1 contains the β -galactosidase gene (Lac Z) within the multiple cloning site (MCS). Cloning in pCR2.1 was performed with isopropyl- β -D-thiogalactoside (IPTG) present in the medium, which functions as the inducer of the Lac operon in the absence of lactose. If no cloned insert was present in the pCR2.1 vector (which would interrupt the Lac Z gene) IPTG triggered expression of β -galactosidase by induction from the T7 promoter. The enzyme then processed 5-bromo-4-chloro-3-indolyl- β -galactoside (X-gal) within the medium (a colourless modified galactose sugar that is metabolized by β -galactosidase). The hydrolysis of colourless X-gal by the β -galactosidase caused the characteristic blue colour indicating that the colonies contained unligated vector. White colonies indicated insertion of HLA sequence and loss of the cells' ability to hydrolyse the marker. An example of colonies grown under conditions for blue/white screening.

Blue/white colony screening

Bacterial colonies with no vector would appear blue. This is because β -galactosidase is a homo-tetramer with each monomer made up of one lac Z- α and one lac Z- ω protein. If only one of the requisite proteins is expressed in the resulting cell, no functional enzyme will be formed. Thus, if a strain of *E.*

coli without lac Z- α in its genome was transformed with a plasmid containing the missing gene fragment, transformed cells did produce β -galactosidase while untransformed cells did not because they were only able to produce the omega half of the monomer. In addition growth of untransformed cells was suppressed by the presence of Kanamycin in the growth medium. A Kanamycin resistance gene on pCR2.1 allowed successfully transformed bacteria to survive despite the presence of the antibiotic.

Glycerol stocks

Glycerol stocks were prepared from bacterial hosts carrying engineered DNA vectors. Therefore single colonies were picked from plates, on which they had been grown on selective medium overnight and were subsequently grown with the appropriate selective liquid medium overnight. 0.5 ml of the overnight culture was then mixed with the same volume of glycerol in a screw cap microcentrifuge tube for storage at -80°C.

Polymerase chain reaction (PCR)

Conventional PCR was performed on a PTC-200 Peltier Thermal Cycler for TCR V β amplifications (primers are shown in Table 2.3 using the following conditions: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds with a final extension step at 72°C for 10 minutes. Therefore agarose gels or denaturing sequencing gels electrophoresis were used for detection of PCR product sizes at the final

phase of the PCR reaction.

Quantitative PCR was used for measuring the kinetics of PCR amplification during the early exponential phase of the reaction when (assuming 100% reaction efficiency) doubling PCR products accumulate at every reaction cycle. It was performed on 96 well optical reaction plates (ABI Prism) using the spectrofluorometric thermal cycler ABI 7300 Real Time PCR instrument (Applied Biosystems) as described below for calculation of the quantitatively related amount of starting material and sensitive detection of variability between samples.

Therefore either an intercalating dye (SYBR Green I) or fluorescent Taqman probes were used. SYBR Green dye bound to the minor groove of double stranded DNA (increasing its fluorescence over a hundred fold upon binding) and as more amplicons were produced during the PCR reaction, the fluorescent signal increased.

Whereas SYBR Green bound to any double stranded DNA molecule, the use of fluorescent probes allowed specific detection of amplification of pre-determined targets. TaqMan probes were designed to anneal to a specific sequence of the template between the forward and reverse primers used for amplification of the target (primers and probes used for TREC and FOXP3 amplification are shown in Table 2.2. They contained a high-energy dye (reporter) at the 5' end and a low-energy molecule (quencher) at the 3' end. Excitation of the intact probe resulted in the reporters dye's emission being suppressed by the quencher dye in close proximity due to fluorescence resonance energy transfer (FRET). During PCR, DNA *Taq* polymerase cleaved the probe that sits in the path of the enzyme that has 5' exonuclease activity.

This stopped the transfer of energy from the reporter to the quencher that were no longer in proximity thereby decreasing the quencher signal and increasing the reporter signal with the latter captured by the sequence detection instrument. Using fluorescent probes, an increase in the product targeted by the reporter probe at each PCR cycle causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter

Gel electrophoresis

Gel electrophoresis is used for the separation of deoxyribonucleic acid or ribonucleic acid using an electric current applied to a gel matrix. Nucleic acids migrate from negative to positive electrodes due to the naturally occurring negative charge carried by their sugar-phosphate backbone.

Gels were prepared by heating 100 ml of 0.5x Tris-Borate-EDTA (TBE) (Cambrex Bio Sciences) buffer containing 2 g (unless stated otherwise) of electrophoresis grade agarose (Gibco BRL) in a microwave oven for 2 minutes or until the agarose solution had began to boil. The mix was cooled down to approximately 50°C before adding 10 µl of 10 mg/ml ethidium bromide solution. The agarose solution was immediately poured into a sealed tray (Bio Rad) with a comb placed to form wells and allowed to set at room temperature. The comb was then removed. 5µl DNA solution of interest were mixed with 3 µl Bromophenol Blue loading dye [containing 0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue and 50% (v/v)

glycerol] before loading into gel wells and run with 1x TBE buffer at 100 Volts direct current in a SUB DNA cell (Bio Rad) for 45 minutes. Bromophenol blue carries a slight negative charge at moderate pH, therefore migrating in the same direction as DNA in the gel (at 2% agarose at the same rate as approximately 500 bp DNA fragments). Xylene cyanol is similarly used to indicate the progress of DNA separation during electrophoresis (at 2% agarose migrating at the same rate as approximately 4000 bp DNA fragments).

DNA separation was visualised and documented using a Gel Doc 1000 single wavelength (312 nm UV-B) mini-transilluminator (Bio-Rad Laboratories Ltd.) and a Charged Coupled Device (CCD) camera using a Wratten 2a filter and 2x magnification lens.

Figure 2.5. Electrophoresis gel of TCRV β PCR products.

The figure shows a gel electrophoresis of 5 μ l of TCRV β PCR products from a healthy control on an 1.5% agarose run at 100 Volts for 45 minutes. The molecular weight of the marker's standards (in bp) is shown on the left side the gel picture (Hyperladder IV from Bioline).



TCR β CDR3 size spectratyping

The size distribution of the T-Cell Receptor β -chain (TCR β) Complementary Determination Region 3 (CDR3) was determined by Reverse Transcript Polymerase Chain Reaction (RT-PCR). RNA was extracted from CD4⁺ and CD8⁺ sorted cells. Complementary DNA was prepared following the protocol described previously. Each TCR V β segment was amplified with one of the V β subfamily specific primer and C β primer recognizing both C β 1 and C β 2 regions. PCR was carried out in a final volume of 12.5ml containing 10X PCR buffer, 50X MgCl₂, sterile water, 1U of Red Hot DNA polymerase (ABgene, UK) 10mM dNTPs (Bioline, UK) and 10pmol of 1 to 26 different TCR V β primers combined with C β primer conjugated with to the fluorescent dye Cy5. The sequences of the TCR V β primers are described in Table 2.3.

The amplification was carried out in a PTC-200 Peltier Thermalcycler (MJ Research, Watertown, Massachusetts, USA). The PCR conditions were denaturation for 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, with a final extension step at 72°C for 10 min.

The PCR products were checked first on an 2% agarose gel stained with 5ml of 10mg/ml Ethidium Bromide, loading 5ml of the product with 1ml of 5X loading buffer (15% Ficoll, 0.25% Xylene Cyanol, 0.25% Bromophenol blue in distilled water). The gels were run at 120 volts for 40 minutes and visualised by ultra violet light using a transilluminator, the size of the product was inferred using a molecular weight marker, Hyperladder IV (Bioline, UK), which has products ranging between 100 to 1000 bp, each

product separated by 100bp.

The size distribution of each fluorescent PCR product was determined by electrophoresis on an automated DNA sequencer ALF-Express (Amersham Pharmacia biotech, UK). The PCR fragments were then denaturalised at 95°C for at least 3 min in the presence of formamide loading dye and then run for 8 hours. Data was collected and analysed by Allelocator Software (Amersham Pharmacia Biotech, UK). The normal TCR β CDR3 size distribution, representing polyclonal TCR distribution is characterised for a Gaussian distribution, containing 8 to 10 peaks for each V β subfamily. The appearance of dominant peaks suggests oligoclonal or clonal distribution. The absence of peaks suggests that there are not T-cells expressing the specific V β subfamily.

Spectratype complexity scoring

The complexity of V β subfamilies was determined by counting distinct peaks per subfamily. These were graded on a score of 0-8. Normal complexity is characterised by a Gaussian distribution that reflect the presence of polyclonal cDNA. A score of 0 was given if no subfamilies were present and 1 for a single peak, etc. The overall spectratype complexity score per sample was calculated by the total number of subfamilies. The complexity score in normal individuals range from 121 to 185 (Fallen, et al., 2003) (Wu, et al., 2000).

Table 2.3. Primers used for V β spectratyping.

The BCint primer was labelled with Cy5 dye.

V β	T _M (°C)	5' to 3'
1	59	GCACAACAGTTCCTGACTTGCAC
2	57	TCATCAACCATGCAAGCCTGACCT
3	59	GTCTCTAGAGAGAAGAAGGAGCGC
4	50	ACATATGAGAGTGGATTTGTCATT
5.1	54	ATACTTCAGTGAGACACAGAGAAAC
5.2-5.3	56	TTCCCTAACTATAGCTCTGAGCTG
6	58	AGGCCTGAGGGATCCGTCTC
7	58	CCTGAATGCCCAACAGCTCTC
8	62	CCATCATGCGGGACTGGAGTTGC
9	50	CCTAAATCTCCAGACAAAGCT
11	56	TCAACAGTCTCCAGAATAAGGACG
12	57	AAAGGAGAAGTCTCAGAT
13.1	48	CAAGGAGAAGTCCCAAT
13.2	53	GGTGAGGGTACAACCTGCC
14	54	GTCTCTCGAAAAGAGAAGAGGAAT
15	56	AGTGTCTCTCGACAGGCACAG
16	49	AAAGAGTCTAACACAGGATGAGT
17	46	CAGATAGTAAATGACTTTCAG
18	56	GATGAGTCAGGAATGCCAAAGGAA
20	56	ACGTCTGAGGTGCCCCAGAAT
21	53	ATTCACAGTTCGGTAAGGATCGA
22	57	GGGCAGAACTCGAGTTTCTGGTT
23	50	TTTATGAAAAGATGCAGAGCGAT
24	57	AAGTCAAGTCAGGCCCAAGCT
BC int	67	CACCCACGAGCTAGCTCCACGTGGTC

Luminex technology for cytokine quantification

The LabMAP technology (Luminex) combines the principle of a sandwich immunoassay with the fluorescent-bead-base technology allowing individual and multiplex analysis for up to 100 different analytes in a single microtiter well (Vignali, 2000).

Cytokine quantification of IL-1, IL-2, IL-4, IL-10, IL-12p70, IL-15, TNF- α and INF- γ

A human cytokine quantification by multiplex Luminex kit assay (8-plex Linco-plex cytokine kit by Linco Research Inc, St. Charles, MO, USA) was used according to manufacturer's instruction, as templates serum samples were used.

The Luminex serum assay was done in a 96-well microplate format. A standard curve ranging from 3.2pg/ml to 10,000pg/ml was generated by serial dilution of reconstituted standard provided with the kit. The filter plate was blocked by adding 200 μ l of assay buffer into each well and incubated on a shaker at room temperature. After 10 minutes the assay buffer was discarded by vacuum aspiration and 25 μ l of assay diluent was added to the wells designated for the samples, standards and quality controls also provided with the kit. Standards, quality controls and patient sera were pipetted at 25 μ l per well in duplicate to the appropriate wells and mixed with 25 μ l of antibody coated fluorescent beads. The microplate was incubated for 2 hours at room temperature on a shaker. Wells were then washed twice with washing buffer using a vacuum manifold. 25 μ l of

Biotinylated secondary antibody (Detection antibody) was added to the appropriate wells and incubated for 1 hour at room temperature in the dark with constant shaking. 25 μ l of Streptavidin-Phycoerythrin (PE) labelled antibodies were added to the plate and after 30 minutes incubation at room temperature wells were washed twice. Finally 100 μ l of sheath fluid was added to the wells and the plate read immediately on the Bio-Plex array reader at high and low RP1 targets. Analysis of experimental data was done using parametric curve fitting.

TGF β 1 cytokine quantification

TGF- β quantification was done using the human TGF β -1 LINCOplex kit (Linco Research Inc, St. Charles, MO, USA).

Quantification of TGF β 1 requires sample pre-treatment. Thus, quantification of TGF β 1 cannot be multiplexed with other analytes.

Serum samples were collected by centrifugation and frozen at -20°C until use.

Before the assay the samples were treated following manufacturer's instruction. The samples were diluted with Sample diluent provided with the kit adding 1 part of sample to 4 parts of Sample Diluent for 1:5 dilution followed by adding 1.0 μ l of 1.0N HCL to each 25 μ l of the diluted sample, samples need to be mixed by shaking at room temperature for 15 minutes and then incubated for 1 hour at room temperature. Finally the acid-treated samples need to be neutralised by adding 1.0N NaOH prior to addition to

sample well.

After sample treatment the assay protocol is the same as the one used for multiple cytokine quantification and for the analysis also a parametric curve was used. For TGF β 1 quantification a standard curve ranging from 40ng/ml to 0.064ng/ml was generated by serial dilution of reconstituted standard provided with the kit.

Statistical Analyses

Several statistical methods were use to analyse the data obtained.

Comparisons

Comparisons between patient and control groups were done using the U Mann-Whitney and the Kaleidagraph software (Synergy Software)

Analysis of Transplant Outcome

Statistical analysis of transplant outcomes and various parameters was carried out using the Kaleidagraph (Synergy Software), R (Free Software Foundation) and SPSS (SPSS Inc) software packages

Overall Survival

Overall survival (OS) was defined as the probability of survival, independent of state of the disease, at any point in time; patients alive at their last follow-up were censored, while only death was considered as an event. Disease free survival (DFS) was defined as the probability of being alive, disease-free at any time point; death, relapse, and graft failure were considered as events, while patients alive and disease free with donor engraftment at their last follow-up were censored. Both these probabilities were analysed by the Kaplan-Meier method, and comparisons between probabilities in different patient groups were performed using the log-rank test.

Relapse

Relapse was defined as the probability of having a relapse before time t ; death without experiencing a relapse was considered a competing event. On the contrary, transplantation-related mortality (TRM) was defined as the probability of dying without previous occurrence of a relapse, which was the competing event. Both these probabilities were estimated as cumulative incidence curves.

Graft versus Host Disease (GvHD)

Also, the probability of chronic GVHD was estimated as cumulative incidence. For chronic GvHD, only patients surviving in remission and with

donor engraftment for at least 100 days were evaluated. In this case, relapse or death was considered competing events, data being censored at time of last follow-up for patients who did not experience chronic GVHD.

All results were expressed as 5-year probability or 5-year cumulative incidence (%) and 95% confidence interval (95% CI).

A univariate analysis of DFS, Relapse, and TRM was performed for the whole study population considering the following variables: patient and donor age; stem cell source; recipient and donor HCMV serology; type of disease; disease status.

Chapter 3

Analysis of the T cell receptor repertoire and TCR reconstitution after HSCT

Introduction

T cell repertoire

In normal individuals the TCR repertoire is relatively stable, diverse and polyclonal, creating the ability to respond to a wide variety of antigens. The stability of the repertoire, given the tendency of the body to maintain homeostasis is not static and immobile, on the contrary, it is a dynamic process with a fluctuation in the numbers, diversity and functions within the T cell compartment during immune responses (Jameson, 2002) (Mahajan, et al., 2005). The shape of the TCR repertoire in the peripheral pool is influenced by intrinsic and extrinsic factors that together can affect the representation of some TCR families. The intrinsic factors that have been associated with TCR expression are: the germline preference of usage of particular V β genes; the HLA antigens that contribute to the negative and the positive selection of T-cells; TCR gene polymorphisms also have been implicated as influencing the TCR repertoire (Melenhorst, et al., 2008).

The extrinsic factors associated with modulation of functional TCR repertoire are: infections, the overall antigenic experience and the presence of superantigens (viral or bacterial proteins that bind to and expand T cells

carrying specific V β chains). Both intrinsic and extrinsic factors contribute to establish the TCR repertoire.

Clonal or oligoclonal expansions of T cells may occur in different situations, including malignant and non-malignant diseases and also during the immune reconstitution after an HSCT. Thus the analysis of TCR repertoire and the clonality (or otherwise) of responses is an area of great interest in immunology, and different methods have been developed to accomplish this task.

The analysis of T-cell repertoire

The peripheral pool of T cells expresses a highly diverse $\alpha\beta$ TCR repertoire. The theoretical diversity of the TCR $\alpha\beta$ repertoire was estimated to be 10^{15} unique receptors (Davis & Bjorkman, 1988). However, based on analysis made of β chain diversity, Arstila et al., (Arstila, et al., 1999) estimated that humans have repertoires of approximately 2.5×10^7 unique T-cell clonotypes. Whatever the true extent of T cell diversity, it is the case that the breadth of the T cell repertoire defines the extent of the individual's capacity to respond to antigens, while clonal expansion within that repertoire reflect the effects of prior exposure to antigens. To examine these questions, there are different methods to analyse TCR diversity and to identify clonal expansions.

Methods to analyse the TCR repertoire

Southern blot

Southern blotting has been considered as the gold standard method to establish T cell clonality in different situations including T cell leukaemia and in lymphomas (Droese, et al., 2004) (Yang, et al., 2005) (Langerak, et al., 2001) (Langerak, et al., 1999). It is highly reliable and in principle it can detect every clonal TCR rearrangement by using optimally positioned probes and appropriate restriction enzymes. Therefore, it is the method used to validate other methods for clonal detection. Despite that, the use of Southern blot in routine laboratories is limited due to its many disadvantages. Southern blotting is a laborious, expensive and time-consuming technique that requires large amounts of good quality DNA to get reliable results.

Phenotype: flow cytometric analysis

Flow cytometric analysis is a method that has many advantages over others for the analysis of the TCR repertoire; it is fast, relatively easy to perform, very reproducible between different laboratories, fairly inexpensive in comparison with other methods, allows the quantification of the different TCRV β receptors and allows the analysis of the TCR repertoire within T cell subsets. The analysis is based on TCR recognition by a panel of antibodies and can be done using multi-colour flow cytometry and fluorescent labelled monoclonal antibodies. (Pannetier, et al., 1993) (Hodges, et al., 2003) Up to now monoclonal antibodies cover more than the 80% of the whole TCRV β

repertoire. However, only a few antibodies have been developed that recognise the TCRVA gene products, therefore, the information given by flow cytometry indicates only the usage of each V β family. Also, each V β has many possibilities of V, D and J recombination such complexity cannot be effectively analysed by flow cytometry. The advantage of this technique is the direct visualization of the protein of interest on the cell surface allowing a direct determination of the percentages of V β -expressing cells in a given population (Lang, et al., 1997) (van den Beemd, et al., 2000).

However, due to the lack of more complex repertoire of monoclonal antibodies, a confirmation of clonal expansions or the clonality of a response is required using other molecular methods. Nevertheless, the method is an alternative approach for initial screening of clonality and once confirmed, it can be used for monitoring (Langerak, et al., 2001).

PCR methods

The development of the polymerase chain reaction (PCR) has replaced the use of the Southern blot, due to its many advantages, in particular that is faster and does not necessarily require high quality DNA. PCR analysis can be done from either DNA or RNA as a starting point. The most widely method used for the analysis of the expressed TCR repertoire utilises TCR β mRNA and requires a coupled reverse transcription PCR (RT-PCR) involving the amplification of all V β segments using primers complementary to all TCRV β genes and a constant region primer (C β). The disadvantage of this

method is that it only provides a semi-quantitative profile of the TCRV β usage (Langerak, et al., 2001) (Pannetier, et al., 1995). This method has been used successfully to assess clonality of the TCR repertoire. Other PCR methods using DNA and consensus primers can suffer from false negativity as result of improper annealing of the primers with gene segments. Other approaches have tried to use nested PCR, but have been proved to experience contamination.

TCR PCR fragments can be detected in different ways in order to discriminate between clonal and polyclonal expansions. The methods used for discrimination include: fluorescent gene scanning (Immunoscope/Spectratyping); heteroduplex analysis; single strand conformation polymorphism (SSCP); denaturing gradient gel electrophoresis (DGGE); cloning and sequencing.

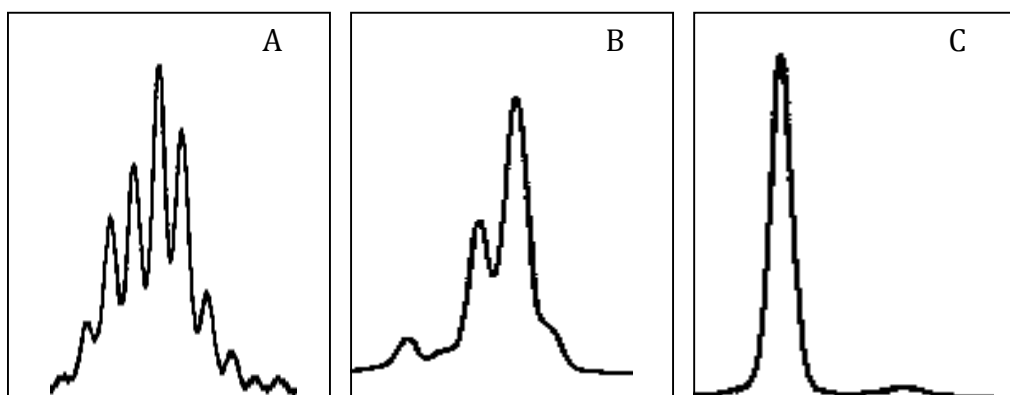
Fluorescent gene scanning

Fluorescent gene scanning consists in the amplification of specific TCR genes using fluorescently labelled primers the resulting PCR products are run on a gene sequencer and analysed through suitable software, in which the yield of transcripts with different CDR3 lengths can be estimated (GeneScan software, Applied Biosystems or Alleleloc software by ALF-Express, Amersham Pharmacia). The technique involves the amplification of cDNA using C and V specific primers or genomic DNA between V and J specific

primers, providing an estimation of specific expansion, complexity and stability of a particular V gene family relative to others as well as the different TCR clones based on their CDR3 length. The study of CDR3 length or CDR3 spectratyping has been proven to be a powerful tool to analyse T cell repertoire under normal or pathological conditions, providing a profile of the T cell repertoire and detecting oligoclonal expansion of T cell bearing a particular TCR phenotype. Polyclonal populations show a normal bell-shaped Gaussian distribution that can be seen as a series of peaks (between 8 to 10 peaks per family). Oligoclonal expansion can be seen as distinct peaks, and monoclonal distribution is observed as a single peak in the family being analysed as can be observed in Figure 3.1. (Assaf, et al., 2000) (Even, et al., 1995) (Gorski, et al., 1994) (Maślanka, et al., 1995). However, because the information given by fluorescent gene scanning (spectratyping) is semi-quantitative its utility is limited.

Figure 3.1. TCR diversity analysis by CDR3 length V β spectratyping.

Panel A shows a normal bell-shaped Gaussian distribution representing polyclonal profile of the T cell repertoire. Panel B shows an example of an oligoclonal profile. Finally panel C shows a monoclonal expansion of a TCRV β family.



Heteroduplex analysis

This method also requires PCR amplification and relies on conformational changes in DNA heteroduplexes rates caused by denaturation and renaturation of mixtures of products that contain mismatches. In the case of TCR β chains, these mismatches between members of the same V β family occur at the VDJ junctional regions. The conformational differences between heteroduplexes can be read out as differential migration of renatured PCR products after by separation under non-denaturing conditions. True clonal expansion results in the predominant formation of homoduplexes, whereas polyclonal distributions result in the formation of heteroduplexes. The homoduplex represents PCR products with identical sequences with a uniform conformation that migrates rapidly through the gel, whilst heteroduplexes formed by the random association of different products with mis-matches run more slowly than the homoduplex. The heteroduplexes are detected as a smear of bands on a gel, while the homoduplex can be detected as a unique, clear band (Marguerie, et al., 1992) (Martinelli, et al., 1997) (Langerak, et al., 1997) (Sottini, et al., 1996). The advantages of heteroduplex analysis are; that it is not restricted to the CDR3 length as spectratyping, but also by the conformation and differential migration time that can distinguish differences in CDR3 region sequences that are hidden within a single peak in spectratyping. In an heteroduplex analysis, clones could be separated into different bands using a gel with higher resolution. However, in comparison with other techniques this is laborious and time consuming (Connelley, et al., 2008).

Single Stranded Conformation Polymorphism (SSCP)

The single strand conformation polymorphism (SSCP) method is based on non-denaturing gel electrophoresis and relies on mobility shifts of single stranded DNA, which reflects the different conformations adopted by strands differing in their nucleotide sequence (Orita, et al., 1989) (Offermans, et al., 1996) (Yamamoto, et al., 1996).

Denaturing gradient gel electrophoresis (DGGE)

This method analyses single base changes at the CDR3 region based on the migration of double stranded DNA through a polyacrylamide gel containing linearly increasing concentrations of a denaturing agent and it is based on sequence dependent melting behaviour (Fodde & Losekoot, 1994) (Myers, et al., 1987) (Offermans, et al., 1996) (Wood, et al., 1994).

Quantitative Real Time PCR for V β

The quantification of the V β -families can also be achieved by quantitative real-time PCR (qRT-PCR), and it has been proved to be able to detect a higher number of families with higher sensitivity and reproducibility. As for spectratyping, 24 primers for the V β regions were used together with a reverse primer for the constant region. However, here an internal fluorogenic oligonucleotide probe (that is labelled with both a fluorescent

reporter dye and a quencher dye) is also used to anneal to the conserved region of C β 1 and C β 2 close to the C β reverse primer. During PCR amplification the internal oligonucleotide probe is cleaved by the Taq DNA polymerase. After cleavage, the quenching of the reporter dye is lost and fluorescence can be detected and measured (Lim, et al., 2002) (Lang, et al., 1997).

The main limitations of the technique are the establishment of an adequate standard curve to permit quantification (Brewer & Ericson, 2005) (Lim, et al., 2002), and that it provides information on the V β family or subfamily only, rather than at the level of individual clones. Thus it is a technique best used in conjunction with, for example, one of the PCR-based methods which can distinguish clonal heterogeneity but not relative quantity.

Cloning and sequencing

In principle, cloning and sequencing provides the best method to assess clonality of the T cell receptor, allowing a detailed analysis of the CDR3 region, including the analysis of the individual V, D and J gene segments, and allowing the identification of non-template nucleotides. Therefore, not only the diversity but also the exact molecular composition of the TCR repertoire can be evaluated. Nevertheless, this technique has the disadvantages of being time-consuming and expensive (Naumov, et al., 1998) (Wilson, et al., 1993) (Packer & Muraro, 2007) (O'keefe, et al., 2004).

TCR β -based oligonucleotide microarray

This method uses 27 TCRV β probes and 13J β probes to analyse TCR repertoire diversity after a single PCR. Microarray hybridisation and analysis by appropriate software provides sequence-specific information to differentiate between monoclonal and polyclonal expansion and it offers both qualitative and quantitative data. This recently developed method seems to be both sensitive and fast, however more work needs to be done in order to validate its potential (Chen, et al., 2007).

Kinetics of immune reconstitution

In previous studies, it has been shown that Natural Killer cells (NK cells) are the first lymphoid cells to appear (Storek, et al., 2008). They recover to normal levels within 3 months after transplant followed by CD8⁺ T-cells that recover 2 to 3 months post-transplant. The last cells to reconstitute are B-cells, which take 3 to 5 months to recover and finally CD4⁺ T-cells which start to recover 6 months post-transplant (Muraro, et al., 2005). During the first 6 months, the peripheral T-cell pool is re-populated basically through peripheral expansion of cells expressing a memory phenotype (Dumont-Girard, et al., 1998). Therefore, initially most of the cells have a memory and effector phenotypes and they recover quite quickly, reaching normal levels within 6 months. This increase of memory and effector cells is more pronounced in the CD8⁺ compartment (Fallen, et al., 2003) while naïve CD8⁺ T-cells recovery is achieved between 6 to 12 months post-transplant. The CD4⁺ compartment regenerates very slowly reaching normal levels between

6 to 10 months post-transplant (Koehl, et al., 2007) (Kook, et al., 1996) (Kalwak, et al., 2002) (de Vries, et al., 2000) (Kim, et al., 2004). During the first 6 months there is a reduction of the naïve CD4⁺ T cells (CD45RA⁺/CD45RO⁻/CD27⁺), while memory and effector cells reconstitute faster as a result of homeostatic proliferation (Jameson, 2002) (Murali-Krishna & Ahmed, 2000). The levels of memory and effector CD4⁺ T cells start to decline after naïve CD4⁺ T cells start to increase 6 months after transplant through a thymic pathway. TREC levels used as an indicator of recent thymic emigrants (RTE); TREC are detected soon after transplant, however, they do not rise to normal levels until 6 months post-transplant.

The slow recovery of naïve T cells post-transplant particularly affects the CD4⁺ T-cell compartment. It has also been shown that naïve T-cell recovery is faster in younger patients than in older patients, as the thymus involutes with age, indicating the importance of the thymic function for a full T-cell recovery (Mackall, et al., 1995). Furthermore, thymic output might be compromised by damage to the thymus due to chemo- or radio-therapy and graft versus host disease (GvHD) thus decreasing the output of newly generated naïve T-cells, and affecting the rates of T-cell reconstitution after transplant.

T cell receptor (TCR) reconstitution after HSCT

Recovery of immune competence is a key process for the success of an HSCT. However, following HSCT a profound period of immune deficiency appears to comprise this. The recovery of immune competency not only implies the recovery of normal numbers of immunological cells, especially of T cells, but also the recovery of their diversity, and the capacity to respond to a wide range of antigens. Immune reconstitution after HSCT depends on several factors including age of the recipient, type of disease, whether or not the graft was T cell depleted, type of conditioning and the occurrence of post-transplant complications such as GvHD and infectious complications. However, the reconstitution of a wider TCR repertoire is not only dependent on the clinical factors or the type of conditioning, it requires a functional thymus.

Ageing and thymic involution

The thymus involutes with age, and this process become even more pronounced in puberty. At the age of 25, the thymus has decreased by 50% of its size at birth, the involution process continues, finding the thymus at 10% of its original size by the age of 50. Consequently a decrease of the thymic derived naïve T cells is observed. A homeostatic process, which consists of expansion of peripheral T cells, maintains the overall T cell pool. However, an increase in the ratio of memory to naïve T cells occurs resulting

in a higher susceptibility to new infections and a more limited TCR repertoire, with a reduced capacity to recognise novel antigens.

Some factors have been associated with the involution of the thymus:

- a) Reduction in growth hormone
- b) Reduction in insulin-like growth factor 1 (IGF-1)
- c) Increased oncostatin M
- d) Increased Stem Cell Factor
- e) Increased interleukin 6 (IL-6)
- f) Increased Leukaemia Inhibitory Factor
- g) Increased TGF- β production

Enhancing T cell reconstitution after HSCT

The pronounced immunodeficiency after HSCT, resulting from the delay in immune reconstitution, especially the delay in the recovery of CD4⁺ T cells, has led to an intense effort in recent years, to enhance post-transplant T-cell reconstitution, with the goal of improving overall survival after HSCT. Some strategies have proven to improve immune reconstitution and with a better reconstitution has come an increase in the overall survival. Some of the strategies that have been used successfully will be analysed here. The possible strategies to improve T-cell recovery include agents that protect thymus stroma and those approaches that stimulate proliferation and differentiation of T-cells (Wils & Cornelissen, 2005). The therapies that have been used to improve thymopoiesis until now include keratinocyte growth factor (KGF), growth hormone (GH), LHRH agonists, interleukin 7 (IL-7) and interleukin 15 (IL-15).

Growth Factors

Growth hormone

Growth hormone (GH) is produced by the pituitary gland but it has been demonstrated that it is also produced in the thymus by thymocytes and thymic epithelial cells (TEC) and acts indirectly on both thymocytes and TECs to stimulate proliferation (Savino, et al., 2003). In the bone marrow, it drives expansion of T and B progenitors (Knyszynski, et al., 1992) (Tian, et al., 1998) (Sumita, et al., 2005). An association between a decreased secretion of GH or its mediator Insulin-like growth factor 1 (IGF1) and thymic involution has been found (Taub & Longo, 2005). Administration of GH or IGF-1 has been associated with recovery of thymic function and with improved immune reconstitution after HSCT (Chen, et al., 2003) (Jardieu, et al., 1994). GH production decreases with age and it may play a role in thymic involution since treatment with GH reverses age-associated thymic involution in mice models.

Keratinocyte growth factor

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family and was discovered as a stimulator of epithelial proliferation and differentiation; it has been implicated in thymic development (Min, et al., 2002). In normal healthy thymus, KGF seems to have no effect, however, a key role as a cytoprotector has been found in situations of thymic damage. KGF is produced by mesenchymal cells (fibroblast) as an essential growth factor for TEC development, but is also produced by thymocytes. It has been

proposed that KGF protects epithelial cells from damage by chemo- and radiotherapy and from GvHD. The administration of KGF prior to transplantation has been shown to enhance thymopoiesis and peripheral T-cell reconstitution of naïve CD4 and CD8 T-cells with a broad TCR repertoire (Min, et al., 2002) (Alpdogan, et al., 2006) (Kelly, et al., 2008). However the mechanism of KGF protection is not fully understood.

Hormones

Sex steroid blockade

Thymic atrophy has been correlated with the onset of puberty and more directly when an augmentation of sex steroid hormones begins, with a negative effect on thymopoiesis. In animal models it has been demonstrated that sex steroid ablation by castration, either via surgical castration by removal of gonads or chemically castration by using luteinising hormone-releasing hormone analogue (LHRH-A), reverses the thymic atrophy with a full recovery on its function (Mackall & Gress, 1997) (Utsuyama & Hirokawa, 1989) (Flores, et al., 1999) (Bertho, et al., 1997) (Steinmann, et al., 1985). In clinical studies, it has been demonstrated that sex steroid ablation restores thymic function inducing effective thymopoiesis, promoting immune reconstitution with an increase in the production of B and T cell in autologous (Goldberg, et al., 2005) and allogeneic transplant (Goldberg, et al., 2007) (Sutherland, et al., 2005) (Roden, et al., 2004). Also it has been

demonstrated that blocking sex steroids prior to HSCT enhances T-cell recovery, especially naïve CD4⁺ T-cells with a broad repertoire (Sutherland, et al., 2008) (Kelly, et al., 2008).

Cytokines

An improvement in T-cell recovery has also been achieved by administration of cytokines that stimulate T-cell progenitors in thymus. The cytokines that have been implicated with this effect are IL-7 and IL-15.

Interleukin 7

Interleukin 7 (IL-7) is a cytokine produced by stromal cells of the bone marrow and thymus. It is an essential cytokine involved in T and B cell differentiation and proliferation. Pre-clinical studies have shown that the administration of IL-7 after HSCT can enhance immune reconstitution by increasing thymopoiesis, increasing the peripheral proliferation of recent thymic emigrants (RTEs) and by decreasing apoptosis of peripheral T cells, suggesting a potential for clinical use. In clinical studies the administration of recombinant human IL-7 (rhIL-7) enhances T cell reconstitution by different mechanisms: increasing the proliferation of de novo generated T cells and by decreasing T cell apoptosis in the periphery, inducing a sustained increase in peripheral CD4⁺ and CD8⁺ T cells causing a wide T cell repertoire diversity

(Parkman & Weinberg, 1997) (Alpdogan, et al., 2001) (Alpdogan, et al., 2003) (Sportès, et al., 2008).

Interleukin 15

Interleukin 15 (IL-15) is a member of the IL-2 family of cytokines. It stimulates the proliferation of previously activated T cells. In pre-clinical studies IL-15 has shown an increase in the peripheral expansion of CD8⁺ T cells, NK cells and NKT cells (Alpdogan, et al., 2005) (Katsanis, et al., 1996).

In my studies, I have chosen to focus on the effect of sex steroid blockade on thymic function and the output of new naïve T cells.

The beneficial effect on the immune system of sex steroid blockade by luteinizing hormone-releasing hormone analogues (LHRH-A) was demonstrated in patients treated for prostate cancer by an increase in the number of naïve CD4⁺ and CD8⁺ T cells in peripheral blood (Sutherland, et al., 2005). However, its effect on patients treated prior to HSC transplant has not previously been shown. A study was therefore carried out to evaluate the TCR repertoire reconstitution by spectratyping in patients treated with LHRH-A prior to transplantation in comparison to patients who weren't treated, the clinical details presented in Table 3.1.

Materials and Methods

This study was carried out in collaboration with the Department of Immunology of Monash University in Melbourne, Australia. However, all work presented here was carried out by myself at the Anthony Nolan Research Institute in London, UK. The complete study has been published in the *Clinical Cancer Research* journal (Sutherland, et al., 2008).

Patients, disease and treatment regimen

Patient age, sex, disease and pre-transplant regimen are presented in Table 3.1. The study consisted of a nonrandomised pilot study involving patients undergoing an HSCT at the Alfred Hospital and the Peter MacCallum Cancer Institute in Melbourne, Australia from 2001 to 2005. For patients who consented to treatment with the LHRH-A goserelin (Zoladex, AstraZeneca), an initial dose of 10.8mg for men and 3.6mg for women was administered 21 days prior to transplantation. For men, an additional 1-month dose (3.6mg) was given at day 63 post-transplant and women were given monthly injections of 3.6mg with a final dose at day 63 post-transplant. In all treated patients, castrate levels of sex steroids were obtained within the first month of administration. Castrate levels of estradiol were maintained for 6 months post-transplant, whereas testosterone castrate levels were not achieved until 4 months post-transplant and were subsequently maintained for 6 months

post-HSCT. The levels returned to normal after 12 months post-transplant. Patients were analysed pre-treatment and at 3, 6, 9 and 12 months post-transplant. Ethical approval was obtained from the Alfred Committee of Ethical Research on Humans (trial no. 01/006).

The types of transplants performed were both autologous and allogeneic transplants, and a total of four patient groups analysed. The autologous control group without LHRH-A treatment consisted of a total of 16 patients and the autologous LHRH-A group consisted of 7 patients. The Allogeneic control group consisted of 8 patients and the Allogeneic LHRH-A group of a total of 13 patients. Clinical details of the patient cohorts are shown in Table 3.1.

Table 3.1. Patient demographics

Table 3.1	Patient Group			
	Allogeneic control	Allogeneic LHRH-A	Autologous control	Autologous LHRH-A
Patient No.	8	13	16	7
Gender (Male/Female)	4M/4F	10M/3F	9M/7F	4M/3F
Age, years (Median±SE)	40.24±3.55	43±3.02	56.44±2.10	54.5±2.31
Diagnosis				
Acute Leukaemia	4	10	1	0
Chronic Leukaemia	1	2	0	0
Lymphoma	0	1	5	4
Multiple Myeloma	0	0	10	3
Aplastic anemia	3	0	0	0
Conditioning				
Cyclophosphamide/ATG	2	0	0	0
Cyclophosphamide/TBI	0	0	1	0
Cyclophosphamide/TBI/ATG	5	8	0	0
Fludarabine/Melphalan	0	3	0	0
Fludarabine/Melphalan/ATG	1	0	0	0
Melphalan	0	0	9	4
LACE	0	0	4	3
Missing	0	2	2	0
Remission Status at transplant				
Complete Response	1	3	6	2
Partial Response	3	3	8	2
Progressive Disease	2	7	0	1
Stable Disease	2	0	2	2
Relapse refractory	0	0	0	0
Relapse untested	0	0	0	0
Stem Cell Source				
Peripheral blood	8	13	16	7
Bone marrow	0	0	0	0
Donor				
HLA identical related	5	8	NA	NA
HLA identical unrelated	3	5	NA	NA

ATG: antithymocyte globulin; TBI: total body irradiation; LACE: Lomustine, etoposide, cytarabine and cyclophosphamide

Preparation of peripheral blood mononuclear cells

PBMC's were isolated as described previously in Material and Methods they were stored in liquid nitrogen until required.

Spectratyping

Spectratyping analysis was done on both CD4⁺ and CD8⁺ T cells pre-transplant, 6, 9 and 12 months post-transplant. The protocol is presented in chapter 2.

Briefly, PBMC's were separated into CD4⁺ and CD8⁺ T cell populations by EasySep magnetic separation with 95% purity. RNA was extracted from sorted cells and converted into cDNA. The cDNA obtained was used for PCR amplification and the PCR products run in a sequencing gel as mentioned previously. The complexity of the TCR repertoire was determined by counting distinct peaks per V β family on a score of 0 to 8. The overall complexity of TCR β subfamilies was calculated as the spectratype complexity score (SCS) as described by Wu, et al. The complexity score in normal individual ranges from 121 to 185 peaks (Fallen, et al. 2000) (Wu, et al., 2000)

Statistical analysis

The spectratyping scores were compared between the healthy control group and the four patient groups at the different time points using the Mann-Whitney U test with KaleidaGraph 4.03 software (Synergy Software).

Results

V β Spectratyping prior to transplantation

In samples prior to transplantation V β spectratyping was performed in all groups and compared with normal values; in 20 healthy controls (median age 27 \pm 3 years) the normal median \pm SD of the CD4⁺ T cell subset was 170 \pm 19.82 peaks (normal range 129 to 185 peaks), while the normal range of the CD8⁺ T cell subset was 121 to 180 peaks (median \pm SD 143 \pm 19.71 peaks).

In the autologous control group the median value of the V β diversity in the CD4⁺ T cell subset was 90 \pm 45.16 (range 28-149 peaks) while the median of the CD8⁺ T cell subset was even lower 48 \pm 34.58 peaks (range 13-136 peaks). Both groups had statistically significant lower number of peaks compared with healthy controls, $p=0.0003$ and $p<0.0001$ respectively, representing a very restricted T cell repertoire in both CD4 and CD8 compartments pre-transplantation, may be due to the type of disease or the previous treatments.

The median of the autologous LHRH-A treated patients was impossible to determine because only one patient sample was available to perform the analysis. Therefore, a meaningful comparison with healthy controls could not be performed. However, in an analysis with all pre-transplant samples of

the autologous cohort (either treated or control group), both CD4 and CD8 compartments were significantly less diverse than in healthy controls ($p=0.0004$ for CD4 and $p<0.0001$ for CD8), as presented in Figures 3.2 and 3.3.

The median \pm SD value for the CD4⁺ T cell subset in the allogeneic control group was 90.5 \pm 63.58 peaks (range 0-162 peaks). No significant difference was observed between the autologous and allogeneic control groups. However, a statistically significance was observed compared with the healthy control group, $p=0.002$.

The median of the CD8⁺ T cell subset in the allogeneic control group was 25 \pm 49.52 (range 0-152). Again, no difference was observed between the autologous and allogeneic control groups, while a statistically significant difference was observe between the allogeneic control group and the healthy controls, $p=0.001$.

In the LHRH-A treated allogeneic group, the median \pm SD of the CD4⁺ T cell subsets was 76 \pm 38.67 peaks (range 11-124 peaks). While the median value of the CD8⁺ T cell subset was 54 \pm 21.68 peaks (range 13-87 peaks). The diversity of both the CD4⁺ and CD8⁺ T cell subsets were statistically significantly lower than the healthy controls, $p=0.0002$ for each subset. When pre-transplant samples from patients for both allogeneic groups (control and LHRH-A treated) were put together and analysed the number of

peaks was statistically lower (less diverse) than healthy controls ($p < 0.0001$ and $p < 0.0001$, respectively), data presented in figures 3.2 and 3.3.

As for the autologous groups, the T cell repertoire in patients seemed to be limited in the allogeneic setting. Although both groups show very low diversity in both CD4 and CD8 compartments, it is clear by the data showed in Figure 3.3 that the skewed repertoire is more pronounced in the CD8 compartment in the allogeneic group.

Figure 3.2. Comparisons of CD4⁺ T cell V β repertoires between autologous and allogeneic groups and healthy donors.

Both, the autologous and the allogeneic groups had statistically significant lower CD4⁺ T cell V β repertoires pre-transplant than the healthy control group ($p = 0.0004$ and $p < 0.0001$ respectively).

Comparison of Spectratype complexity of CD4 Compartment

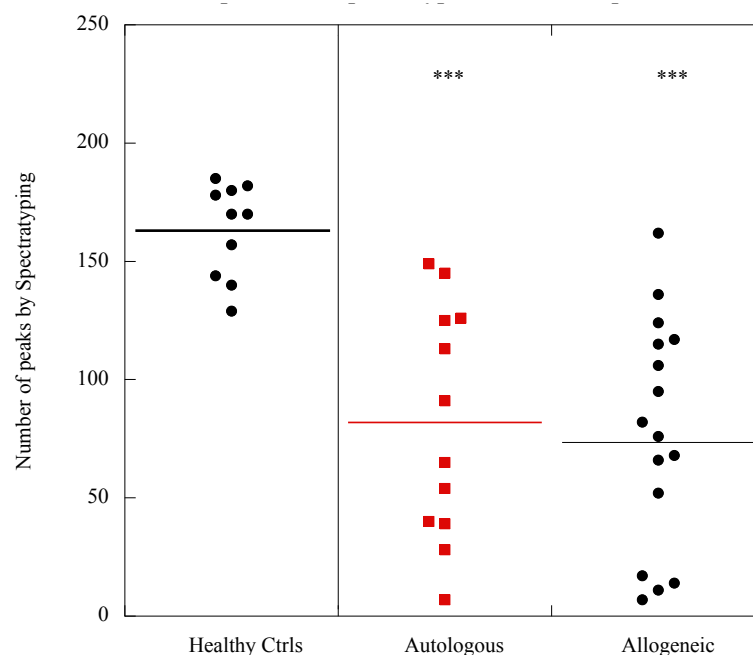
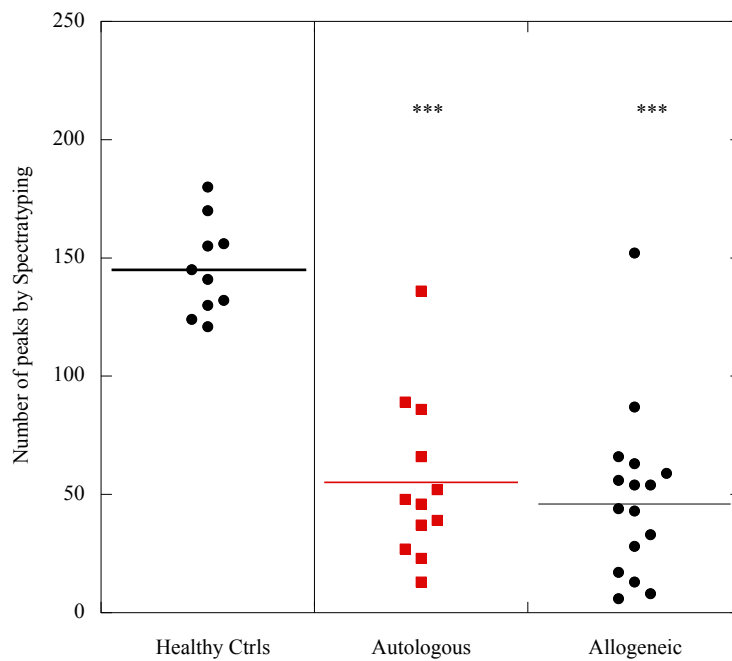


Figure 3.3. Comparisons of CD8⁺ T cell V β repertoires between autologous, and allogeneic groups and healthy donors.

As in the CD4⁺ T cell compartment, the pre-transplant repertoire of the CD8⁺ T cells in both autologous and allogeneic transplant showed a very skewed pattern in comparison with healthy controls ($p < 0.0001$ and $p < 0.0001$).

Comparison of Spectratype complexity of CD8 Compartment



Reconstitution pattern of the autologous transplant group at 6, 9 and 12 months post-transplant

The median \pm SD values of the TCRV β repertoires of the CD4⁺ T cell compartment of the autologous control group at 6 months post-transplant were of 95 \pm 38.06 peaks, at 9 months post-transplant of 114 \pm 58.44 peaks and at 12 months post-transplant of 143 \pm 50.36 peaks, as presented in Table 3.4. Although this shows an increasing trend, it was not statistically significant.

The median values of the CD8⁺ T cell compartment for this control group at 6 months were of 66 \pm 35.74 peaks, showing statistically significant improvement in comparison with the pre-transplant values ($p=0.009$), at 9 months of 87 \pm 32.06 peaks (it was not statistically different to the previous observation) and at 12 months of 82 \pm 57.12 peaks (no statistically significant difference) (Table 3.5).

The median values of the CD4⁺ T cell compartment in LHRH-A treated autologous transplant group were at 6 months of 127 \pm 40.50 peaks, at 9 months of 133 \pm 56.06 peaks and at 12 months of 130 \pm 28.49 peaks, not showing a statistically significant improvement in the pattern of reconstitution. While the median values of the CD8⁺ T cell compartment were at 6 months of 99 \pm 29 peaks, at 9 months of 93.5 \pm 3.69 peaks and at 12 months of 102 \pm 38.19 peaks, not statistically significant, data presented in Table 3.4.

As the comparison of the reconstitution rate between the control- versus the LHRH-A treated group in the autologous setting did not show any statistical significant difference (shown in Table 3.2 and Figures 3.4 and 3.5).

Table 3.2. Comparison of the reconstitution pattern of CD4⁺ and CD8⁺ T cells

Comparison of the reconstitution pattern of both CD4⁺ and CD8⁺ T cells at different time-point between the control group and the LHRH-A treated group in the autologous transplant setting. The patten of T cell reconstitution of both CD4 and CD8 compartment was not different between the control group and LHRH-A treated group.

T cell reconstitution in the autologous setting Controls vs LHRH-A treated group		
	CD4 ⁺ T cell subset <i>(p value)</i>	CD8 ⁺ T cell Subset <i>(p value)</i>
Pre-transplant	<i>p=NS</i>	<i>p=NS</i>
6 Months	<i>p=NS</i>	<i>p=NS</i>
9 Months	<i>p=NS</i>	<i>p=NS</i>
12 Months	<i>p=NS</i>	<i>p=NS</i>

Figure 3.4. *Reconstitution pattern of the CD4⁺ T cell compartment.*

Reconstitution pattern in the CD4⁺ T cell compartment between control group (solid black circles) and LHRH-A treated group (solid red squares) in an autologous setting. The two groups showed very similar pattern of reconstitution, and only few patients reach normal TCR repertoires 12 months post-transplant.

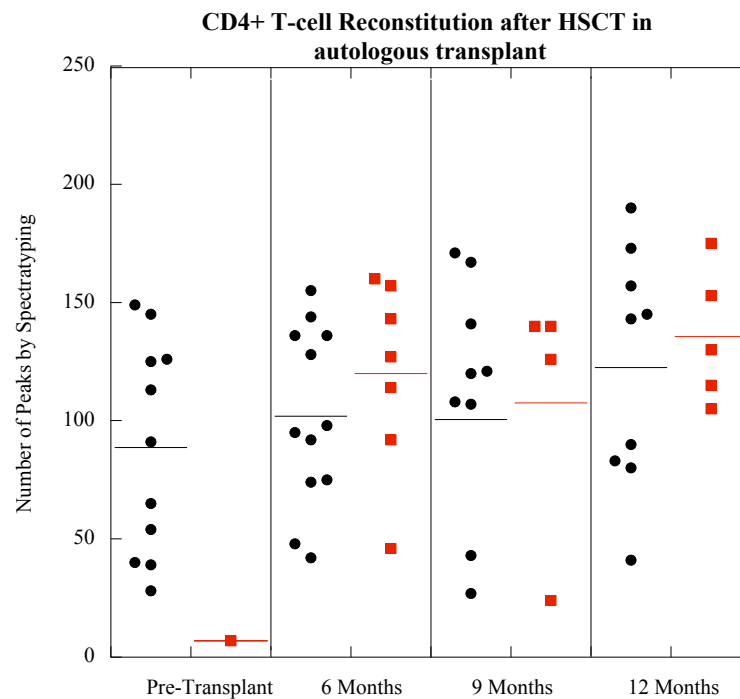
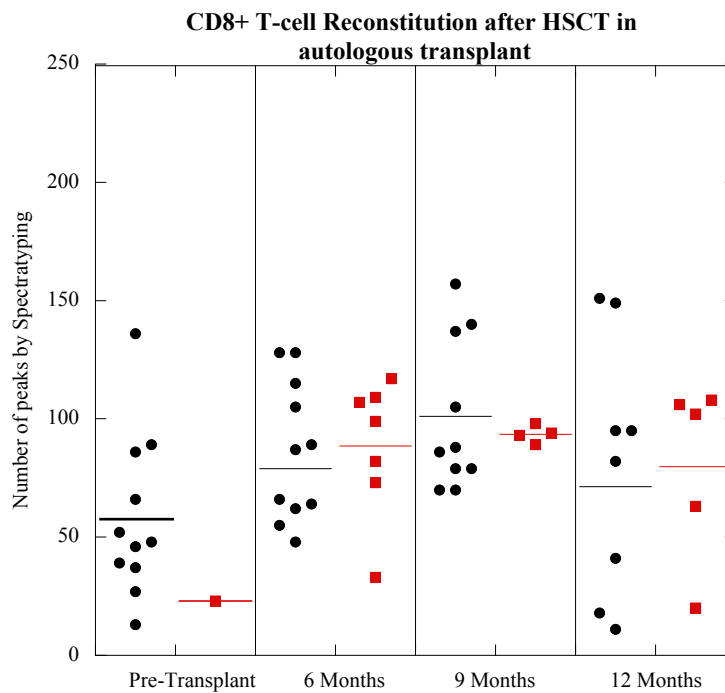


Figure 3.5. *Reconstitution pattern of the CD8⁺ T cell compartment.*

Reconstitution pattern of the CD8⁺ T cell compartment in an autologous control group (solid black circles) and an autologous LHRH-A treated group (solid red squares) who underwent an HSCT. No difference was found in the reconstitution pattern between the two groups. Both groups continued to show oligoclonal patterns 12 months post-transplant.



Reconstitution pattern in the allogeneic transplant groups

The allogeneic transplant control group showed the following pattern of TCRV β reconstitution in the CD4⁺ T cell compartment after HSCT; at 6 months the median number \pm SD of peaks was 76.5 \pm 19.05 peaks, at 9 months 128 \pm 39.73 peaks, (a significant improvement, p=0.05) and at 12 months 89 \pm 31.61 peaks as shown in Table 3.4.

The CD8⁺ T cell compartment reconstituted as follow, at 6 months a median \pm SD of 71.5 \pm 21.03 peaks, at 9 months 60 \pm 16.38 peaks and at 12 months 71 \pm 15.15 peaks, as presented in Table 3.5.

The median values of the CD4⁺ repertoires by V β spectratyping of the allogeneic LHRH-A treated group reconstituted as follows (data presented in Table 3.4), at 6 months 120 \pm 26.95 peaks, showing a significant improvement (p=0.009) in comparison to the pre-transplant repertoire, at 9 months of 130 \pm 38.98 peaks (an increase from 6 months post-transplant, but not statistically significant) and at 12 months of 179 \pm 22.38 peaks (a statistically significant increase from the previous time-point, p=0.04).

The CD8⁺ T cell compartment median repertoire values at 6 months was of 83 \pm 17.66 peaks (a statistically significant improvement in comparison to the pre-transplant repertoire, p=0.002), at 9 months 98.5 \pm 27.07 peaks (not statistically significant) and at 12 months of 113.5 \pm 56.34 peaks (no statistical significance) (Table 3.5).

In the allogeneic transplant setting, the reconstitution pattern showed great differences between the treated and control groups, in particular in the CD4⁺ T cell compartment. The LHRH-A treated group recovered diversity in the CD4 compartment more rapidly, as presented in Table 3.3 and in Figure 3.6. On the other hand, while the CD8 compartment also showed a trend towards faster recovery of diversity in the LHRH-A treated patients, this was not statistically significant, as presented in Table 3.3 and Figure 3.7.

A larger study may be required to demonstrate significant effects within the CD8⁺ compartment, or it may be that peripheral expansion plays a role in early CD8 T cell reconstitution and the effects of thymic reactivation are masked.

Table 3.3. TCRV β reconstitution of CD4 ⁺ and CD8 ⁺ T cells

TCRV β reconstitution of CD4⁺ and CD8⁺ T cells 6, 9 and 12 months post transplant in patients receiving an allogeneic transplant and treated with LHRH-A. The TCRV β of the CD4⁺ compartment reconstitute faster than the CD8⁺ compartment, and at 12 months patients treated with LHRH-A have a wider repertoire of T cell receptors, in particular of the CD4⁺ compartment.

T cell reconstitution in the allogeneic setting		
Controls vs LHRH-A treated group		
	CD4 ⁺ T cell subset (<i>p value</i>)	CD8 ⁺ T cell subset (<i>p value</i>)
Pre-transplant	<i>p=NS</i>	<i>P=NS</i>
6 Months	<i>p=0.0022**</i>	<i>P=0.03*</i>
9 Months	<i>p=NS</i>	<i>P=0.09</i>
12 Months	<i>p=0.008**</i>	<i>P=0.07</i>

Figure 3.6. Comparison of the reconstitution pattern of the CD4⁺ T cell compartment between allogeneic controls and donors.

Comparison of the reconstitution pattern of the CD4⁺ T cell compartment between allogeneic controls (solid black circles) and patients treated with LHRH-A (solid red squares) in an allogeneic setting. The LHRH-A treated group showed faster reconstitution of the CD4 compartment in comparison with the control group, in particular at 6 and 12 months post-transplant. Almost all patients who were treated with LHRH-A reach normal TCR values.

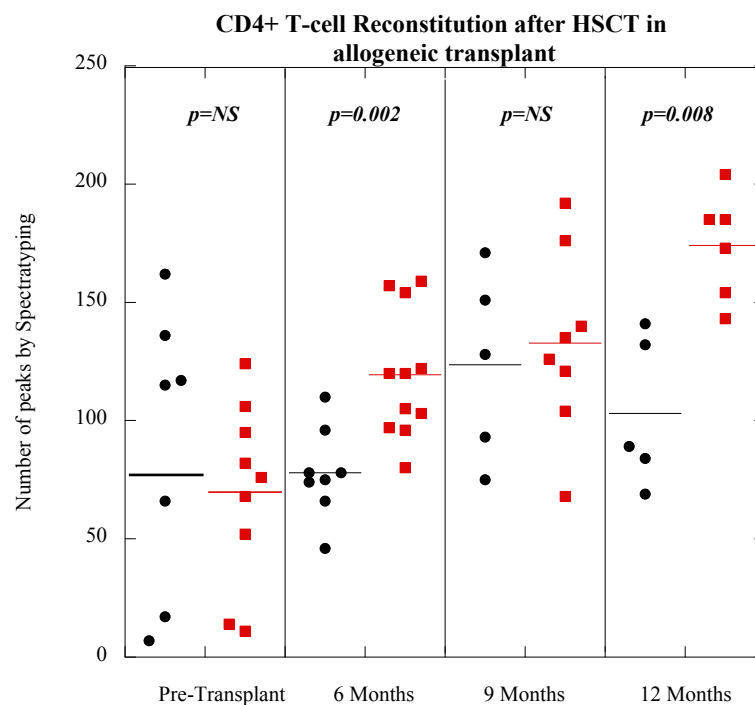


Figure 3.7. Reconstitution pattern of the CD8⁺ T cell compartment in controls and LHRH-A treated patients who underwent an allogeneic HSCT.

Reconstitution pattern of the CD8⁺ T cell compartment in controls and LHRH-A treated patients who underwent an allogeneic HSCT. A trend towards better reconstitution was found in patients receiving an LHRH-A treatment, however it is not statistically significant.

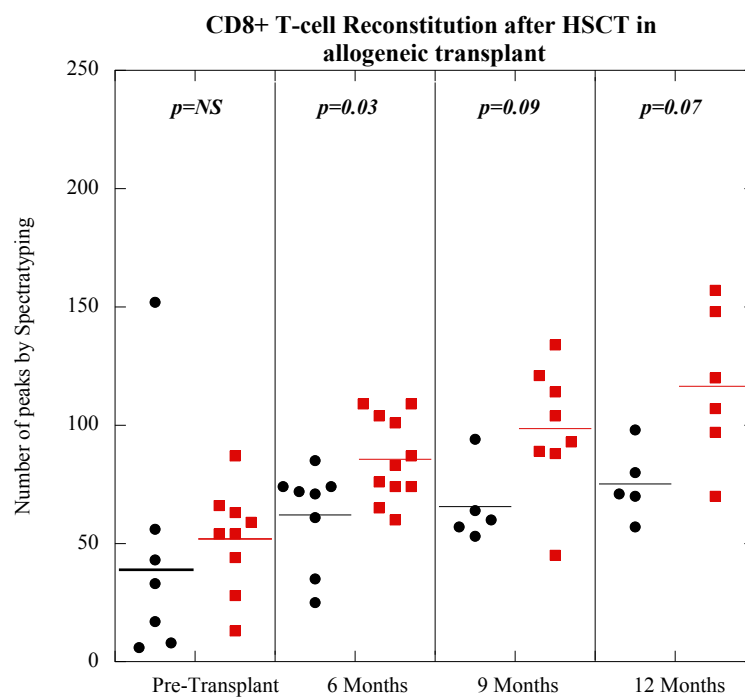


Table 3.4. Median±SD values of the total CD4⁺ T cell repertoires

Median±SD values of the total CD4⁺ T cell repertoires of all groups, and comparisons of improvements on the reconstitution between the different time-points. The greater improvement was seen in the allogeneic LHRH-A treated group from the pre-transplant period to 6 months post-transplant.

Spectratyping of CD4+ T cell compartment (median values)								
	Group	Pre-transplant	6months	p value	9months	p value	12months	p value
Allogeneic	Control	90.5±63.58	76.5±19.05	p=NS	128±39.73	p=0.056	89±31.61	P=NS
	LHRH-A	76±38.67	120±26.95	p=0.009	130±38.98	p=NS	179±22.38	P=0.04
Autologous	Control	90±45.16	95±38.06	p=NS	114±58.44	p=NS	143±50.36	P=NS
	LHRH-A	NA	127±40.50	p=NA	133±56.05	p=NS	130±28.49	P=NS

Table 3.5. Median±SD values of the total CD8⁺ T cell repertoires

Median±SD values of the total CD8⁺ T cell repertoires of all control and LHRH-A treated groups in an allogeneic and an autologous settings, and comparisons of improvements on the reconstitution between the different time-points. The greater improvement was seen in the allogeneic LHRH-A treated group from the pre-transplant period to 6 months post-transplant.

Spectratyping of CD8+ T cell compartment (median values)								
	Group	Pre-transplant	6months	p value	9months	p value	12months	p value
Allogeneic	Control	25±49.52	71.5±21.03	p=0.04	60±16.38	p=NS	71±15.15	p=NS
	LHRH-A	54±21.68	83±17.66	p=0.02	98.5±27.07	p=NS	113.5±56.34	p=NS
Autologous	Control	48±34.58	66±35.74	p=0.009	87±32.06	p=NS	82±57.12	p=NS
	LHRH-A	NA	99±29	p=NA	93.5±3.69	p=NS	102±38.19	p=NS

Figure 3.8 CD4⁺ reconstitution 12 months post-transplant in allogeneic settings in control and LHRH-A treated patients.

The LHRH-A treated patients showed significant better reconstitution rate, having a normal and wide repertoire of TCRV β at 12 months post-transplant, while the patient without treatment showed oligoclonal patterns at 12 months post-transplant.

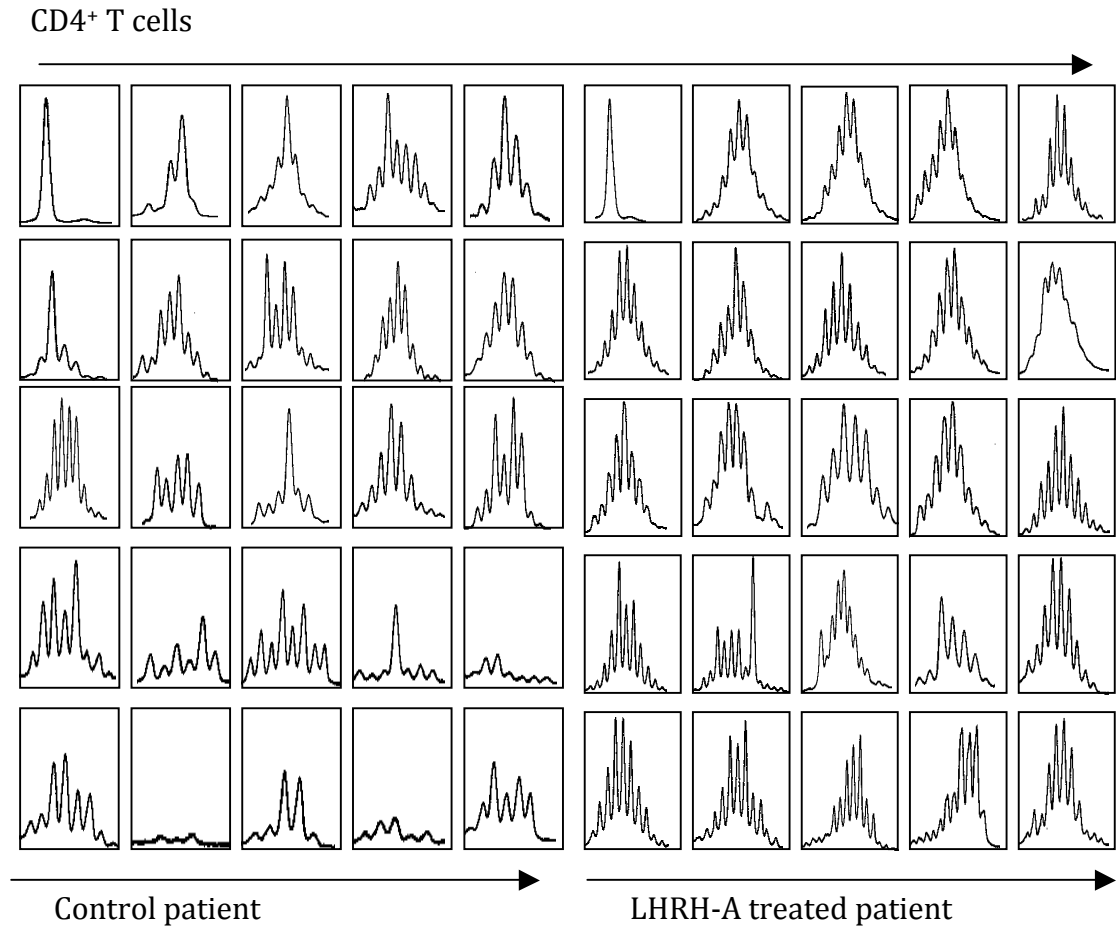
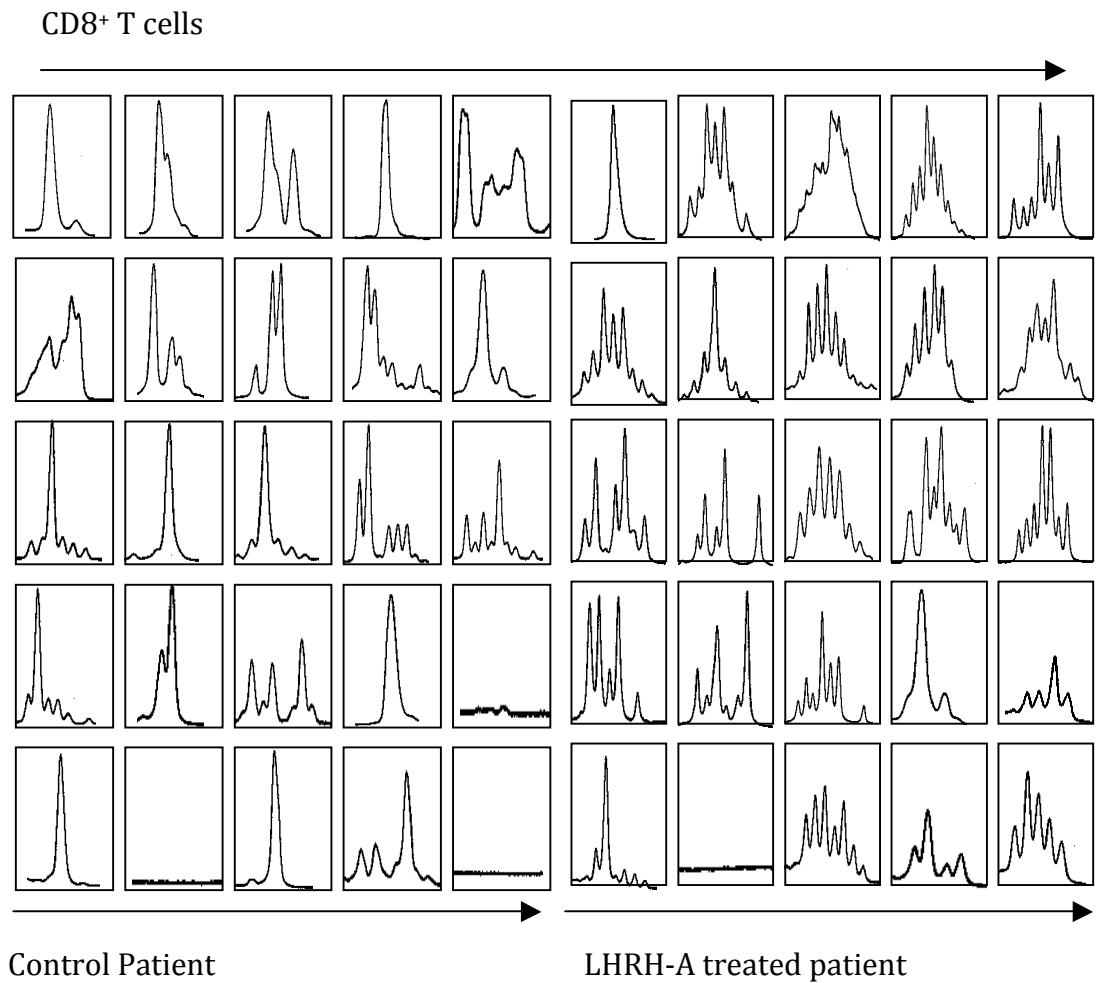


Figure 3.9. CD8⁺ reconstitution 12 months post-transplant in an allogeneic settings in control and LHRH-A treated patients.

The LHRH-A treated patients showed better reconstitution rate. However, at 12 months post-transplant the CD8⁺ T cell compartment still shows skewed repertoires with oligoclonal or monoclonal patterns in both groups.



Discussion

The ability to regenerate the peripheral T cell pool, in particular the CD4⁺ T cell pool, after an HSCT is a key element for the success of the transplant. It has been reported previously that adults can take up to two years to regenerate normal numbers of naïve CD4 T cells, mainly due to the atrophy of the thymus (Dumont-Girard, et al., 1998) (Fallen, et al., 2003). Several strategies have been used to try to improve reconstitution after transplant, and some of these have shown a beneficial effect and have improved survival after HSCT by decreasing the incidence of infectious complications and by improving anti-tumour responses. In this study the effect of LHRH-A treatment on the reconstitution of T cell diversity was analysed in both autologous and allogeneic transplant settings. This study was part of a prospective trial aimed to assess the impact on immune reconstitution of LHRH-A administration before and after an HSCT; my role in this study was to carry out the measurement of TCR V β diversity, other measurements were carried out by other members of the collaborative group.

As previously reported for this cohort (Sutherland, et al., 2008), LHRH-A treated patients showed a faster engraftment in both autologous and allogeneic settings. Moreover, a very striking finding was a significant increase of TREC in the CD4⁺ T cell compartment together with a faster reconstitution of the CD4⁺ naïve T cell subset in both autologous and allogeneic groups. However, it was particularly important in the allogeneic group, in which I have shown that the reconstitution of the TCRV β diversity in the CD4 compartment was also improved.

An interesting finding was that a restricted diversity was found in all patients pre-transplantation in comparison to healthy controls. The restricted repertoire may be due to the nature of the disease or the prior treatments received by the patients. However, T cell reconstitution shows a significant increase in the diversity as early as 6 months post-transplant. Moreover, the LHRH-A treated patients in particular in the allogeneic setting show a completely normal T cell repertoire 12 months post transplant. This is most striking in the CD4⁺ compartment in contrast with previous publications, where a slow recovery of the CD4 compartment has been observed.

After transplantation, the reconstitution of a broad T cell repertoire requires a thymic-dependent pathway. After 6 months post-transplant the autologous LHRH-A treated group showed a good reconstitution in both CD4⁺ and CD8⁺ T cells, however, it was not statistically significant when compared with the control group. At 9 and 12 months the LHRH-A groups showed also an improvement in the reconstitution of the diversity of CD4 and CD8 T cells, as the control group continued to show restricted repertoires. However, statistical differences were not found in the reconstitution pattern between the two groups. At 12 months both groups showed almost normal pattern of T cell receptor diversity.

In contrast, in the allogeneic group, the T cell diversity of the CD4 compartment reached almost normal level within 6 months, while the control group remained restricted. The reconstitution followed the same pattern, and at 12 months all the patients studied to whom LHRH-A was administered had normal TCR repertoires. The repertoire of the control

groups continued with improvements but they remained lower than the LHRH-A treated group.

The CD8 compartment also showed a trend towards better reconstitution in the LHRH-A treated group at the different time points, but the differences with the control group were not statistically significant.

The mechanisms of thymic recovery after LHRH-A administration have not yet been elucidated due to the complex mechanisms involved in both thymic atrophy and thymic regeneration. However, many studies have been carried out by different groups in order to understand this process. One mechanism suggests that sex steroid ablation affects the reorganization of the thymic stromal cell microenvironment, increasing the proliferation of thymic epithelial cells and inducing the production of IL-7 and GH by stromal cells that also contribute to thymic expansion (Ann Chidgey, Monash University, unpublished observations). It is important to considerate that a critical requirement for thymic regeneration is to have some remnant thymic function, because it has been demonstrated that young castrated mice regenerate larger thymuses than older mice (Sutherland, et al., 2005). In considering the mechanism of sex steroid ablation it has been observed that many thymic cells, including thymic epithelial cells (TEC), thymic dendritic cells, CD31⁺ thymic endothelial cells and thymocytes all have androgen receptors (AR). Unfortunately, this broad expression of AR makes it difficult to confine the mechanism of regeneration to only one cell type or one mechanism. However, AR deficient mice show thymic enlargement in comparison to non-deficient mice. The depletion of sex steroids may result in changes in proliferation of both thymocytes and TEC, or a reduction in the

release of pro-apoptotic molecules after stimulation of the androgen receptors. Also it has been observed that steroids kill thymocytes, and that blockading sex steroid production may allow more thymocytes to survive and become T cells. It is important to mention that the steroid ablation effect in mice is only temporary and wears off after a year (Fletcher, et al., 2009). However, even a transient increase in thymic output would be of benefit to the patient. Studies on immune reconstitution in younger patients show that normal levels of naïve CD4 and CD8 T cells are obtained some 12-15 months after transplant (Fallen, et al., 2003) (Paul Travers, unpublished data).

Other candidates for mediating thymic regeneration after sex steroid ablation treatment are: growth hormone (GH) and Insulin-like growth factor 1 (IGF-1) through the stimulation of TEC-produced growth factor thymulin (Taub & Longo, 2005); antiapoptotic molecules such as IL-7 and keratinocyte growth factor (KGF) which have shown to induce thymic regeneration in mice (Min, et al., 2002) (Pido-Lopez, et al., 2002); or reduction in proapoptotic molecules such as TGF- β . Due to the complex and largely unknown mechanisms that underlie thymic atrophy and regeneration, one can only speculate on the molecular mechanism that drives thymic regeneration after treatment with LHRH-A.

It is possible then that a steroid ablation effect persisting for only 1 year would allow the patients to establish normal levels of T cells; a subsequent decrease in the thymic output would simply return the immune system to the state it would normally be in individuals of that age.

Chapter 4

Prognostic analysis of pre-transplant peripheral T cell levels and TRECs in patients receiving an allo-HSCT

Introduction

Haematopoietic stem cell transplantation is a potentially curative treatment for many haematological, hereditary and immunological disorders. Even though more than 30,000 transplants are performed every year, the full range of factors that determine the successful outcome of the transplant are yet to be elucidated. The major factor implicated in the success of an HSCT is to find a fully compatible HLA-match donor. However, even in a 10/10 HLA-matched setting, complications have not been avoided completely and patients transplanted with fully matched donors still die after transplant. This suggests that there must be other factors affecting the outcome of the transplant.

Many groups (Gaziev, et al., 2002) (Savage, et al., 1997) (Enright, et al., 1996) have suggested different prognostic factors that may be correlated with transplant success, such as:

- the patient's clinical status prior to transplantation (a stable stage of the disease is preferred over other disease stages);
- patient age (younger patients have better outcomes);
- toxicity of the conditioning regimen,
- success of engraftment;

- the level and duration of immunosuppression;
- the development of GvHD; and
- the relapse of the primary tumour.

However, all the previous elements are not independent factors but represent a combination of pre-transplant and post-transplant variables, which could complicate the selection of the best possible factors to predict the transplant outcome. Therefore, I have focused on parameters that could be measured prior to the transplant to ask whether any of these are predictive factors for the outcome of the HSCT. The identification of such pre-transplant prognostic factors may help to identify patients who are at higher risks of transplant complications and poorer transplant outcome and who are more likely to benefit from alternative or intensified therapeutic approaches.

Graft characteristics and transplant outcome

The composition of the stem cell graft has been associated with the clinical outcome after transplantation, and some cells are in particular associated with the development of GvHD.

Vela-Ojeda et al. showed that the transfer of over 6×10^6 CD34⁺ cells/kg is associated with the onset of acute GvHD (Vela-Ojeda, et al., 2006). It was also shown by Vela-Ojeda et al. that better overall survival was associated with the infusion of fewer than 8×10^6 CD34⁺ cells/kg, along with fewer than 3×10^7 NK cells/kg and fewer than 1.5×10^7 NKT cells/kg. Likewise, Przepiorka and

cols found that a high dose of CD34⁺ cells was an independent predictive factor for the onset of aGvHD (Przepioka, et al., 1999). On the other hand, Gorin et al. found better outcome in patients with acute myeloid leukaemia who received higher doses of mononuclear cells (greater than $2.7 \times 10^8/\text{kg}$) (Gorin, et al., 2003).

Pre transplant immune status of the patient

The pre-transplant immune status of the patient has been recently used to evaluate the ability of the patient to reconstitute a fully functional immune system and thus minimise transplant complications. Thymic function and T cell subsets in patients prior to transplantation have been proven to be good indicators of immune function and have been correlated with the transplant outcomes.

Thymic function

As mentioned in previous chapters, thymic function plays an essential role in immune reconstitution. After transplantation there are two main routes to repopulate the immune system: a thymic independent pathway, which relies on the expansion of transplanted mature donor T cells, which is therefore only capable of generating a limited TCR repertoire, and a thymic dependent pathway, which involves the generation of new T cells from engrafted progenitor cells (haematopoietic stem cells and any committed lymphoid

progenitor cells present in the graft) and recapitulates T cell ontogeny in the thymus. This second pathway can therefore generate the broad TCR repertoire required for adequate immune responses. The thymic dependent pathway clearly requires a functional thymus, suggesting that pre-transplantation thymic activity may be analysed to predict engraftment and reconstitution, independently of the age of the recipient (Mackall, et al., 1993).

Thymic function and the thymic dependent pathway of reconstitution can be assessed by the analysis by flow cytometry of recent thymic emigrants (RTE), initially identified as naïve T cells that express the CD45RA⁺ marker. However, some disadvantages to this method have been reported. On one hand, it has been showed that some CD45RO⁺ memory T cells can re-express the CD45RA isoform, but remain as functional memory cells (Haynes, et al., 2000); and on the other hand, some CD45RA⁺ T cells can undergo extensive division while retaining the expression of CD45RA (Kimmig, et al., 2002), thus complicating the use of CD45RA for analysis of thymic function.

Thymic function can also be evaluated throughout the quantification of T cell receptor excision circles (TRECs) by quantitative real time PCR (qRT-PCR). As mentioned in the introductory chapter, TRECs are episomal circles of DNA that are formed from the deletion of the TCR δ region during the rearrangement of the TCR α locus. TRECs are not replicated during each cell division but on the contrary they become diluted out as T cells divide (Douek, et al., 1998).

It has been suggested by several groups that assessment of the status of the thymus prior to transplant can be used as a good indicator of recovery of

immune competency after the transplant and therefore could be used as predictor of the transplant outcome (Talvensaaari, et al., 2002) (Clave, et al., 2005).

Chen et al. (Chen, et al., 2007) showed that in paediatric patients, the quality of the thymic function before HSCT was correlated with the rate of T cell reconstitution. Those patients with a higher thymic output pre-transplant reconstituted their immune system faster than the patients with low pre-transplant thymic output. Also, they proposed that the rate of reconstitution influenced the outcome of the transplant, suggesting that the assessment of TREC levels as a marker of thymus function prior to transplant could be used as a good predictor for post-transplant reconstitution and consequently of transplant outcome.

Others have shown a direct impact of the pre-transplant levels of TRECs on transplant outcome. Talvensaaari et al. showed that low levels of TRECs pre-transplant correlated with higher incidence of both acute and chronic GvHD post-transplant in an allogeneic transplant setting (Talvensaaari, et al., 2001). Also, Svaldi et al. showed a correlation of high levels of TRECs pre-transplantation with better overall survival (OS) and better disease free survival (DFS) in patients with multiple myeloma (MM) who received an autologous transplant (Svaldi, et al., 2003). Interestingly, Clave et al. also showed a correlation between high TREC levels prior to transplantation with better overall survival (OS) and fewer infectious complications, which was confirmed in a multivariate analysis of the outcome after allogeneic transplant from HLA-identical sibling donors (Clave, et al., 2005) (Clave, 2006).

In conclusion, all previous work suggested that the assessment of the thymic function of the recipient prior to transplantation could be used as a predictive prognostic factor of HSCT outcome and also could be used to identify those patients requiring a closer monitoring.

I therefore undertook to assess the contribution of other pre-transplant immune parameters to the overall outcome of unrelated donor transplantation.

T cell subsets

Pre-transplant T cell status is an important component of the immunological status of the patient that can be assessed to evaluate its correlation with transplant outcome.

Parameters of immune status that can be evaluated are, for example, the distributions of different T cell subsets, such as: CD4 (helper) T cells in their different stages of differentiation (naïve, memory and effector), and of CD8 (cytotoxic) T cells also in their different stages of differentiation. In addition other important immune cell subsets can be defined and evaluated, including dendritic cells (DC), natural killer cells (NK) and natural killer T cells (NKT). The identification of these different immune cell subsets can be carried out by the expression of different cell surface markers, analysed by flow cytometry.

T cells can be identified by the expression of the CD3 marker. Two functional subsets of CD3⁺ T cells have been identified, the helper subset, which

expresses the CD4 marker on the cell surface and the cytotoxic subset, which expresses the CD8 surface marker, as mentioned previously in the description of T cell ontogeny in Chapter 1 (*T cell ontogeny*). Moreover, the stage of differentiation of either CD4⁺ or CD8⁺ T cells is important in their function and has been categorised into naïve, central memory, effector memory and terminal effector stages (Sallusto, et al., 1999). Cells at each of these stages express different homing and cell surface receptors, which can help to identify their phenotype.

In the introduction it was mentioned that many studies have analysed the cellular composition of the haematopoietic stem cell graft and have investigated its impact on transplant outcome. However, just a few have analysed the impact of the immune cell status of the recipients and its correlation with transplant outcome.

Rosinski et al. evaluated the impact of different T cell subsets on transplant outcome in patients receiving an autologous HSCT, showing the following findings:

1. All patients evaluated that were undergoing an HSCT had CD4⁺ T cell levels below those observed in healthy controls, showing a immunodeficiency within the population of patients studied.
2. Higher pre-transplant levels of CD4⁺ T cells correlated with better disease free survival (DFS) and better overall survival.
3. Higher pre-transplant levels of memory CD4⁺ T cells correlated with better DFS, which was confirmed by a multivariate analysis.

Arpinati et al. also analysed the levels of other immune cells, in particular antigen-presenting cells (APCs) such as dendritic cells (DCs), and monocytes and their correlation with the onset of GvHD (Arpinati, et al., 2007).

Dendritic cells are the most potent antigen-presenting cells. Different subtypes of DC have been described (Shortman & Liu, 2002), with a major division into two functional subsets: DC1 or myeloid (mDC) expressing the CD11c marker, which reside in the peripheral lymphoid organs, and DC2 or plasmacytoid (pDC) subsets expressing the CD123 marker, which are present in secondary lymphoid organs while their numbers in peripheral blood remain low.

Another important cell type are monocytes, which express the surface marker CD14 and can differentiate either into DC or macrophages depending on the cytokine environment (Geissman, et al., 2010). In both cases, they will function as antigen presenting cells (APC).

The development of acute GvHD is initiated through the activation of donor derived mature T cells by host APC, through either direct or indirect presentation of alloantigens. Thus these cells play a pivotal role in the onset of acute GvHD; the assessment of APC in the patient prior to transplant may be an important determinant of transplant outcomes.

The role of dendritic cells in HSCT has been evaluated recently, and it was observed that low DC levels after transplantation are found in patients with severe aGvHD (Vakkila, et al., 2005). Moreover, Reddy et al. showed that low DC levels after HSCT could predict the onset of GvHD, and could even predict relapse and death (Reddy, et al., 2004). These results appear contradictory,

since GvHD is usually considered to contribute to the graft-*versus*-leukaemia response. The resolution of the contradiction may be that DC are susceptible to allo-killing, and will be eliminated early in the course of GvHD, perhaps before the onset of other symptoms, hence low DC levels can be not really predictive but an early warning that GVHD taking place. The paradox is that GvHD would normally be associated with less relapse, but here it may be that low levels of DC – in this case probably of donor DC – fail to stimulate an effective anti-tumour response through a failure in crosspresentation (Rock & Shen, 2005)

An evaluation of the pre-transplant status of APC, which included mDC, pDC and monocytes was done by Arpinti, and a correlation between pre-transplant levels and transplant outcomes was investigated, in particular with respect to the onset of GvHD. However, no correlation was found between the levels of either mDC or pDC and the development of acute or chronic GvHD. Surprisingly, the levels of monocytes did influence the onset of GvHD and high levels of monocytes correlated with higher incidence of acute GvHD in allogeneic HSCT from related donors. However, that observation was not reproducible in HSCT from unrelated donors, which may be due to the different conditioning therapies received.

All these observations suggest that there is a relationship between the pre-transplant immune status of the recipient and the transplant outcome. This may be due to an impact on GvHD, on early infection control, or on immune mediated tumour control after HSCT. Therefore, in this study the aim was to analyse further the pre-transplant immune status of the recipients,

specifically by evaluating T cell subset levels and the level of TREC, and to correlate these with transplant outcomes.

Clearly there are other subsets of T cells - NKT cells and $\gamma\delta$ T cells - as well as NK cells themselves that might also play a role in the transplant outcome. Analysis of the reconstitution and role of these cells was the subject of a separate PhD project (Andrea Knight, UCL PhD Thesis, 2010). In the work presented here the focus is on the role of CD4 and CD8 T cells and the association that different T cell subsets might have with the outcome of transplantation.

Materials and Methods

Patients and controls

The study group consisted of 113 recipients who had received an HSCT from an unrelated donor identified through the Anthony Nolan Trust (ANT) donor registry. 64 donors from the registry with the same age range as the patients were analysed as a control group. Both recipients and donors were required to have 4-digit allele typing results at six HLA loci (HLA-A, -B, -C, -DRB1, -DQB1, -DPB1). Because the study was registry based, blood samples and clinical data were provided by the individual transplantation centres (TCs). Clinical data were collected on standard forms, which were collected from data managers at the individual TCs.

The transplants in the cohort took place between September 1996 and January 2003. A-broad ranges of diseases were indications for transplantation and the patient and donor pre-transplantation demographics can be seen in Table 4.1. The majority of the transplantation protocols included T-cell depletion (86.7%), in most cases using Alemtuzumab (Campath 1H) *in vivo* (81.4%). A roughly equal number of patients received transplants for each of the main diagnoses: 29 patients with AML, 25 patients with ALL, 26 patients with chronic myeloid leukaemia (CML), 28 patients with other malignant diseases and only 5 patients with non-malignant diseases (Table 4.1). Early disease stage was defined as first complete remission (CR1) in acute leukaemia and first chronic phase (1CP) in CML. Untreated patients (e.g., with MDS) were included in this definition. All patients with a disease beyond this were grouped as late-stage disease. The median age of the patients was 32.31±16.41 years with a broad range,

including infants. The donors had a median age of 34.24 ± 6.29 years, also with a relatively broad range.

Table 4.1 Patient demographics

	Patients <i>n=113 (%)</i>	Donors <i>n=68 (%)</i>
Age, years (Median\pmSD)	32.14 \pm 16.41	34.24 \pm 6.29
Gender		46 (67.6) M/22 (32.4)F
Sex Matched	67 (59.3)	
Male patient/Female donor	11 (9.7)	
Female patient/M donor	35 (31.0)	
CMV		
Low risk	71 (62.8)	
High risk	37 (32.8)	
Missing	4 (4.4)	
Diagnosis		
Chronic myeloid leukaemia (CML)	26 (23.0)	
Acute myeloid leukaemia (AML)	29 (25.7)	
Acute lymphoblastic leukaemia (ALL)	25 (22.1)	
Other malignant	28 (24.8)	
Non-malignant	5 (4.4)	
Stage of the disease		
Early	60 (53.1)	
Late	46 (40.7)	
Missing	7 (6.2)	
Conditioning Regimen		
Myeloablative	78 (69.0)	
RIC	30 (26.5)	
Missing	5 (4.4)	
Stem cell source		
Bone Marrow (BM)	95 (84.1)	
PBSC	18 (15.9)	
T cell depletion		
Campath in vivo	92 (81.4)	
No T cell depletion	9 (8.0)	
Missing	12 (10.6)	
Abbreviations: CMV: cytomegalovirus; PBSC: Peripheral blood stem cells; RIC: Reduce intensity conditioning		

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from peripheral blood samples by standard Ficoll density gradient centrifugation. Briefly, whole blood was diluted with an equal volume of RPMI164 medium and overlaid on 25 ml of Ficoll (density 1.077, Sigma, UK) in a 50ml Falcon Tube. Tubes were centrifuged at 4°C for 30 min at 700g, decelerating without brake. The white interphase containing nucleated cells (between the plasma and the Ficoll fractions) was removed with a pipette and transferred into a fresh tube, then washed twice with RPMI1640. At this point the number of cells in the sample was counted using a haemocytometer.

Aliquots of 1×10^6 PBMC were resuspended in freezing medium, consisting of 90% heat inactivated foetal calf serum and 10% DMSO, before storage at -196°C in liquid nitrogen vapour phase. For flow cytometric analysis, cells were thawed, washed and counted. Cell viability was analysed using Trypan blue exclusion.

Flow Cytometry

Blood was taken at the pre-conditioning period by venepuncture. Peripheral mononuclear cells (PBMC's) were isolated as described in Materials and Methods and T-cell subsets were analysed phenotypically by 4-color flow cytometry using a titrated panel of directly conjugated antibodies to CD3, CD4, CD8, CD27, CD45RO, CCR7 and CD45RA. All the antibody dilutions were titrated to give equivalent levels of staining and the working concentrations are shown in Table 4.2 (BD BioScience, San Jose, California, USA). Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PerCP and

allophycocyanin (APC) were used as fluorochromes. The acquisition was performed using a FACSCalibur flow cytometer and CellQuest software (BD Bioscience). Acquired data was analysed with Flow Jo software (TreeStar Inc, USA). The frequency of each subset was calculated as a percentage of positive cells relative to the total lymphocyte gate.

Table 4.2. Antibody dilutions for staining PBMC populations

Antibody	Manufacturer	Label	Final dilution
CD3	BD	APC	1:10
CD4	BD	PerCP	1:10
CD8	BD	PerCP	1:10
CD45RO	BD	PE	1:25
CD27	BD	FITC	1:10
CCR7	BD	PE	1:25
CD45RA	BD	FITC	1:25

T cell subset analysis

The combinations of antibodies used to define naïve, memory and effector

CD4⁺ and CD8⁺ T-cells are shown in Tables 4.3 and 4.4 respectively.

Table 4.3. Immunophenotypic analysis of CD4⁺ T cells

Immunophenotypic definitions of naïve, memory and effector CD4⁺ T cells

	CD4 ⁺ T cells			
	Naïve	Memory	Effector	Terminal Effector
CD45RA	+	-	-	+
CD45RO	-	+	+	-
CD27	+	+	-	-
CCR7	+	+	-	-

Table 4.4 Immunophenotypic analysis of CD8⁺ T cellsImmunophenotypic definitions of naive, memory and effector CD8⁺ T-cells

	CD8 ⁺ T cells			
	Naïve	Memory	Effector	Terminal Effector
CD45RA	+	-	+	+
CD45RO	-	-/int	int/-	-
CD27	+	+/int	low/-	-
CCR7	+	+	-	-

TREC quantification

TREC quantification was performed as described in Chapter 2, Detection and Quantification of TREC.

DNA isolation

DNA isolation was performed as described in Chapter 2, Preparation of cell lysates.

Quantitative Real Time-PCR

Real-Time PCR was carried out using an ABI/PRISM 7500 sequence detection system (Taqman®) and SDS software (Applied Biosystems, Foster City, CA) to quantify TREC in cell lysates from donor and patient samples DNA.

The PCR reactions were set up in 96-well optical reaction plates (Applied Biosystems, Foster City, CA, USA); sealed using Optical Adhesive Covers (Applied Biosystems, Foster City, CA, USA) and transferred to the ABI/PRISM 7500 Sequence Detector for analysis.

The PCR was carried out using Platinum Taq (Invitrogen) and ROX dye (Invitrogen) was employed in each reaction as a background reference to control for sample to sample variation in pipetting or in fluorescence detection.

Each 25 μ l reaction contained 5 μ l of cell lysates or 5 μ l of cDNA or 5 μ l of the specific standard (TREC standard), and the final concentration of 1U Platinum Taq (Invitrogen), 3.5mM MgCl₂, 0.25mM dNTPs, 12.5mM of each primer (Table 2.6), 3.75mM fluorescent labelled probe (Table 4.5), 2.5 μ l Platinum buffer (Invitrogen) and 0.5 μ l of ROX reference dye. One cycle of denaturation (95° for 5 minutes) was performed, followed by 40 cycles of amplification (95°C for 30 sec, 60°C).

Table 4.5. Primers and probes used for TREC quantification
--

Gene	5' primer	3' primer	Probe
TREC	cacatcccttcaacctgct	gccagctgcagggttagg	FAM- acacctctggttttgtaaagggtgccact- TAMRA

Detection and quantification of TREC by Real-Time PCR

To quantify TREC a standard consisting of a series of dilutions of a known quantity of plasmid containing the signal-joint junction was used. Each test sample was run in triplicate and each run included the dilution standard series of TREC plasmid and a no-template control (NTC). Data was normalised based on the percentage of CD3⁺ T cells in total PBMCs and calculated as number of TREC per 1.5x10⁵ CD3⁺ cells.

Statistical Methods

Comparison of the levels of each T-cell subset between patients and healthy controls were made by Student T test or Mann-Whitney U test using Kaleidagraph (Synergy Software version 4.03).

Associations between the pre-transplant counts for the naïve and memory lymphocyte subset with TREC level were assessed by the Spearman correlation test. All p values were two-tailed.

Disease free survival (DFS) was defined as the time from study entry to a documented progression or death without progression. Overall survival (OS) was defined as the time from study entry to death from any cause. Both survival times were analysed using the Kaplan-Meier method using SSPS Software. Univariate analysis of the different immunologic counts with DFS and OS were made using the two-sided log-rank test. Patients were segregated into two groups based on the mean count of each lymphocyte subset.

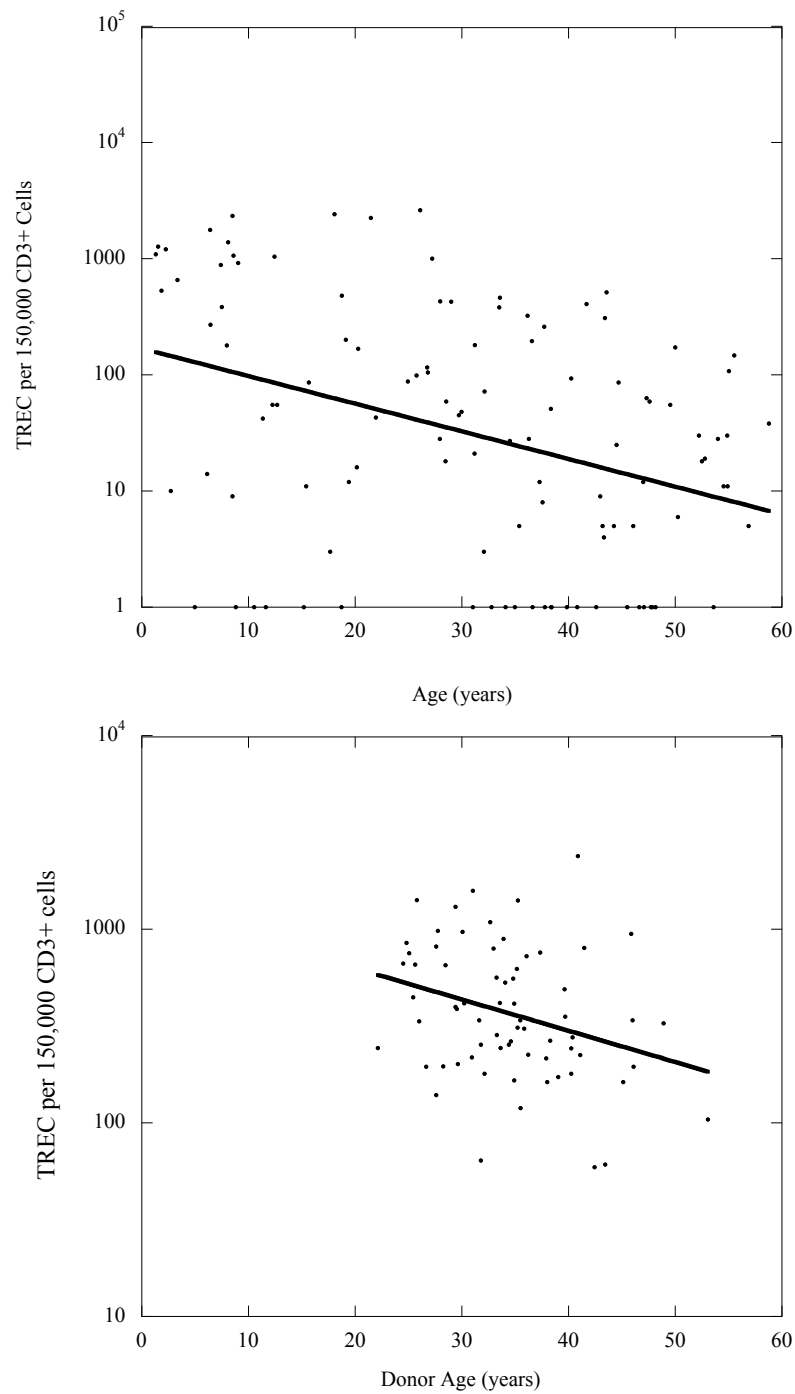
Results

TREC values pre-transplantation

The number of TREC copies per 150,000 CD3⁺ cells was measured by quantitative real-time PCR in preconditioning samples from 113 patients who underwent an HSCT from unrelated donors (UD) and in a group of 68 healthy donors. A statistical analysis was performed as shown in figure 4.1, and a significant correlation between TREC values prior to transplantation and age was found in the patient group ($r=-0.416$, $p<0.001$), despite the great variability in TREC values. However, in the control group the same effect could not be demonstrated ($r=-0.185$, $p=0.129$), although a slope can be clearly observed, the lack of correlation could be due the difference in donor and patient ages, where in the patients' group we can observe a wider age range, the donor group was more restricted in age, between 20 and 50.

Figure 4.1 Association between pre-transplantation TREC per 150,000 CD3⁺ cells and age.

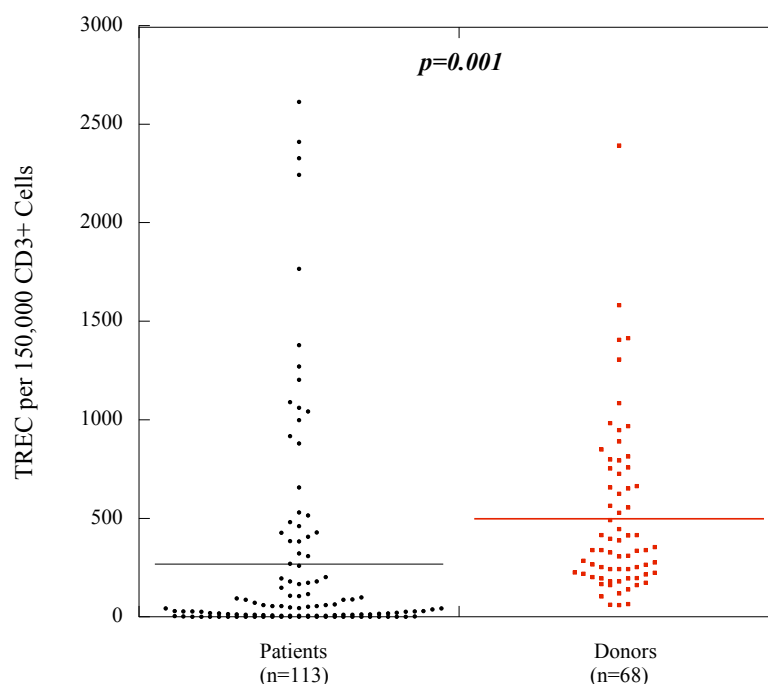
In the patient group (upper panel) an inverse correlation was seen between TREC level prior transplantation and their age ($p < 0.001$). However, in the donor group (lower panel), although the trend appeared similar, a significant association could not be found. The lack of significance may result from a sampling artefact in this cohort possibly due to the fact that all donors were over 20 years old.



The mean and standard error of the TREC values for the 62 healthy controls was 498.10 ± 51.37 TREC per 150,000 CD3⁺ cells (range 59-2391), statistically significantly higher ($p=0.001$) than the mean TREC per 150,000 CD3⁺ cells of 313.13 ± 67.87 (range 0-2613) in the patient group, as presented in Table 4.6. Patients with malignant diseases have statistically significant lower values of TREC per 150,000 CD3⁺ cells ($p=0.03$) than patients with non-malignant diseases. However, only 5 patients out of the 113 have non-malignant disease, and the majority of them were children, so it is difficult to draw significant conclusions about the impact of malignancy per se. It could be thought that the effect of malignancy may simply be that the presence of malignant cells in the blood sample reduces the effective concentration of TREC, leading to the lower levels being measured. However, the TREC levels are normalised to a cell count of 150,000 CD3⁺ cells, and so the presence of variable numbers of malignant cells between samples should not be a significant factor in the interpretation of the results.

Figure 4.2. Comparison of TREC levels pre-transplant between patients and healthy controls.

TREC levels were measured by quantitative Real-Time PCR. Patients pre-transplant have significantly lower levels of TRECs per 150,000 CD3⁺ cells compared with healthy donors.



TRECs and Transplant outcome

Based on the mean value of TREC pre-transplant, patients were separated into two different groups: the high TREC level group with TREC values above the mean value and the low TREC level group with TREC values below the mean, and a correlation with the transplant outcome was performed in each of these groups.

TREC values pre-transplantation did not affect overall survival, disease free survival or the incidence of relapse, in contrast with previously published

observations; the reasons why the results reported here differ from those reported earlier by others will be discussed in a later section. Furthermore, the values of TREC did not show an association with the onset of either acute or chronic GvHD. However, the incidence of extensive cGvHD was slightly higher in patients with lower values of TREC ($p=0.048$).

An analysis between the TREC values pre-transplant and the incidence of infections was also performed, but again no association was found.

T cell subsets pre-transplantation

The distribution of separate CD3⁺, CD4⁺ and CD8⁺ T cell subsets, and of functional T cell subsets (naïve, memory and effector phenotypes) were analysed by flow cytometry in a group of 113 patients and in 66 healthy controls, the results of statistical analyses are given in Table 4.6. Patients were undergoing an HSCT and samples were taken prior to the start of the conditioning regimen to evaluate their T cell status. The samples were collected as part of a long term project to identify immunogenetic markers for transplant outcome; sample collection began over 10 years ago and no absolute quantification of cell numbers in the peripheral blood was done at the time of collection, therefore, only percentages can be presented in this study.

As illustrated in Figure 4.3, which in a dot plot format shows the distribution of values for each sample, it is clear to see that the distribution of CD3⁺ cells in the patients is lower than in the controls. The mean \pm SE percentage of

CD3⁺ cells in the patient group was of 38.11±2.01% (range 0-89), which was significantly lower ($p<0.0001$) than in the control group (mean±SE of 62.79±1.71%, range 26-86). Within the CD3⁺ cells, the CD4⁺ T cell subset was also significantly lower in the patient group with a mean±SE value of 30.77±1.78% (range 0-60) compared with the control group which had a mean±SE value of 54.27±1.28% (range 36-77) CD4⁺ T cells ($p<0.0001$). In contrast, the CD8⁺ subset was not different between the patient and the donor groups. The patients showed a mean±SE of CD8⁺ T cells 33.18±2.14 (range 1-75), while the control group showed a mean±SE of 34.39±0.94 (range 21-52) CD8⁺ T cells. This observation argues that the decrease in the CD3⁺ and CD4⁺ T cell percentages are not simply due to an expansion of another cell population, since an expansion of a CD3⁻ population would be expected to drive down the percentages of both CD4⁺ and CD8⁺ T cells.

Looking in more detail at the CD4⁺ T cell subset, as shown in Figure 4.4, the lower levels in this subset were largely due to a significantly lower naïve (CD4⁺CD45RA⁺CD27⁺CD45RO⁻) CD4⁺ T-cell percentage in the patient group, with a mean±SE of 31.88±2.21% (range 0-90) compared to the control group mean of 40.76±1.56% (1-69), ($p=0.004$). The distribution of values of naïve CD4⁺ within the patient group does not appear to be unimodal, and while the upper part of the range in patients overlaps the upper range in the controls, the patient group appears to have a discrete population in which low levels of naïve CD4⁺ T cells are found. The CD4⁺ memory (CD4⁺CD45RO⁺CD27⁺) T cell subset was not significantly different between the patients and the donor groups (mean±SE of 45.77±1.94% versus 41.88±1.23%). However, the

effector (CD4⁺CD27⁻CD45RO⁺) CD4⁺ T cell subset was significantly higher ($p=0.02$) in the patient group (mean $17.94\pm 1.46\%$ versus 14.04 ± 0.73 respectively).

When analysed in more detail, the percentages of the naïve, memory and effector CD8 subpopulations were not different between patients and donors, as presented in Figure 4.5.

Table 4.6. Summary of statistics

Summary of statistics (mean±SE values) and comparison of TREC and T cells subsets between healthy donors and patients. Patients had significantly lower values of TRECs, and lower percentages of CD3⁺ cells and CD4⁺ cells.

	Patient (n=113) (Mean±SE)	Donors (n=68) (Mean±SE)	<i>p</i> value <i>T</i> test
TREC/150,000CD3 ⁺ cells	313.13±67.87	498.10±51.37	<i>p=0.001</i>
CD3 ⁺ cells (Percentage of Lymphocytes)	28.11±2.01	62.79±1.71	<i>p<0.0001</i>
CD4 ⁺ T cells (percentage of CD3 ⁺ T cells)	30.77±1.78	54.27±1.28	<i>p<0.0001</i>
CD8 ⁺ T cells (percentage of CD3 ⁺ T cells)	33.18±2.14	34.39±0.94	<i>p=NS</i>
CD4 Subsets			
Naïve (percentage of total CD4 ⁺ T cells)	31.88±2.21	40.76±1.56	<i>p=0.001</i>
Memory (percentage of total CD4 ⁺ T cells)	45.77±1.94	41.88±1.23	<i>p=NS</i>
Effector (percentage of total CD4 ⁺ T cells)	17.94±1.46	14.04±0.73	<i>p=0.02</i>
CD8 Subsets			
Naïve (percentage of total CD8 ⁺ T cells)	36.27±2.45	38.29±1.62	<i>p=NS</i>
Memory (percentage of total CD8 ⁺ T cells)	27.89±2.04	29.07±1.24	<i>p=NS</i>
Effector (percentage of total CD8 ⁺ T cells)	17.30±1.82	16.51±1.43	<i>p=NS</i>
NS: Not significant			

Figure 4.3. Comparison of pre-transplant percentages of CD3⁺, CD4⁺ and CD8⁺ cells between patients undergoing an HSCT and donors.

Comparison of pre-transplant percentages of CD3⁺, CD4⁺ and CD8⁺ cells between patients undergoing an HSCT (solid black squares) and donors (solid red circles). Mean values within the groups are indicated by the horizontal lines. Patients had significantly lower percentages of CD3⁺ ($p < 0.0001$) and CD4⁺ cells ($p < 0.0001$), while no difference was found in the CD8⁺ subset. Absolute quantification of CD3⁺, CD4⁺ and CD8⁺ T cells was not performed due the lack of material, as the collection of the samples started more than 10 years ago for immunogenetic analyses.

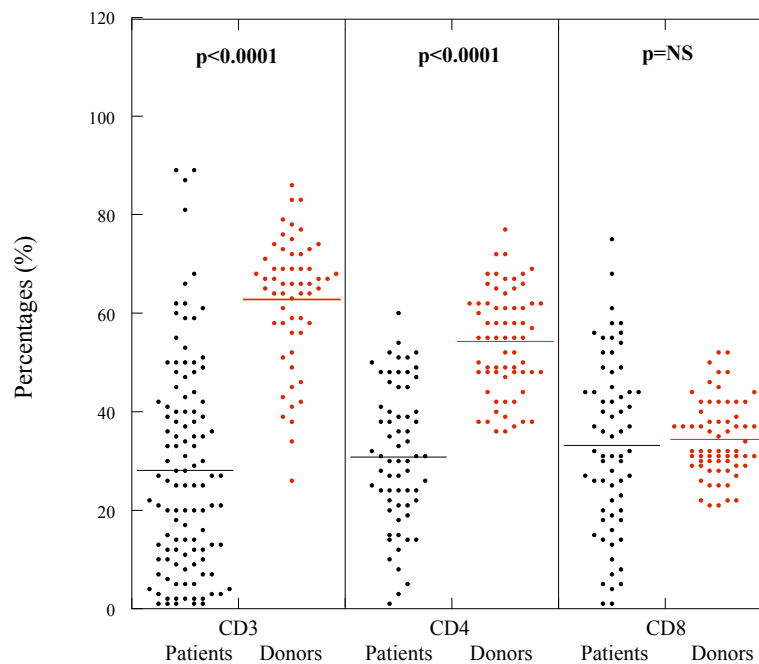


Figure 4.4. Comparison of the levels of subpopulations of the CD4⁺ compartment prior to transplantation.

Pre-transplant percentages of naïve CD4⁺ cells were significantly lower in the patient group (solid black squares) than the control group (solid red circles). The mean values in each group are indicated by the horizontal lines. The memory and the effector cells did not show any statistically significant difference between the two groups. Only percentages were analysed and no absolute quantification due the lack of material.

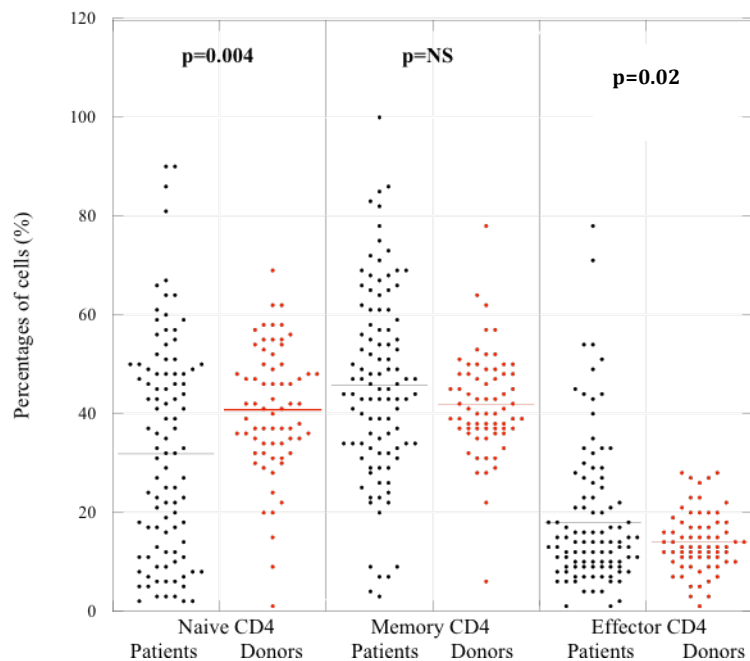
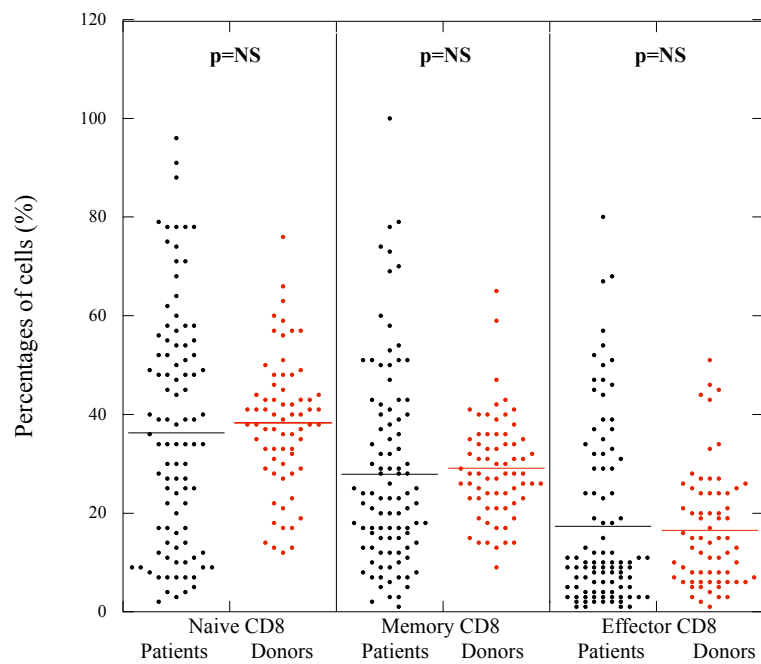


Figure 4.5. Comparison of subpopulations of the CD8⁺ compartment prior to transplantation.

The percentages of CD8⁺ T cell subsets prior to transplant showed no significant differences between patients receiving a HSCT and donors. Only percentages were analysed due the lack of material to perform absolute quantification.



Transplant outcome and T cell subsets

A correlation of the distribution of T cell subsets with the transplant outcome was performed. The mean follow-up of the patients in the cohort was 1206 days, which is sufficient to allow the identification of most significant post-transplant events.

Pre-transplant levels of CD3⁺ T cells in patients did not appear to have any relationship with the transplant outcomes. However, CD4⁺ T cell levels did show a correlation with transplant outcome. Two groups of patients were formed as before, either above or below the mean level of CD4⁺ T cells, and an analysis of the transplant outcome was performed. Patients with higher proportions of CD4⁺ T cells pre-transplant showed a trend towards lower relapse rates (log rank 0.063) than patients with lower proportions of CD4⁺ T cells. Overall survival, disease free survival (DFS) and the incidence of cGvHD were not correlated with the pre-transplant levels of CD4⁺ T cells. The same analysis was performed with the different CD4⁺ T cell subpopulations to identify the subset with the highest association with the transplant outcome.

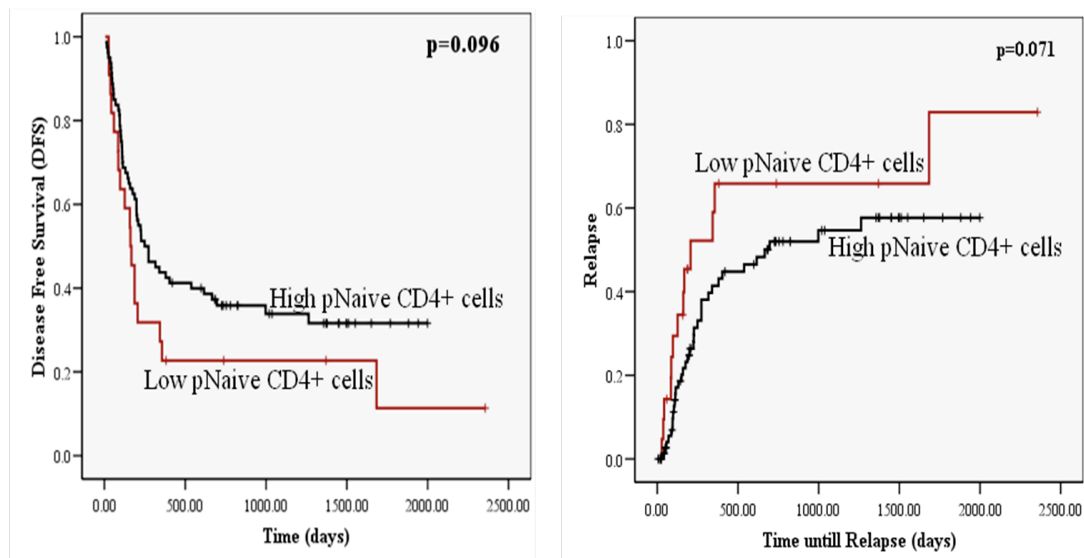
Patients with higher proportions of naïve CD4⁺ T-cells showed a trend towards improved DFS (log rank test, 0.096), conversely patients with low naïve CD4⁺ T-cells showed a trend towards increased risk of relapse (log rank test, 0.071); this data is presented in Figure 4.6 However, overall survival and the onset of cGvHD were not affected by the levels of naïve CD4⁺ T cells.

Neither the levels of memory nor effector CD4⁺ cells appear to have an impact on the transplant outcome. An analysis of donor CD4⁺ T cells

percentages and transplant outcome was also performed, but no association was found.

Figure 4.6. Naïve CD4⁺ levels and DFS and Relapse.

A trend towards lower relapse rates and improved disease free survival (DFS) was observed in patients who had higher levels of naïve CD4⁺ T cells prior to transplantation.

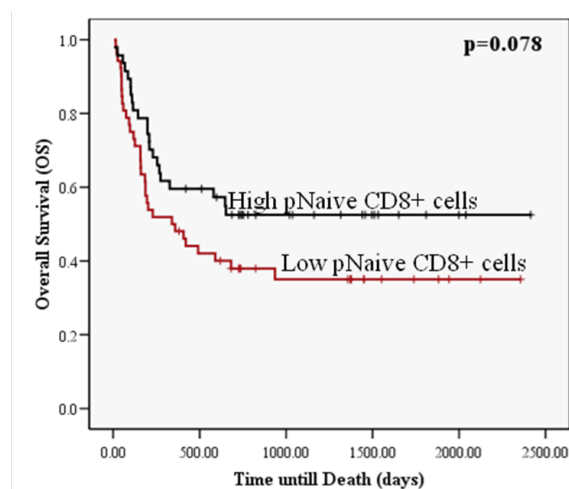


As for the CD4⁺ T cell subset, patients were divided into two groups based on the mean level of the CD8⁺ T cells: patients with percentages of CD8⁺ T cells pre transplant higher or lower than the mean.

In contrast to the results with the CD4⁺ T cells, the levels of CD8⁺ T cells were not correlated with the transplant outcome. Although no impact of the overall CD8⁺ T cells was observed, an analysis was also made with the different CD8⁺ subpopulations. Only high levels of naïve CD8⁺ T-cells pre-transplant showed a correlation, with a trend towards improved overall

survival (log rank test, 0.078). Memory and effector CD8⁺ T cell proportions did not affect the transplant outcomes. As for the CD4⁺ T cells, donor CD8⁺ T cells percentages and transplant outcome was analysed, but again no association was found.

Figure 4.7. High levels of naïve CD8⁺ T cells pre-transplant in patients have a positive impact on overall survival.



Correlation between TREC values and T-cell subsets

A positive correlation was found between high TREC levels per 150,000 CD3⁺ cells pre-transplant and both naïve CD4⁺ T-cells and naïve CD8⁺ T-cells pre-transplant ($r^2= 0.476$, $p<0.0001$ and $r^2= 0.352$, $p=0.0003$, respectively). Such a correlation is to be expected, since the population of naïve T cells exiting from the thymus has the highest concentration of TREC, which will become somewhat diluted by homeostatic proliferation within the naïve T

cell compartment and more significantly diluted during T cell expansion after activation and thus will be low in memory and effector T cell subsets. Conversely, a negative correlation was found between TREC levels per 150,000 CD3⁺ cells and both memory CD4⁺ and memory CD8⁺ T-cells ($r^2 = -0.330$, $p=0.0006$ and $r^2 = -0.257$, $p=0.01$). This is to be expected since only percentages could be analysed and of course a higher percentages of naïve CD4 or CD8 T cells implies a corresponding lower percentage of memory and effector cells.

Donor TREC and T cell subset influence on Transplant Outcome

Analysis of the impact of the donor T cell subsets and TRECs and transplant outcome was also performed. However, donor T cell subsets and donor TREC showed no significant associations with any outcome measure, suggesting that the donor immune status pre-transplant does not affect the success of the HSCT, whereas, the patients immune status plays an important role regulating the immune responses following an HSCT.

Discussion

During the past decades, much effort has been made to improve the outcome of allogeneic HSCT. However, infections, relapse and graft versus host disease are still significant causes of morbidity and mortality in transplanted patients. New efforts to identify those patients at greater risk of poor outcome are required in order to determine if further therapeutic strategies, such as Donor Lymphocytes Infusion (DLI), are necessary. Recently, some studies have analysed the impact of various recipient and donor T-cell subsets as well as overall thymic output in allogeneic HSCT from siblings. Those studies have shown that the status of the immune system prior to transplantation could be used as a predictive tool for HSCT outcome. In this study an evaluation of T-cell subsets and TREC levels as an indicator of thymic function pre-transplant were analysed and compared with healthy individuals.

Percentages of CD3⁺ T-cells were significantly lower in patients pre-transplant than in healthy controls; however, it is necessary to bear in mind that here only percentages have been analysed and thus it is possible these changes may be a consequence of previous treatments or of the remission state of the patient. For further studies absolute quantification will be needed. The low CD3⁺ percentages in patients were due to a decrease in the CD4⁺ population consistent with data that has previously been published (Rosinski, et al., 2005). Within the CD4 T-cells, the naïve CD4⁺ subset was significantly lower in patients pre-transplant. Therefore, the low overall CD4⁺ cells percentages are principally due to a decrease in the naïve subset.

On the other hand, the overall percentages of CD8⁺ T-cells and the CD8⁺ subsets were comparable between donors and patients.

Rosinski et al. (Rosinski, et al., 2005) published that pre-transplant levels of memory CD4⁺CD45RA⁻CD62L⁻ T-cell had a prognostic effect on transplant outcome in haematological malignancies and breast cancer, independently of other known factors. However, in the study shown here the prognostic effect of the CD4⁺ memory subset was not demonstrated. On the contrary, a prognostic effect of the naïve subset of both CD4 and CD8 was found. A trend towards better survival was observed in patients who had higher naïve CD8⁺ T-cells pre-transplant. While higher levels of naïve CD4⁺ cells was associated with a decrease in the risk of relapse and with a trend towards enhanced disease free survival without reaching statistical significance. It seems that while there is an influence of the naïve T cells on outcome, the mechanism of this effect is not yet clear.

One question to ask is why the levels of naïve CD4⁺ T cells should be lower in the patient cohort than in the controls, while there was no significant difference in the levels of naïve CD8⁺ T cells. One possibility is that, since survival of naïve T cells depends on tonic signalling through the T cell receptor (Seddon & Zamoyska, 2002), and since the MHC class II⁺ APC capable of such signalling to CD4⁺ T cells are of haematopoietic origin, alterations in haematopoiesis as a consequence of the underlying malignancy or its treatment may impact on the numbers of APC and thus indirectly influence the number of naïve CD4⁺ T cells. Since the CD8⁺ T cells receive signals from MHC class I molecules, which are ubiquitous, there may then be no observable effect on the naïve CD8⁺ T cell compartment.

Chapter 5

Regulatory T cells in HSCT

Introduction

Allogeneic Haematopoietic Stem Cell Transplantation (allo-HSCT) is a well-established therapy for a variety of malignant and non-malignant diseases of the haematopoietic system (Gratwohl, et al., 2002). Although HLA matching has improved allo-HSCT outcome, recipients remain susceptible to life-threatening post-transplant complications, including infections, disease relapse and the development of Graft versus Host Disease (GvHD) (Shaw, et al., 2001). Donor T cells facilitate cell engraftment, protect against opportunistic infections and promote anti-tumour activity, protecting the patient from disease relapse (through the graft versus leukaemia effect, GvL). However, donor cells also attack host tissues producing graft versus host disease, GvHD (Ferrara, 2000). One of the major challenges in allo-HSCT has been to find the optimal balance between the beneficial and harmful effect of T cells (Edinger, et al., 2003). Recently, advances in the understanding of regulatory T cells (CD4⁺CD25^{hi} T cells) has started the development of new methodologies and new strategies to improve the outcome of HSCT using Tregs as modulators of immune responses after transplantation. The new knowledge has given new insights about the immune responses following an HSC transplant.

Definition of CD4⁺CD25^{hi} Treg population

Recently, a distinct subset of the CD3⁺CD4⁺ compartment subset with immune suppressive capacity has been defined as regulatory T cells (Treg). They appear to have an important role in controlling allo-reactive T cells and maintaining peripheral tolerance to self-antigens. These regulatory T cells constitutively express the α -chain of the high-affinity IL-2 receptor (IL-2Ra, CD25) (Sakaguchi, et al., 1996) and they represent 5 to 10% of the normal T cell compartment in mice and humans (Sakaguchi, et al., 1996) (Zorn, 2006) (Itot, et al., 1999). In a normal thymus, 2-5% of CD4⁺CD8⁻ thymocytes express CD25; those cells are functionally competent (Itot, et al., 1999). They also express the transcription factor FOXP3 (Hori, et al., 2003) (Fontenot, et al., 2003). Their most characteristic feature is their impaired proliferative response (anergy) to standard stimuli, and their suppressive activity.

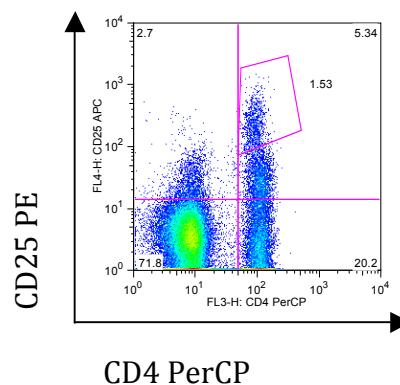
Studies indicate that thymus is continuously producing potentially self-reactive CD4⁺CD25⁻ T cells as well as functionally mature CD4⁺CD25⁺ Tregs. Consequently, the thymus contributes to the maintenance of self-tolerance, not only by the central process of negative selection but also by producing CD4⁺CD25⁺ Tregs that act in peripheral sites to control unwanted self-reactivity.

CD4⁺CD25⁺ Tregs are pluripotent suppressors modulating many different immune reactions. They inhibit naïve CD4⁺ T cell proliferation and differentiation; prevent cytotoxic activity of CD8⁺ T cells both in vivo and in vitro (Piccirillo & Shevach, 2001) (Murakami, et al., 2002) (Suvas, et al., 2003) (Dittmer, et al., 2004); suppress the activation and antibody

production of B cells (Sakaguchi, et al., 1995) (Bystry, et al., 2001) and limit the activity of cells from the innate immune system, such as NK cells, neutrophils and monocytes (Maly, et al., 2000). Moreover, they can efficiently limit the stimulatory capacity of antigen-presenting cells (APC's) by down-regulating cell surface expression of co-stimulatory molecules such as CD80 and CD86 (Cederbom, et al., 2000).

Figure 5.1. Gating strategy for the analysis of CD4⁺CD25^{hi} Regulatory Cells.

Peripheral blood mononuclear cells (PBMCs) were stained with PerCP-conjugated anti-CD4, PE-conjugated anti-CD25 and FITC-conjugated anti-CD3. Lymphocytes were gated via their forward- and side-scatter properties, then on the basis of CD3 expression before being analysed on the basis of CD25 expression and CD4 expression. The population of CD4⁺CD25^{hi} cells selected as Treg is indicated and corresponds to 1.53% of the CD3⁺ lymphocytes.



Expression of FOXP3

The forkhead box transcriptional regulator, FOXP3, has been shown to be expressed almost exclusively in CD4⁺CD25^{hi} T cells, and to be essential for both their generation and function (Hori, et al., 2003) (Khattari, et al., 2003) (Fontenot, et al., 2003). This FOXP3 expression has been used recently as specific marker that better defines the regulatory subset of CD4⁺ T cells, and discriminates them from activated effector T cells, which temporarily express the high affinity IL-2 receptor, CD25. In peripheral blood of normal donors, up to 96% of CD4⁺CD25^{hi} T cells expressed FOXP3, whereas only 22-47% of CD4⁺CD25^{int} T cells stained positive for FOXP3 by flow cytometry (Roncador, et al., 2005). Activation of CD4⁺CD25⁺ T cells increases FOXP3 expression. In contrast, activated effector T cells, which up-regulate CD25, do not express FOXP3.

Retroviral transfection of CD4⁺CD25⁻ T cells with the FOXP3 transcription factor induces Treg-like cells both phenotypically, with the induction of expression of CD25, CTLA-4, GITR and CD103; and functionally, by acquiring a suppressor function, indicating that the transcription factor itself is sufficient to induce Tregs (Hori, et al., 2003) (Fontenot, et al., 2003) (Khattari, et al., 2003). Over-expression of FOXP3 induces suppressive activity in both CD4⁺CD25⁻ and CD8⁺ T cells. Therefore, regulatory T cells can be defined as CD3⁺CD4⁺CD25^{hi}FOXP3⁺ T cells, which have suppressive functions and which control alloreactive and autoreactive immune responses. Moreover, a role of Tregs in controlling and inhibiting anti-tumour responses has been identified (Dranoff, 2005).

Expression of other markers in Tregs

Several studies have been carried out to better characterise and to identify new markers for regulatory T cells. Those studies have revealed a high expression of the negative regulator of T cell activation CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4 or CD152); the glucocorticoid-induce tumour necrosis factor receptor, (GITR) (McHugh, et al., 2002), and expression of membrane-bound tumour growth factor (TGF- β 1) (Green, et al., 2003).

In contrast to the CD28 receptor to which it is evolutionary related, ligation of cytotoxic T-lymphocyte antigen (CTLA-4) on the surface of activated T cells by its ligands CD80/86 expressed on APC's, delivers a negative signal leading to inhibition of T cell activation (Chambers, et al., 2001). GITR function is not completely understood in humans, however, it seem to down-regulate Treg function (Bushell & Wood, 2007) (Kanamaru, et al., 2004) (Ko, et al., 2005). The expression of the membrane-bound tumour growth factor TGF- β 1 has been associated with the specific mechanism of immunosuppression, which in Tregs is dependent on cell-cell contact (Nakamura, et al., 2001).

Mechanisms of suppression of Tregs

The precise molecular mechanisms of Treg suppression are currently controversial. In vitro studies have shown that Tregs have to be activated through their T cell receptor (TCR), but once they have been activated they can suppress responses to other antigens non-specifically (Takahashi, et al., 1998) (Thornton & Shevach, 1998).

In vitro Tregs suppress their targets in a cell-cell contact dependent manner via the Fas/FasL (CD95/CD95L) signaling pathway for inhibition of proliferation with or without inducing cell death. Other pathways may be involved since even in vitro Tregs require stimulation via TCR to exert their regulatory function (Baatar, et al., 2007) (McIver, et al., 2008). However, in vivo regulation seems to be more complicated and several suppressive cytokines have been implicated in Treg suppressor function (Asseman, et al., 1999) (Annacker, et al., 2001).

So far, it is not completely understood whether CD4⁺CD25^{hi} Tregs can use different suppressor mechanisms or whether individual specialised regulatory subsets exist that exert suppression just via one mechanism.

Although the mechanism of Treg suppression remains unclear, the following conclusions have been accepted:

- Tregs require T-cell receptor (TCR) ligation to antigen-specific or polyclonal stimuli for activation (Takahashi, et al., 1998) (Thornton and Shevach 1998).
- Once activated, Tregs are able to suppress without regard to antigen specificity (Takahashi, et al., 1998) (Thornton & Shevach, 1998).

- In vitro, Tregs are anergic to antigenic stimulation; the anergic state corresponds to their suppressive activity (Takahashi, et al., 1998).

Clinical impact of regulatory T cells in transplantation

Tregs are actively involved in immune mediated mechanisms after allogeneic stem cell transplantation and a potential clinical benefit of Tregs in controlling GvHD and reducing the complications of allogeneic HSCT has been suggested. However, a role of Tregs in suppressing anti-tumour immune responses has also been proposed contributing to increase relapse. Thus, their role in the overall transplant outcome remains controversial (Meignin, et al., 2005) (Zorn, et al., 2005).

The majority of the published work to date has focused on the post-transplant period, analysing the impact of donor derived Tregs on transplant outcome, as mentioned in chapter 1. Tregs have been associated with lower incidence of GvHD, though, data obtained until now, have not been conclusive (Cohen, et al., 2002) (Clark, et al., 2004) (Sanchez, et al., 2004) (Zorn, et al., 2005). Recent data have shown that Tregs can survive and can even expand after aggressive conditioning and after T cell depletion before an HSCT (Bloom, et al., 2008) (Bayer, et al., 2009), indicating that they may be capable of playing an important role in the early post-transplant events. Therefore, in this study we aimed to examine the impact of pre-transplant Treg levels in patients undergoing HSC transplantation from an unrelated donor and analyse their association with overall survival (OS), disease relapse, disease free survival (DFS) and GvHD.

Patients, Materials and Methods

Patients and controls

The study included two separate, independent cohorts: an initial cohort (n=90) of patients receiving an HSCT from unrelated donors through the Anthony Nolan Trust (ANT) in the UK; and a second cohort (n=24) from an independent centre (The Alfred Hospital in Australia) receiving either autologous or allogeneic transplants.

For the first group, both recipient and donor were required to have 4-digit allele typing results at six HLA loci (HLA-A, -B, -C, -DRB1, -DQB1, -DPB1). Because the study was registry based, the blood samples and clinical data were provided by the individual transplantation centres (TCs). Clinical data were collected on standard forms, which were sent to data managers at the individual TCs.

The transplantations took place between September 1996 and January 2003. A broad ranges of diseases were indications for transplantation, and the patient and donor pre-transplantation factors can be seen in Table 5.1. The majority of the transplantation protocols included T-cell depletion (85%), in most cases using Alemtuzumab (Campath 1-H) in vivo (80%). A roughly equal number of patients received transplants for each of the diagnoses: AML, ALL, chronic myeloid leukaemia (CML), and other malignant diseases. A small number received transplant for non-malignant diseases, patient information is presented in table 5.1. Early disease stage was defined as first complete remission (CR1) in acute leukaemia and first chronic phase (1CP)

in CML. Untreated patients (e.g., with MDS) were included in this definition. All patients with disease beyond this were grouped as late-stage disease. The median age of the patients was 30 years with a broad range, including infants. The donors had a median age of 34 years, although once again with a relatively broad range.

The second study group comprised patients from The Alfred Hospital and the Peter MacCallum Cancer Institute in Melbourne Australia. All patients gave written informed consent to treatment with LHRH-A. Pre-treatment samples were taken from 2001 to 2005 from patients undergoing an HSCT.

Transplant type included both allogeneic and autologous. For the allogeneic transplants, both recipient and donor were required to have 4-digit allele-level typing results at six HLA loci (HLA-A, -B, -C, -DRB1, -DQB1, -DPB1), and be fully matched at HLA-A, -B, -C, -DRB1, and -DQB1 (10/10 match).

1 patient with chronic myeloid leukaemia (CML), 8 patients with acute myeloid leukaemia, 2 patients with acute lymphoblastic leukaemia (ALL), 13 with other malignancies (including Hodgkin and non-Hodgkin lymphoma, multiple myeloma and aplastic anemia) were analysed.

Early disease stage was defined as first complete remission (CR1) in acute leukaemia and first chronic phase (1CP) in CML. Untreated patients (e.g., with MDS) were included in this definition. All patients with disease beyond this were grouped as late-stage disease. The median age of the patients was 51.5 years, ranging between 2 to 59. Patient demographics are summarised in Table 5.1.

Table 5.1 Patient demographics		
	Anthony Nolan Trust UK group n= 90 (%)	The Alfred Hospital Australian group n= 24 (%)
Age, years	29.39±1.77	51.5±1.98
Gender		
Male	49 (54.4)	17 (70.8)
Female	41 (45.6)	7 (29.2)
Diagnosis		
CML	22 (24.4)	1 (4.2)
AML	22 (24.4)	8 (33.3)
ALL	19 (21.1)	2 (8.3)
Other malignant	23 (25.6)	13 (54.2)
Non-malignant	4 (4.4)	0 (0)
Conditioning regimen		
Myeloablative	61 (67.8)	13 (54.2)
RIC	24 (26.7)	11 (45.8)
Missing	5 (5.5)	0 (0)
Stem Cell Source		
Peripheral blood	15 (16.7)	24 (100)
Bone Marrow	75 (83.3)	0 (0)
Type of Transplant		
Autologous	0 (0)	12 (50)
Allogeneic related donor	0(0)	8 (33.3)
Allogeneic UD	90 (100)	4 (16.7)
T cell depletion		
Yes	80 (89.9)	0 (0)
No	10 (11.1)	24 (100)
Abbreviations: CML: chronic myeloid leukaemia; AML: acute myeloid leukaemia; ALL: acute lymphoblastic leukaemia, RIC: reduce intensity conditioning; UD: unrelated donor		

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from peripheral blood samples by standard Ficoll density gradient centrifugation. Aliquots of 1×10^6 PBMC were frozen in liquid nitrogen in freezing media, consisting of 90% foetal calf serum and 10% DMSO. For flow cytometric analysis, cells were thawed, washed and counted. Cell viability was analysed using trypan blue.

RNA extraction and cDNA synthesis

RNA isolation was performed using RNeasy mini kit (Qiagen, Valencia, CA). Total RNA was eluted with water and store at -70°C . For reverse transcription of mRNA and cDNA synthesis, 1mg of total RNA was reversed transcribed and stored at -20°C until quantitative real-time polymerase chain reaction (qRT-PCR) was performed.

Flow Cytometry

Cell phenotypes were analyzed by 4-color flow cytometry using a titrated panel of directly conjugated antibodies to CD3, CD4 (BD BioScience, San Jose, California, USA) and CD25 (2A3 clone, eBioSciences). Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PerCP were used as fluorophores. The acquisition was performed using FACSCalibur flow cytometer and CellQuest software (BD Bioscience). Acquired data was

analysed with Flow Jo software. The frequency of Tregs was calculated as a percentage of positive cells in the CD4⁺ population. The percentage of CD4⁺ lymphocytes expressing high levels of CD25 was assessed by flow cytometry, the gating strategy used is shown in Figure 5.1.

Quantitative PCR

FOXP3 gene expression was measured from PBMC's using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously. GAPDH was used as an internal control gene for mRNA expression. Primer and probe sequences for GAPDH were as follows: 5'-GGA GTC AAC GGA TTT GGT CGT ATT-3' (forward) 5'-GGC AAC AAT ATC CAC TTT ACC AGA GT-3' (reverse) FAM-CTG GTG ACC AGG CGC C-3'TAMRA (probe). Primer and probe sequences for FOXP3 were as follows: 5'-GCC TCC TCT TCT TCC TTG AA-3' (forward), 5'-GTG AGG CTG ATC ATG GCT-3' (reverse), 3'-VIC-CCA TCG CAG CTG CAG CTC TCA A-TAMRA-5' (probe). To create a standard curve, GAPDH and FOXP3 cDNA were amplified by PCR using the same primers designed for qRT-PCR and cloned into a TOPO vector (Invitrogen) following manufacturer's instruction. Plasmid DNA was extracted and quantified by UV spectrophotometry. The number of copies was calculated by using the molecular weight of the vector and each gene amplicon. Serial dilutions of the amplified genes at known concentrations were tested by qRT-PCR. qRT-PCR of cDNA from patients, standards, and water as a negative no-template control (NTC) were carried out in a total volume of 25 μ l with Platinum Taq (Invitrogen). Thermal cycler conditions

were as follows: 5min at 95°C and 50 cycles of 95°C for 30seconds and 60°C for 40 seconds. Standard curve extrapolation of copy number was performed in both FOXP3 and GAPDH. Sample data was normalised by dividing the number of copies of FOXP3 transcripts by the number of copies of GAPDH transcripts. All PCR assays were performed in triplicates and reported as the mean.

Statistical analyses

Analysis was performed using either SPSS version 14.0 (SPSS, Chicago, IL) or R version 2.2.1 (<http://www.r-project.org>) with the help and advice of Dr Rafael Duarte and Dr. Hazael Maldonado of the Anthony Nolan Trust. The association between variables was assessed using chi-squared test or Mann-Whitney test. Correlation analyses were performed using Pearson correlation test.

Acute (a)GVHD was analysed as a binary variable using logistic regression models. The probabilities of Transplant Related Mortality (TRM), disease relapse and chronic GvHD (cGvHD) were compared using Gray's test and analysed using the Cumulative Incidence (Cum Inc) method (Gooley, 1999) (Szydlo, 2004)

The probabilities of Overall Survival (OS) and Disease Free Survival (DFS) were analysed by the Kaplan-Meier methods and compared using log-rank statistic (Szydlo, 2004). For time-dependent variables, multivariate analysis was performed using Cox-regression analysis. Outcomes were considered to be significant when $p < 0.05$. A trend was identified when $0.05 < p < 0.1$.

Results

CD4⁺CD25^{hi} regulatory T cells

Treg levels in patients and controls: gating CD25^{hi} T cells

Precise definition of Tregs is not conclusive as yet. However, they are naturally occurring T cells, predominantly confined to the CD25^{hi} subset of CD4⁺ T cells (Baecher-Allan, et al., 2001) (Baecher-Allan, et al., 2003). They express the transcription factor FOXP3 along with varying levels of cytotoxic T lymphocyte associated antigen 4 (CTLA-4), glucocorticoid induced tumour necrosis factor receptor (GITR), and programmed death 1 (PD-1) (Hori, et al., 2003) (Fontenot, et al., 2003). Upon stimulation, they produce regulatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor β (TGF- β) (Viguiier, et al., 2004) (Liyanage, et al., 2002). They represent 1 to 2% of the peripheral CD4⁺ T cells.

Our first definition of Tregs was CD4⁺CD25^{hi} cells. We assessed the percentage of CD4⁺ lymphocytes expressing high levels of CD25 by flow cytometry, following the gating strategy showed in Figure 5.1.

In the last couple of years it has been described that CD4⁺CD25^{hi} Tregs are increased in cancer patients. We compared the frequency of Tregs in patients prior to UD/HSCT (n=90) and in healthy controls (n=49). In contrast to previous publications but consistent with Clark et al and Nadal et al, we found that the mean percentage of CD4⁺CD25^{hi} T cells in patients prior to transplantation was slightly but significantly lower (2.23%±0.29%) than that in healthy controls (3.03%±0.19%) ($p=0.03$) as is shown in Figure 5.2. However, the percentages in both healthy controls and patients were within the normal range. This observation was confirmed in a second independent cohort from an Australian group, where a mean of 1.66%±0.17% of CD4⁺CD25^{hi} T cells was observed, which was significantly lower than in the healthy control group ($p<0.0001$), as presented in Table 5.2 and in Figure 5.2.

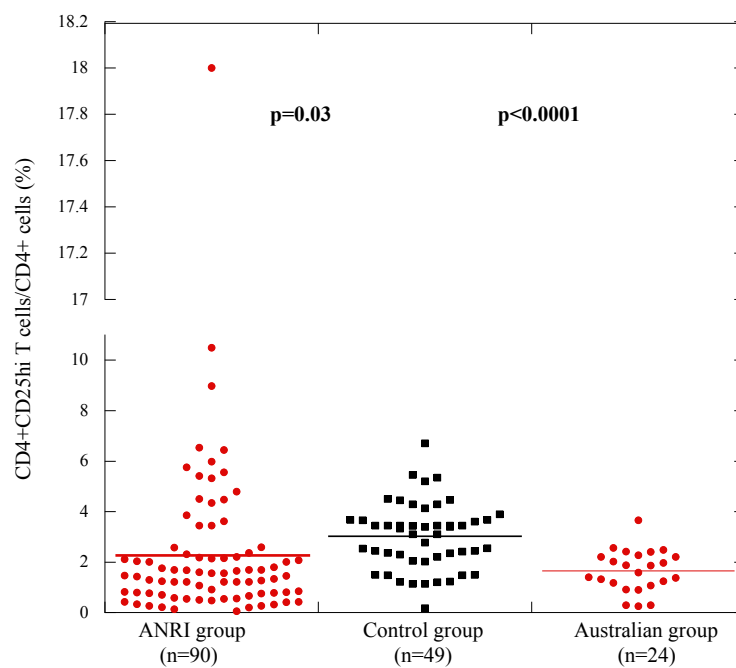
Table 5.2. Comparing the expression of Tregs

Comparing the expression of Tregs (CD4⁺CD25^{hi} T cells) in a control group and two independent patient cohorts. Both patient groups had statistically significantly lower Tregs when compared with the control group.

Comparison between the patient groups and controls		
Group	Mean±SE	<i>p value</i>
Control group (n=49)	3.03±0.19%	
ANRI group (n=90)	2.23±0.29%	$P=0.03$
Australian group (n=24)	1.66±0.17%	$p<0.0001$

Figure 5.2. CD4⁺CD25^{hi} T cells between different groups.

The percentage of CD4⁺CD25^{hi} T cells in peripheral blood were found to be lower in patients undergoing either an allogeneic or an autologous HSCT in two independent groups of patients in comparison with a healthy control group. The ANRI group comprised allogeneic transplants only while the Australian group included both autologous and allogeneic transplants.



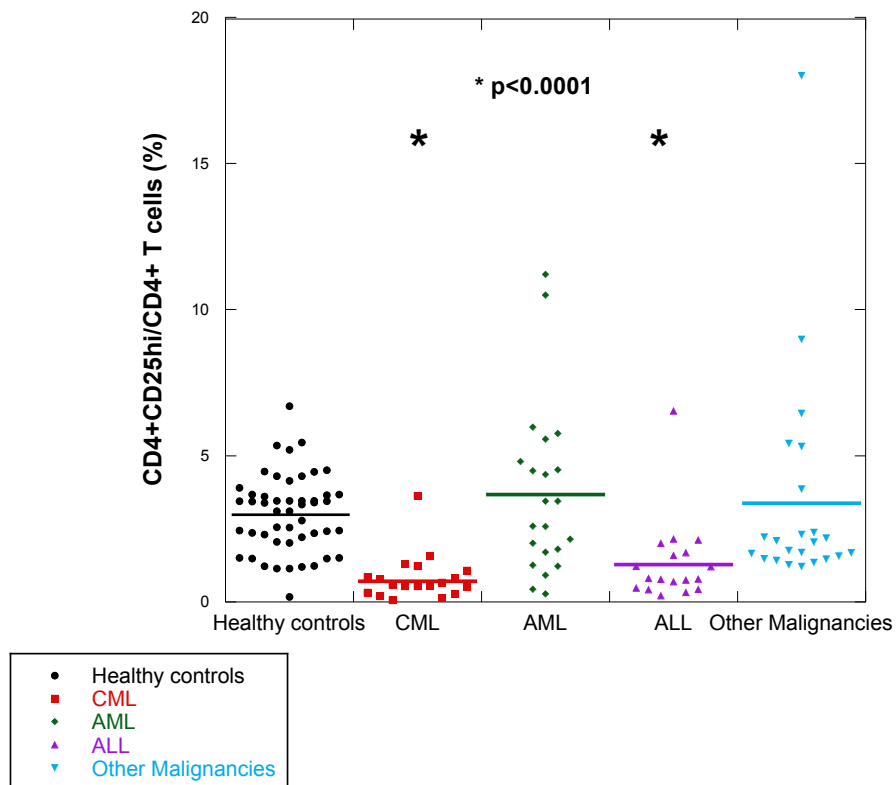
Expression of Tregs in different diseases

As mentioned previously, CD4⁺CD25^{hi} regulatory T cells were significantly lower in patients with haematological malignancies (n=90) when compared with healthy controls (n=49). However, when we looked at the frequencies of Tregs in the different diseases we found significantly lower Tregs in CML patients (0.71% ± 0.17%) compared with AML patients (3.68% ± 0.62%) ($p < 0.0001$) and with healthy controls (3.03% ± 0.19%) ($p < 0.0001$) as Nadal's group have previously described. Low Treg levels equivalent to those seen in CML patients in the case of ALL patients (Nadal, et al, 2007).

It has also been described by Wang et al (Wang, et al., 2005), that AML patients have higher frequencies of Tregs than healthy controls. However, although we found higher frequencies of Tregs in the AML patient group compared with healthy controls it did not reach a statistical significance ($p = 0.19$). The analysis couldn't be performed in the Australian group due to the lower number of patients included in the study group.

Figure 5.3. The frequencies of CD4⁺CD25^{hi} T cells in different disease types.

The percentage of CD25^{hi} T cells within all CD4⁺ T cells was assessed in different disease types, chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML), acute lymphocytic leukaemia (ALL) and a in group of “Other Malignancies”, each occurring at low frequency. Significantly lower expression of Tregs was observed in CML and ALL patients compared with healthy controls ($p < 0.0001$). AML patients showed higher frequencies of Tregs in comparison with healthy controls, however, this difference was not statistically significant.



Pre-transplant patient Tregs levels and clinical outcome

Overall Survival and other non-relapse outcomes

Based on the mean value of the CD4⁺CD25^{hi} T cells (Tregs) the patients were separated into two different groups, Treg^{Low} group in which those patients with Tregs below the mean (less than 2.31%) were included, and the Treg^{High} group including the patients with Tregs over the mean.

The median time of follow-up of recipients in the first cohort was three-years. For the whole population, the estimated three-year OS and DFS were 47.8%, and 31.8%, respectively. As is shown in Figure 5.4, the outcome was significantly poorer in the Treg^{High} group than in their Treg^{Low} counterpart (OS, 28% vs. 55.4%; p=0.030; DFS, 12% vs. 39.7%; p=0.017, respectively). We then investigated the reasons for the inferior OS and DFS. There was no association between Treg expression and non-relapse mortality (all cases: 32.9%; n/s.). In addition, there was no association between pre-transplant Treg levels and the incidence of acute GvHD (all cases: overall 45.6%, grades III-IV 3%; n/s.) or chronic GVHD (all cases, overall 36.1%, extensive 7.8%; n/s.).

In the second cohort, the median follow up was one year and the estimated one-year OS and DFS were 87.5% and 54.2% respectively. In contrast with the first cohort the overall survival was not different between the Treg^{High} group and the Treg^{Low} group (100% vs. 75%). However, supporting the data of the first cohort the DFS was significantly lower in the Treg^{High} group than in the Treg^{Low} group (16.7% vs. 91.7%, p<0.001), data presented in Figure 5.5.

Because the donor graft is the one that reconstitute the recipient's immune system an analysis of donor Tregs and transplant outcome was carried out; unlike the situation with patient Treg levels, the donor Tregs showed no correlation with the overall survival, DFS or the relapse rate in this analysis.

Figure 5.4. Overall survival (OS) and Disease Free Survival (DFS) in patients from the Anthony Nolan Group.

The patients were stratified into two groups based on their levels of Tregs, either above or below the mean level for the cohort. Patients with high levels of Tregs have worse OS (log rank, 0.030) and DFS (log rank, 0.017) than those patients with low Treg levels.

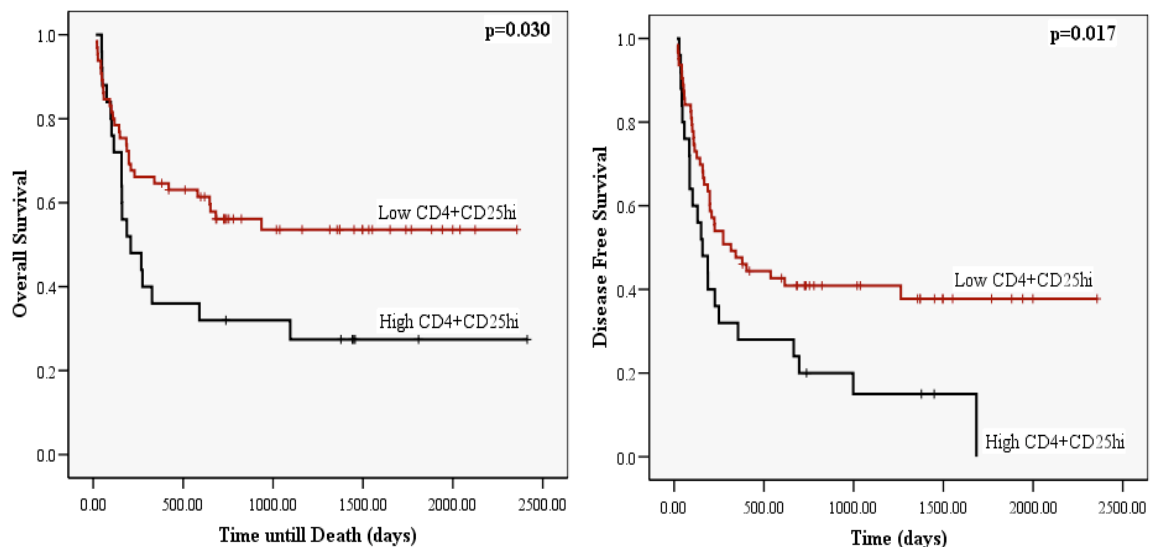
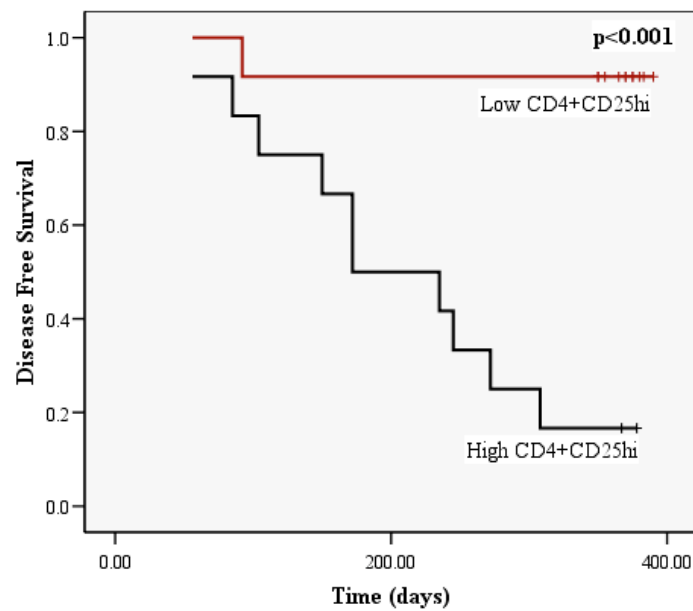


Figure 5.5. Disease Free Survival (DFS) in patients from the Australian cohort.

Patients were stratified into two groups based on their expression of Tregs, either above or below the mean level for the cohort. Although, the OS was not different between the two groups, in concordance with the data showed in the cohort from the UK, patients with high levels of Tregs showed significantly lower DFS (log rank <0.001).

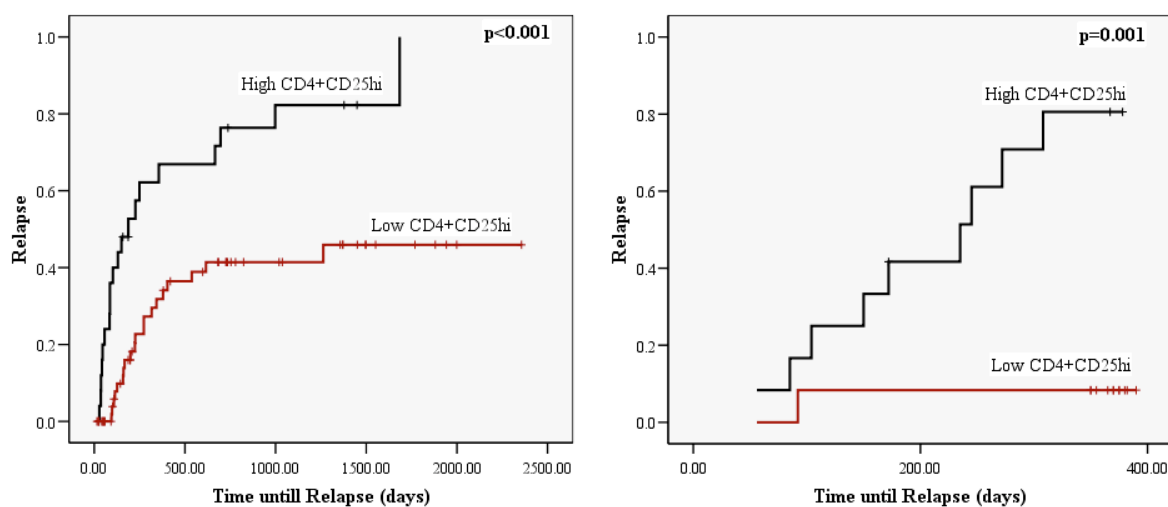


Disease Free Survival (DFS) and Relapse

Higher CD4⁺CD25^{hi} Tregs levels, however, did associate with an increased risk of disease relapse in both cohorts. Treg^{High} patients had a significant increase in the incidence of disease relapse compared to Treg^{Low} patients. The estimated three-year risk of relapse in the first cohort was 80% in Treg^{High} and 31.7% in Treg^{Low} patients ($p < 0.0001$) as presented in Figure 5.6; and the estimated one-year risk in the second group was 75% in the Treg^{High} group and 8.3% in the Treg^{Low} group ($p = 0.001$) as can be seen in Figure 5.6, suggesting an effect of Tregs on graft versus tumour responses in these populations.

Figure 5.6. Relapse rate in patients after HSCT. Both groups showed higher incidence of relapse associated with high levels of Tregs.

Both the UK (panel A) and the Australian (panel B) patient cohorts were stratified in the same fashion, into groups with Treg levels either above or below the mean for the cohort. A comparison of relapse rates in the two patient cohorts examined showed that in both cohorts, patients with high levels of Tregs showed higher relapse rates.



A. Group from the ANT, UK
(n=90)

B. Group from the Alfred
Hospital, Australia (n=24)

Specific markers for Tregs: FOXP3 expression

While the study had clearly shown an association between high levels of CD4⁺CD25^{hi} T cell subsets, this broad phenotypic definition did not clearly discriminate between inflammatory and regulatory CD4 T cells. Since activated T cells are also known to express CD25, relying on CD25 as a marker of CD4⁺CD25^{hi} regulatory T cells could overestimate the true number. To assess whether this subpopulation of T cells was indeed regulatory, we also measured the expression of the Treg specific transcription factor FOXP3, which is thought to be a reliable marker for Tregs (Hori, et al., 2003). Thus, we analysed the expression of FOXP3 by qRT-PCR in patients where samples prior to transplantation were available. Patients prior to transplantation also had significantly lower expression of FOXP3 (27.18 ± 5.28 FOXP3 copies/GAPDH) in comparison with healthy controls (49.57 ± 4.05 FOXP3 copies/GAPDH) ($p=0.012$).

We then examined the correlation of the expression of FOXP3 and CD4⁺CD25^{hi} populations in donors and patients. As expected, we found a positive correlation between the proportion of CD4⁺CD25^{hi} T cells and FOXP3 transcripts in donor samples as is shown in Figure 5.7 panel A. However, the correlation between CD4⁺CD25^{hi} T cells proportion and FOXP3 transcripts in patients' samples was not particularly strong ($r=0.518$) (Figure 5.7 Panel B). This relatively poor correlation was surprising, especially in view of the good correlation of CD4⁺CD25^{hi} T cells with TGF- β expression (this would be described in more detail later). One possibility is that in the patient group, there are cells that express FOXP3 and are committed to becoming Tregs but which do not yet express the CD25^{hi}TGF β ⁺

phenotype.

The intranuclear expression of FOXP3 by flow cytometry (Figure 5.8) was also tested in 11 samples from the first cohort and in the 24 patients from the second cohort and a correlation with the CD4⁺CD25^{hi} expression was analysed; a strong correlation was found in both groups ($r^2=0.623$ and $r^2=0.766$, respectively).

As an indicator of Treg function we also quantified TGF- β levels in plasma by Luminex® technology. We analysed the correlation between CD4⁺CD25^{hi} T cell phenotype and FOXP3 expression and as expected, we found a positive correlation ($r^2=0.518$, $p<0.0001$). We also found a positive correlation between CD4⁺CD25^{hi} T cells and TGF- β production ($r^2=0.884$, $p<0.0001$) and between FOXP3 expression and TGF- β ($r^2=0.733$, $p<0.0001$).

Figure 5.7 Correlation between percentages of CD4⁺CD25^{hi} T cells among total CD4⁺ T cells and FOXP3 expression in peripheral blood (PB).

The relative level of FoxP3 expression amongst total PBMC was plotted against the percentage of CD4⁺CD25^{hi} cells amongst total CD4⁺ T cells in normal controls (panel A) and in patients pre-transplant (panel B). A) A positive correlation was found in healthy controls as shown by the trend line. B) A positive correlation was also found in the patient group. However, in the patient group a different pattern of FOXP3 expression can be observed, where low CD4⁺CD25^{hi} T cells express high FOXP3, that could be explained by the fact that there are some CD4⁺CD25⁻ T cells that express FoxP3, and possess similar regulatory function as the CD4⁺CD25⁺ Tregs. Patients with high CD4⁺CD25^{hi} T cells and low FoxP3 transcripts may reflect those in which an expanded cell population in the periphery (eg tumour cells) decreases the average FoxP3 level, but is not considered with the percentage of CD4⁺CD25^{hi} T cells, which is the percentage within total CD4⁺ T cells only.

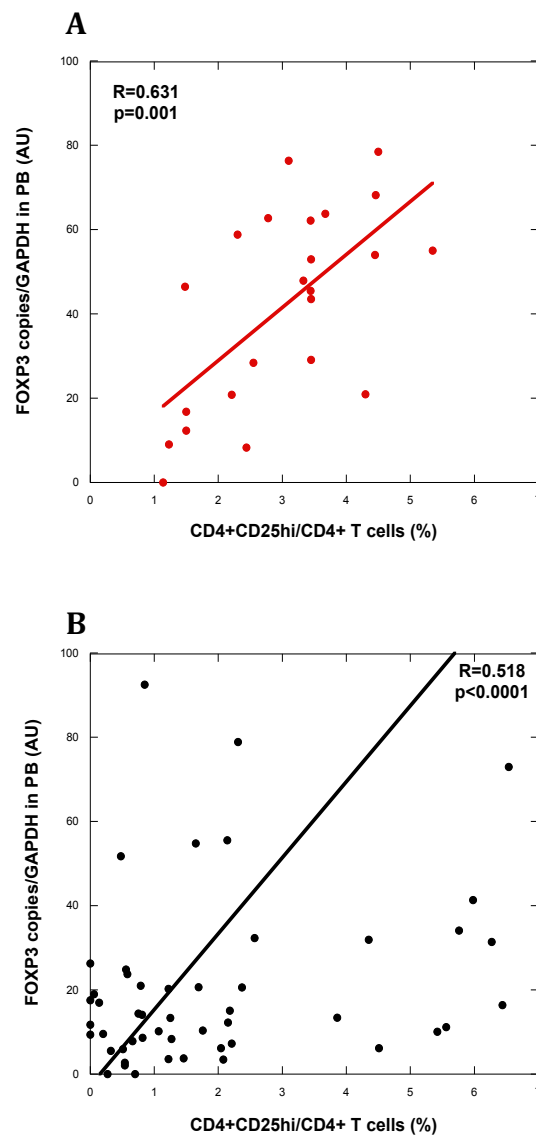
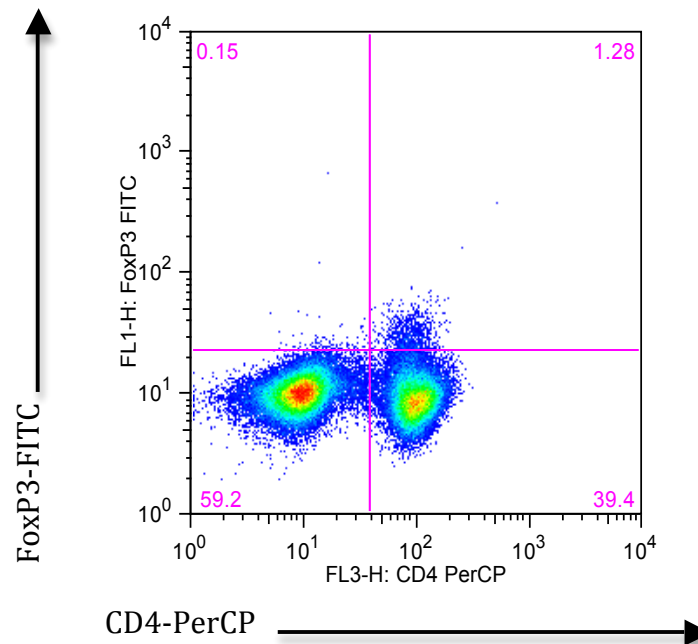


Figure 5.8 Intracellular staining of FOXP3.

Pretransplant patient cells were permeabilised and stained with antibodies to FoxP3 and CD4; FoxP3⁺ cells were found only within the CD4⁺ fraction. The percentage of FoxP3⁺CD4⁺ cells – in the right upper quadrant – was taken to represent Treg.

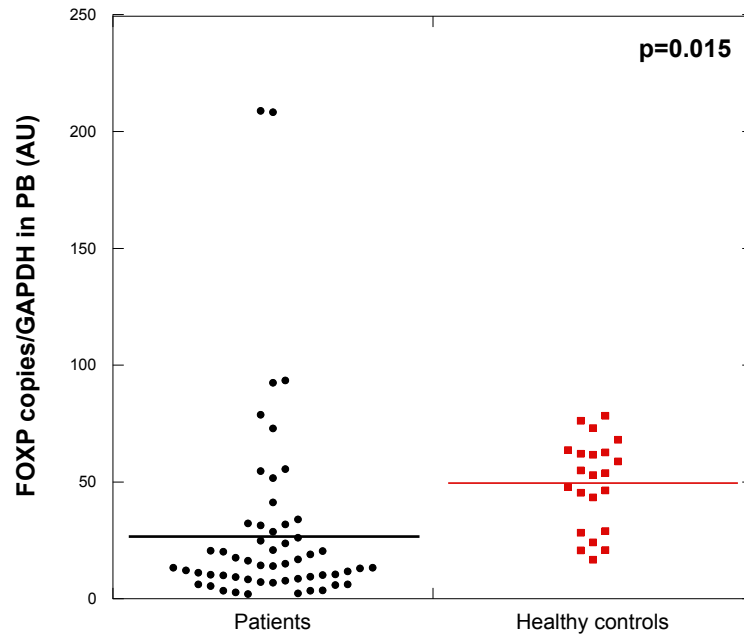


mRNA levels of FOXP3 in patients and controls

The expression of FOXP3 by quantitative PCR in healthy controls as in the percentages of Tregs was higher (49.57 ± 4.05 FOXP3 copies/GAPDH) than in patients with haematological malignancies (27.18 ± 5.28 copies FOXP3/GAPDH) ($p=0.015$) as shown in Figure 5.9

Figure 5.9 Comparison of the expression of FOXP3 between controls and patients.

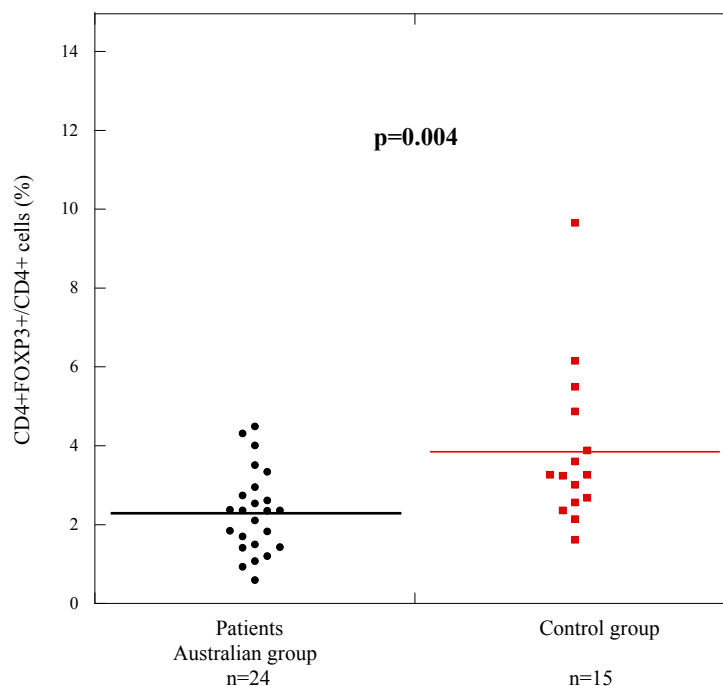
The expression of FOXP3 was quantified by a semiquantitative real-time PCR protocol, healthy controls had significantly greater FOXP3 expression compared to patients with haematological diseases ($p=0.015$).



The quantification of FOXP3 by flow cytometry was only possible in the Australian group of patients (n=24) and in 15 healthy controls. As in the quantification of FOXP3 mRNA and in accordance with the results of CD4⁺CD25^{hi} quantification, patients also showed statistically significant lower expression of FOXP3 than healthy controls (median 2.36 ± 1.05% vs. 3.25 ± 2.03, p=0.004) as presented in Figure 5.10. In this case, since the measurements in patients and controls are both within the gated CD4⁺ lymphocyte population, artefacts that arise from expansions of other non-CD4⁺ cells can be excluded. Specifically, the presence of malignant cells in the patients cannot lower the percentage of FoxP3⁺CD4⁺ cells unless the malignant cells themselves are CD4⁺, which in this cohort they were not. While this cannot be conclusive proof, it is suggestive that the decreased FoxP3 levels seen in the Anthony Nolan cohort is also a real decline and not an artefactual one.

Figure 5.10 Comparison of the expression of intracellular FOXP3 in healthy controls and patients prior to HSCT.

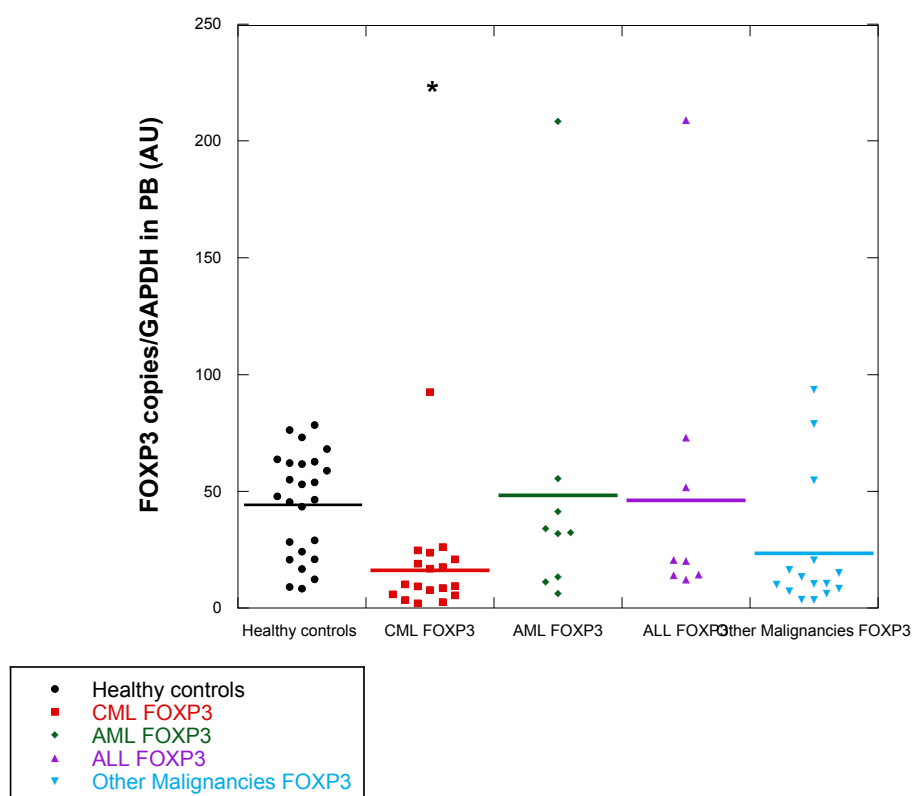
The percentage of CD4⁺ cells staining for the presence of the FoxP3 protein intracellularly (measured as in Figure 5.8) was compared between a cohort of patients prior to transplant and a set of normal, healthy controls. Lower levels were found in the patient cohort, and this was statistically significant, with a p value of 0.004.



The comparison of FOXP3 expression between the different diseases showed essentially the same results as observed for the frequencies of Tregs; CML patients have statistically significant lower expression of FOXP3 (16.18 ± 4.54 copies of FOXP3/GAPDH) in comparison with AML patients (48.23 ± 20.68 copies of FOXP3/GAPDH) ($p=0.04$) and with healthy controls (49.57 ± 4.05 copies of FOXP3/GAPDH) ($p<0.0001$) as shown in Figure 5.11.

Figure 5.11. *FOXP3* expression in different diseases.

The expression of *FOXP3* by quantitative real-time PCR showed that healthy controls (n=26) had significantly greater *FOXP3* expression compared to patients CML (n=19) ($p < 0.0001$). The mean expression of *FOXP3* was similar between healthy controls and AML patients (n=9) as was the level in ALL patients. However, the number of AML and ALL patients analysed may be too small to make any meaningful conclusion.



*Pre-transplant patient *FOXP3* levels and clinical outcome*

Overall Survival and other non-relapse outcomes

Following the same strategy used for CD4⁺CD25^{hi}, patients were separated into two groups based on the mean value of *FOXP3* mRNA expression for the ANT groups and *FOXP3* intracellular expression for the Australian group, a

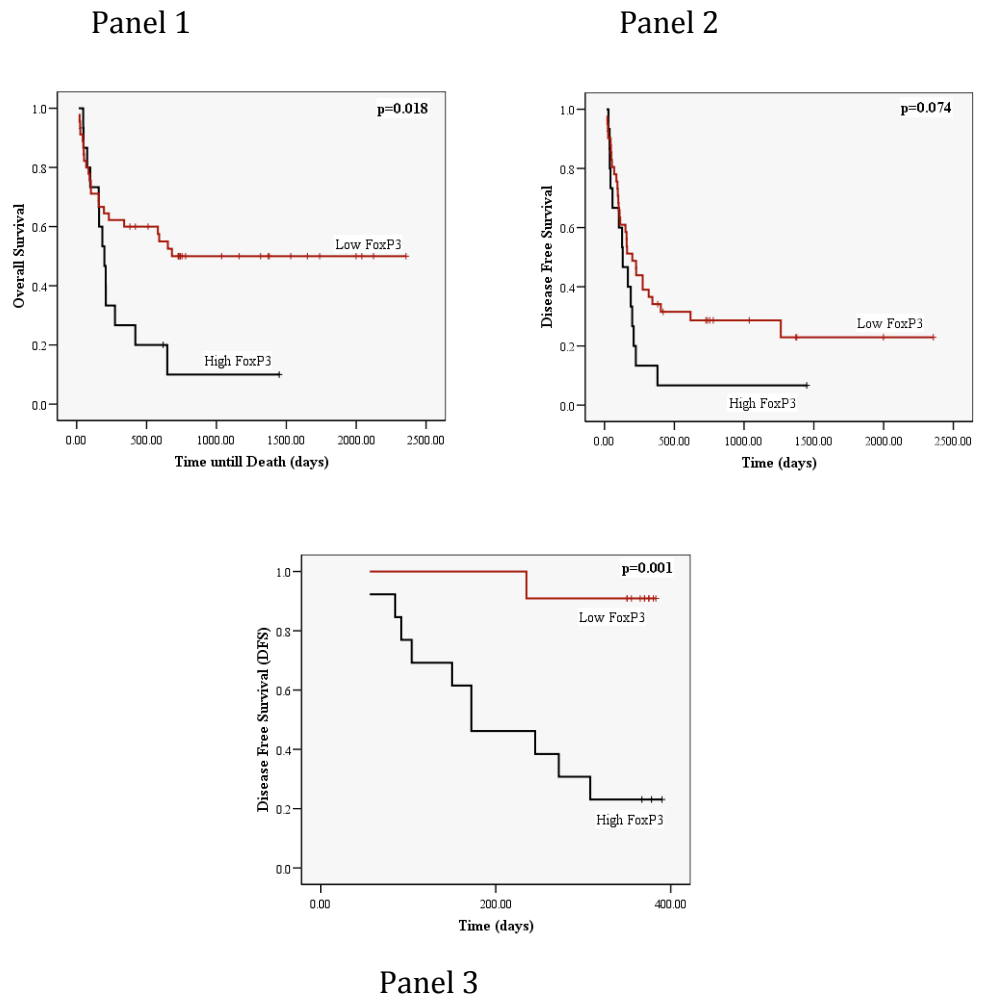
FOXP3^{Low} group of those patients with FOXP3 below the mean (less than 27.18 copies and less than 2.36% respectively) and the FOXP3^{High} group including the patients with FOXP3 levels over the mean.

As mention before, the median time of follow-up of recipients in the ANT cohort was three-years. For the whole population, the estimated three-year OS and DFS were 47.8%, and 31.8%, respectively. As is shown in Figure 5.11, and supporting the previous data of Tregs the outcome was significantly poorer in the FOXP3^{High} group than in their FOXP3^{Low} counterpart (OS, 48.9% vs. 86.7%; $p=0.018$; DFS, 6.7% vs. 26.8%; $p=0.074$, respectively). As shown in the analysis of Tregs, an analysis was performed to find the reason for the inferior OS and DFS, and again the results failed to show an association between FOXP3 expression and non-relapse mortality. In addition, there was no association between pre-transplant FOXP3 levels and the incidence of acute GvHD in the patients.

In the Australian cohort the median follow up was one year as previously presented. The estimated one-year OS and DFS were 87.5% and 54.2% respectively. As in the previous analysis, the overall survival was not different between the FOXP3^{High} group and the FOXP3^{Low} group (100% vs. 76.9%). However, the DFS was significantly lower in the FOXP3^{High} group than in the FOXP3^{Low} group (23.1% vs. 90.9%, $p=0.001$), data presented in Figure 5.12.

Figure 5.12. Overall survival and Disease Free Survival base on FOXP3 expression prior to transplantation.

Expression of FOXP3 in the patients in the Anthony Nolan cohort was measured by qRT-PCR and normalised against GAPDH expression. Patients were stratified based on values above or below the mean for the cohort. As can be seen in the first panel, high expression of FOXP3 was associated with worse overall survival ($p=0.018$). High FOXP3 expression was also associated with worse DFS in two independent cohorts, (panel 2: ANT group, $p=0.074$; panel 3: Australian group, $p=0.001$).

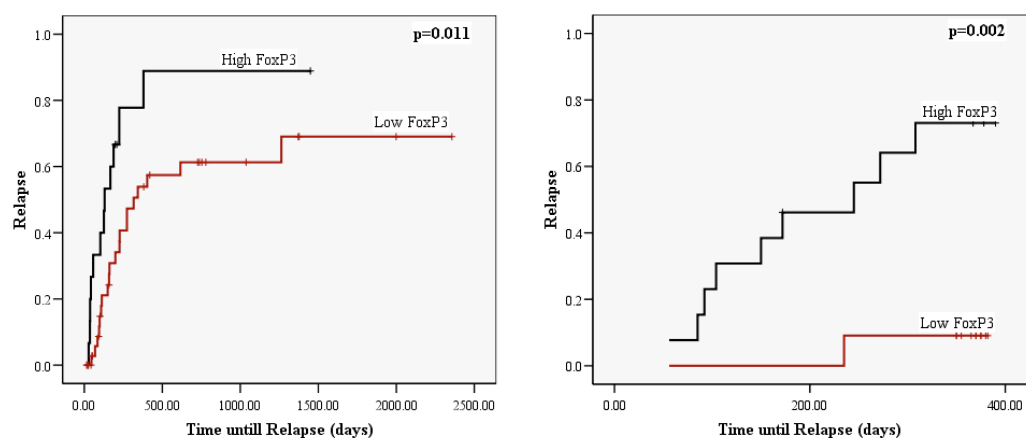


FOXP3 and Relapse

Results with FOXP3 analysis also supported the previous data based on the CD4⁺CD25^{hi} phenotype; high FOXP3 levels were associated with an increased risk of disease relapse in both cohorts. FOXP3^{High} patients had a significant increase in the incidence of disease relapse compared to FOXP3^{Low} patients. The estimated three-year risk of relapse in the first cohort was 80% in FOXP3^{High} and 48.8% in FOXP3^{Low} patients ($p=0.011$) as presented in Figure 5.14; and the estimated one-year risk in the second group was 69.2% in the FOXP3^{High} group and 9.1% in the FOXP3^{Low} group ($p=0.002$) as can be seen in Figure 5.13, supporting the hypothesis that Tregs might be involved in suppressing anti-tumour responses.

Figure 5.13 Relapse rate in patients based on the expression of FOXP3 before transplantation.

An increase of relapse was associated with high expression of FOXP3 in two independent cohorts, the panel on the left is showing the ANT group ($p=0.011$) and the panel on the right is showing the Australian group ($p=0.002$)



Multivariate Analysis

Separate multivariate analyses of transplant outcome were carried out including as factors the pre-transplant CD4⁺CD25^{hi} T cells levels along with clinical variables that have been proven before to be major factors for UD HSC transplant outcome, such as age (>/< 30 years), disease type (CML, AML, ALL and other malignancies), disease status at transplant (early, late or relapse), myeloablative vs. reduced intensity conditioning, stem cell source, and HLA-DPB1 matching status (previously described in our patient group; BE Shaw et al, Blood 2006;107:1220-6). As shown in Table 5.3, the effect of pre-transplant Treg levels on relapse rate (RR= 3.42; P<0.001), DFS (RR= 1.92; P=0.024) and OS (RR= 2.06; P=0.028) remained independent from other factors, and was the most significant factor predicting disease relapse.

Table 5.3 Multivariate analysis of OS, DFS and Relapse.

In a multivariate analysis including clinical factors and level of CD4⁺CD25^{hi} Tregs was the most significant factor involved in transplant outcome.

	<i>Univariate</i>	<i>Multivariate</i>	<i>RR</i>	<i>95% CI lower</i>	<i>Upper</i>
<i>Overall Survival</i>					
Age	<0.001	0.055	1.96	0.99	3.89
Stem cell source	0.011	NS	1.35	0.63	2.89
Conditioning	0.077	NS	1.21	0.61	2.43
Stage	0.002	NS	1.29	0.79	2.1
Treg level	0.024	0.028	2.06	1.08	3.91
<i>Disease Free Survival</i>					
Age	0.002	NS	1.46	0.81	2.65
Stem cell source	0.003	NS	1.51	0.75	3.05
Conditioning	0.053	NS	1.39	0.76	2.56
Stage	<0.001	NS	1.05	0.67	1.65
Treg level	0.017	0.024	1.92	1.09	3.38
<i>Relapse</i>					
Age	0.004	NS	1.44	0.7	2.98
Stem cell source	<0.001	0.023	2.52	1.13	5.61
Conditioning	0.03	NS	1.28	0.62	2.62
Stage	<0.001	NS	1.07	0.62	1.85
Treg level	<0.001	<0.001	3.42	1.78	6.58
NS: Not significant					

The effect of pre-transplant Treg levels on the outcome was virtually identical when FOXP3 levels (higher or lower than the mean) rather than CD4⁺CD25^{hi} phenotype was used for the analysis. Patients with high Treg levels based on FOXP3 expression had an impaired OS (RR: 2.68; p=0.014) and DFS (RR: 2.21; p=0.027) and also an increased risk of relapse (RR: 3.61; p=0.003) as seen in Table 5.4.

Table 5.4 The multivariate analysis.

The results were virtually identical to the multivariate analysis using CD4⁺CD25^{hi} T cell levels, indicating that in fact Treg are playing a pivotal role in transplant outcome

	<i>Univariate</i>	<i>Multivariate</i>	<i>RR</i>	<i>95% CI</i>	<i>lower</i>	<i>upper</i>
<i>Overall Survival</i>						
Age	<0.001	NS	1.86	0.81	4.29	
Stem cell source	0.011	NS	1.19	0.48	2.96	
Conditioning	0.077	NS	0.79	0.32	1.97	
Stage	0.002	NS	0.27	0.69	2.33	
FOXP3 expression	0.024	0.014	2.68	1.22	5.87	
<i>Disease Free Survival</i>						
Age	0.002	NS	1.22	0.61	2.43	
Stem cell source	0.003	NS	1.42	0.62	3.25	
Conditioning	0.053	NS	1.04	0.47	2.26	
Stage	<0.001	NS	1.07	0.62	1.83	
FOXP3 expression	0.017	0.027	2.21	1.09	4.45	
<i>Relapse</i>						
Age	0.004	NS	0.95	0.41	2.22	
Stem cell source	<0.001	0.028	2.96	1.13	7.78	
Conditioning	0.03	NS	0.9	0.35	2.33	
Stage	<0.001	NS	1.24	0.65	2.36	
FOXP3 expression	<0.001	0.003	3.61	1.55	8.42	
NS: Not significant						

Effect of Regulatory T cells by Disease

To better understand the effect of Tregs on the overall outcome of the transplant, the group of patients was divided into two different groups, patients with acute leukaemias and patients with chronic leukaemias, and an analysis of relapse was performed, correlating this independently with CD4⁺CD25^{Hi} T cells and with the expression of FOXP3.

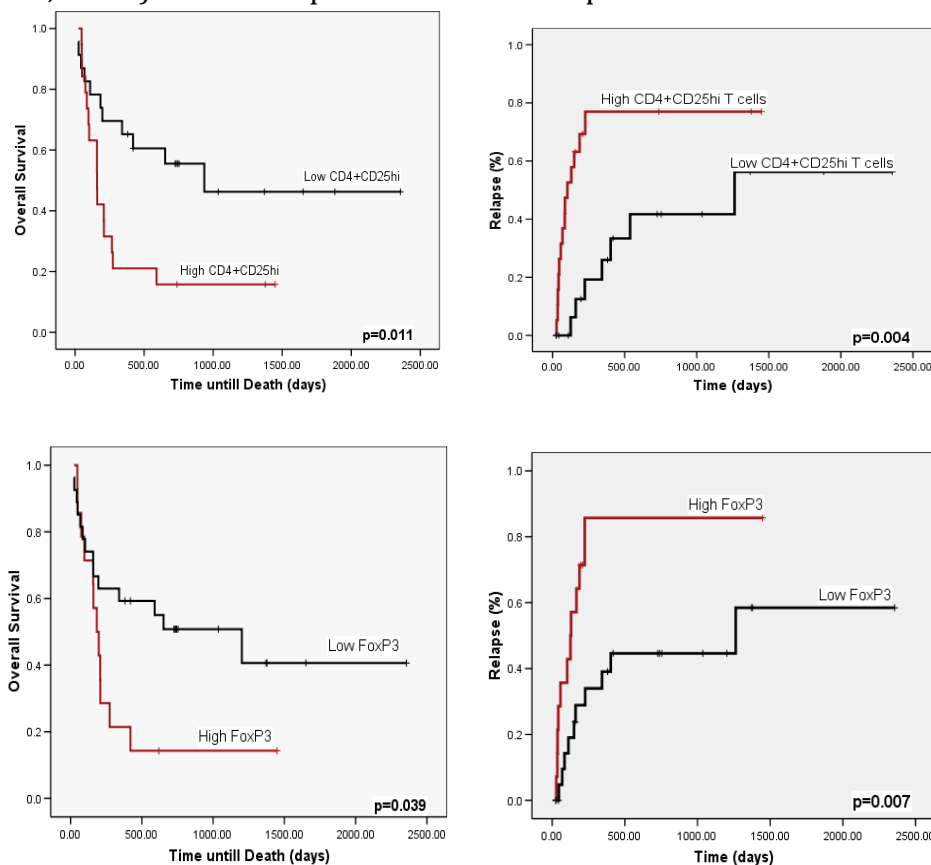
The results showed that the estimated three-year OS for the population of patients with acute leukaemias (n=42) was 36%. The outcome was poorer for the patients with either high CD4⁺CD25^{Hi} Tregs or high expression of FOXP3, at 15.8% and 14.3% respectively overall survival at 3 years compared with patients with low CD4⁺CD25^{Hi} or low FOXP3 expression, whose overall survival was 52.2% and 48.1% respectively. These data are presented in Figure 5.14.

The worse outcome in these patients was due to a high incidence of relapse. The overall relapse rate for the population with acute leukaemias was 45%, with a higher incidence in those patients with high percentages of CD4⁺CD25^{Hi} (65% relapse) or high expression of FOXP3 (58% relapse) in comparison with patients with low levels.

The same analysis was done in patients with chronic leukaemias, unfortunately only one patient had high levels of CD4⁺CD25^{Hi} T cells and high expression of FOXP3, therefore, it was not useful to draw any conclusion. However, this one patient did suffer a relapse and died at 1-year post transplant.

Figure 5.14 Overall Survival (OS) and Relapse in patients from the Anthony Nolan Group with Acute Leukaemias.

A study for survival and relapse was performed in patients with acute leukaemias stratified into two groups based on the levels of CD4⁺CD25^{hi} T cells (Tregs; in the upper two panels) or the expression of FOXP3 (in the lower panels). Results are shown separately for Overall Survival, in the left hand column, and Relapse, in the right hand column. Patients with high levels of Tregs had worse OS (log rank, 0.011) and greater relapse rates (log rank, 0.004) than those patients with low levels. Similar results were found when analysing the expression of FOXP3, patients with high expression of FOXP3 had worse outcome (log rank, 0.039) and greater relapse rates (log rank, 0.007) than those patients with low expression.



Regulatory and Inflammatory cytokines

The mechanism of suppression by Tregs has not been completely understood. Some studies show that the mechanism of suppression is by a contact-dependent manner, while another studies have demonstrated that the suppression can be also in a contact-independent manner. However, it is thought that the main mechanism is cell contact dependent and therefore, the best way to analyse their role is by functional assays.

However, *in vitro* and *in vivo* studies have shown different ways of suppression. On the one hand, *in vitro*, cytokine production has not been proved to be involved in Treg function. On the other hand, *in vivo* studies have shown the opposite, that cytokine production plays an important role in their suppressive functions. The soluble factors that have been involved in Treg suppression by a cell contact-independent manner are suppressive cytokines such as IL-10 and TGF- β .

Due to the lack of material to perform functional assays, we decided to examine the production of suppressive cytokines such as TGF- β and IL-10 that have been proved *in vivo* to contribute to the suppressive function of Tregs and to analyse their correlation with the expression of CD4⁺CD25^{hi} T cells. The analysis of TGF- β along with other cytokines will be reviewed in the next chapter.

Discussion

Natural occurring Tregs have been extensively studied in the context of clinical HSCT mostly in the post-transplant period, where an imbalance of donor derived Tregs and effector T cells has been associated with the occurrence of GvHD. Those studies have led to an increased interest in the potential use of regulatory T cells to modulate post-transplant allo-reactive phenomena. In the context of UD-HSCT, T cell depletion is used to avoid these allo-reactive phenomena. However, the consequence of using this approach may be also detrimental for the transplant outcome, increasing the rates of disease relapse and opportunistic infections. Numerous recent publications have identified donor derived Tregs as an important tool to decrease the incidence of GvHD while preserving the GvL effect. However, very little work has been done in the context of patient derived Tregs. In this study we aimed to analyse the effect of recipient's derived Tregs levels on the post-transplant allo-reactive phenomena and transplant outcome. In contrast to previous publications, our results did not show any impact on preventing GvHD. However, our results do show that high recipient levels of Tregs prior to transplantation predict an increased incidence of disease relapse, which translates into poorer DFS and OS in the UD-HSCT setting. Some questions have arisen trying to understand the mechanism of this Treg effect. A key question that needs to be addressed is whether or not Tregs can survive patient conditioning and T cell depletion? And if they survive, what is the mechanism of their response that causes an increased relapse rate?

Recent data has indicated that T cell depleting agents such as Campath or ATG can spare Tregs from depletion through the induction of anti-apoptotic signals. However, while they are more resistant to apoptosis they retain their

suppressive capacity (Pearl, et al., 2005) (Watanabe, et al., 2006). Along with those studies, it has also been published that Tregs are preferentially expanded under lymphopenic conditions (Cox, et al., 2005). Together, these observations may explain why patient Tregs could be implicated in regulating post-transplant phenomena. In our cohort 90% of the transplants were performed using Campath for T cell depletion, therefore, Tregs may be expanded faster post-transplant.

The other observation from our data was that Tregs were not associated with the incidence of GvHD, but with the incidence of relapse, which was confirmed in a second independent cohort. This would appear paradoxical, but similar results have been obtained by others (Nadal, et al., 2007), which may suggest difference in the capacity of Tregs to regulate limited antigen-specific responses versus complex allogeneic responses.

There have been interesting data published, demonstrating that tumours can induce or recruit Tregs to inhibit anti-tumour responses in murine models (Shimizu, et al., 1999).

In humans, an increased number of Tregs has been found in peripheral blood in many different types of cancers (head and neck, hepatocellular, gastric, breast, ovarian, lung, melanoma, etc.) (Wolf, et al., 2005) (Ascierto, et al., 2010) (Deng, et al., 2010) (Kordasti, et al., 2007) (Kobayashi, et al., 2007) and the level of Tregs is associated with the progression of the disease. Such observations support the idea that tumours can evade immuno-surveillance through the induction of regulatory T cells that can suppress anti-tumour responses. Also very recently data have been published showing that increased Tregs post-transplant in CML patients are associated with an

increase in relapse rate (Nadal, et al., 2007). Whether these Tregs are patient derived or donor derived expanded under the lymphopenic conditions or tumour induced we still do not know.

Another theory is that tumours can produce cytokines such as TGF- β or IL-10 that can induce regulatory T cells to evade tumour-specific responses. In our cohort, we found a good correlation between the frequency of Tregs in peripheral blood and the levels of TGF- β in plasma ($r=0.884$; $p<0.0001$). However, whether this production of TGF- β is Treg derived or tumour derived is not known.

Altogether these findings suggest that an imbalance in favour of recipient-derived Tregs prior to UD-HSCT might have an impact on the outcome through the suppression of donor-derived GvT responses, depending not only on the presence of Tregs but also on the individual microenvironment, such as type of T cell response (Th1 vs. Th2) and cytokine production.

We also found in our data, that patients can be stratified into high and low risk of disease relapse sub-groups based on their level of Treg cells prior to transplantation.

Consequently, it is possible that a strategy based on the depletion of patient Tregs prior to transplant may be beneficial to reduce the risk of relapse and improve the outcome of HSCT in such high-risk patients.

Although our study has opened new questions that need to be resolved, it has reinforced the little knowledge we have on Tregs and has confirmed the position of Tregs as a potential target for therapeutic intervention in the transplantation-field.

Chapter 6

Cytokine profiles and the outcome of haematopoietic stem cell transplant (HSCT)

Introduction

Haematopoietic stem cell transplant (HSCT) is a well-established treatment for many haematological diseases. However, complications such as infections, relapse of the underlying disease and graft versus host disease are still major problems that render difficult the wider use of HSCT. Although significant advances in HLA-matching, along with better patient and donor selection and new conditioning regimes, have improved the outcome, these advances have not completely prevented the development of transplant complications and still there is a significant compromise in the success of the transplant.

Many non-HLA factors have been proposed as being responsible for this lack of complete success, and most likely there is not one single factor responsible, but an interaction between different factors.

Some of the immunological factors that have been suggested as correlating with post-transplant complications and poor transplant outcome include

- delay in immune reconstitution,
- polymorphisms of non-HLA genes including pro-inflammatory cytokines and their receptors and their antagonists,
- cytoplasmic proteins such as NOD2/CARD15 family,
- receptors such as the killer immunoglobulin-like receptor (KIR), immunoregulatory molecules,
- regulatory T-cells.

As shown in the previous chapter, an analysis of the effect of regulatory T cells and its correlation with transplant outcome found that high numbers of regulatory T-cells in the pre-transplant period correlated with worse transplant outcome, poorer disease free survival and a higher incidence of relapse. However, the mechanism of this effect was not clear. In this chapter we will focus in analysing the effects of the pre-transplant cytokine levels seeking to understand the possible effect of regulatory T-cells on cytokine production and the contribution of these cytokines to transplant outcome.

Effects of cytokine levels, cytokine polymorphisms and HSCT outcome

Cytokine polymorphisms affecting the level of cytokine expression, and the dysregulation of the cytokine network that occurs during the transplant procedure, have been associated mainly with the development of acute graft versus host disease (aGvHD), worse transplant outcome and greater transplant related mortality (TRM) (Ferrara, 2000). Host cell damage induced during the conditioning regimen, coupled with the release of endotoxin and other innate stimuli as a consequence of breaching the integrity of the gut, results in an acute inflammatory stimulus that causes T cell activation, the release of pro-inflammatory cytokines and the upregulation of HLA molecules on host cells. The high level of cytokine production that occurs through T-cell activation is called the cytokine storm (Cohen, et al., 2000) (Antin & Ferrara, 1992) (Ferrara, et al., 1993). Proinflammatory cytokines produced during the cytokine storm, such as IFN- γ , TNF- α and IL-6, are implicated in the pathogenesis of aGVHD. On the

other hand anti-inflammatory and immunomodulatory cytokines such as IL-10 and TGF- β are associated with transplant tolerance. Consistent with this, the production of cytokines such as TNF alpha, in the pre-transplant period has been implicated in the induction of transplant complications (Remberger, et al., 1995) (Holler, et al., 1990).

The evidence until now points towards the fact that variation in the level of expression of cytokines, either drug-induced variation or innate variation resulting from polymorphisms in the cytokine genes (and promoters) has an impact on the transplant outcome. Therefore, measurement of the cytokine levels both in the pre-transplant period and the post-transplant period could be an important tool in predicting the overall outcome.

Cytokines and transplant outcome

Pro-inflammatory cytokines such as TNF- α , IL-2, IL-6 and IL-12 are associated with the pathogenesis of GvHD but also with complete remission of advanced tumours (Nair, et al., 2006). There is evidence that IFN- γ has protective role in anti-tumour immunity, exerting anti-proliferative, anti-angiogenic and pro-apoptotic effects in different tumour cells (Beatty & Paterson, 2001) (Ikeda, et al., 2002). Consequently, cytokine therapy has been applied using the anti-tumour cytokines mentioned before (IL-2, IL-12, TNF- α and IFN- γ) in both mice and human with success in eliminating established tumours through the induction of enhanced anti-tumour

responses, promoting activation of T-effector/memory cells and of NK-cells, and increasing secretion of IFN- γ (Egilmez, et al., 2007).

However, long-term efficacy of this treatment has not been achieved. The failure of the cytokine therapy is due an augmentation of suppressive activity post-treatment that initiates the decline of the anti-tumour effect (Egilmez, et al., 2007).

The relationship between transplant outcome and cytokine production has been further complicated by the suggestion that tumours secrete anti-inflammatory cytokines such as IL-10 and TGF- β that induce the recruitment of regulatory T cells, including naturally occurring Tregs, Tr1 and Th3 (Nair, et al, 2006). Moreover, some tumours have been shown to express indoleamine-2,3-dioxygenase (IDO), an enzyme which depletes tryptophan from the local environment and induces the production of regulatory T cells (Curti, et al., 2006). Regulatory T cells secrete more anti-inflammatory cytokines that further induce the conversion of CD4 T-cells into Tregs. The infiltration of regulatory T cells and the production of an anti-inflammatory environment decrease immune surveillance and neutralise and reduce potential anti-tumour activity inducing relapse or recurrence of the original tumour (Nair, et al, 2006). Regulatory cells also have a key role in regulating cancer progression by inhibition of CD4⁺ or CD8⁺ T cell responses. Another mechanism of Treg suppression which requires TGF- β and IL-10 (Larmonier, et al., 2007) is by inhibiting dendritic cell function (DC's) causing down-regulation of TNF- α and IL-12 expression. In addition, it has been demonstrated that depletion of regulatory T cells enhances anti-tumour responses, inducing complete remission. In the previous chapter, a

correlation of regulatory T cells and transplant outcome was demonstrated. To understand how this might be mediated, and to verify that the increased Treg numbers seen in some patients correlated with increased Treg function, an analysis of cytokine profiles in the pre-transplant serum samples was carried out, concentrating on pro-inflammatory cytokines versus anti-inflammatory cytokines.

The range of cytokines that were included in the analysis were: tumour necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 17 (IL-17), interleukin 10 (IL-10) and transforming growing factor beta (TGF- β). These cytokines were chosen to be representative of inflammatory (TNF- α , IFN- γ , IL-1, IL-6, IL-12, IL-17), helper (IL-4, IL-10) and regulatory (TGF- β , IL-10) immune responses. Note that not all of these cytokines are necessarily expressed by T cells; for example, IL-1 can be produced by a wide variety of cells including endothelial cells, while IL-10 can be produced by monocytes and cytotoxic CD8⁺ T cells as well as Th2 and Treg cells.

In the next section, the known associations of these cytokines with HSCT outcome will be outlined.

Pro-inflammatory cytokines

Tumour Necrosis Factor alpha (TNF- α)

TNF- α is a pro-inflammatory cytokine implicated in the pathogenesis of inflammatory or autoimmune diseases; anti-TNF- α has been used as

treatment for rheumatoid arthritis (Bongartz, et al., 2006). It is also implicated in tumour growth control and, furthermore TNF- α has been associated with early stages of aGvHD. High levels of TNF- α in serum are correlated with the severity of aGvHD and elevated plasma levels during pre-transplant conditioning are associated with the development of aGvHD, worse transplant outcome and increased TRM (Remberger, et al., 1995). Furthermore, it has been reported that neutralization of TNF- α reduces complications after HSC transplantation (Holler, et al., 1993). The relative concentration of TNF- α has also been correlated with the severity of both acute and chronic GvHD. (Holler, et al., 1990) (Hägglund, et al., 1995) (Cavet, et al., 1999) (Takahashi, et al., 2000) (Rocha, et al., 2002) (Ochs, et al., 1996). A polymorphism has been identified in the TNFA gene associated with a change in TNF- α secretion; there is a single base polymorphism (G/A) in position -308 of the promoter region (Wilson, et al., 1992) and the (A) allele is correlated with increased production of TNF- α caused by higher transcriptional activation. Thus, genetic factors as well as other factors that cause increased production of TNF- α could be implicated in the development of acute or chronic GvHD, TRM and poorer transplant outcome (Holler, et al., 1993) (Holler, et al., 1990).

However, as with many immune phenomena, there may be benefits as well as disadvantages associated with elevated TNF- α production; there is evidence that TNF- α can be used as a therapy to promote tumour regression by induction of effector T-cells (Egilmez, et al., 2007).

Interferon gamma (IFN- γ)

IFN- γ is a pro-inflammatory cytokine that has been associated with transplant complications. Polymorphisms in the IFN- γ gene (IFNG) generate changes in levels of production (Pravica, et al., 1999) (Reynard, et al., 2000), giving rise to a variation in how individual patients might respond to the same set of conditions. High serum levels of IFN- γ have been implicated in the pathogenesis and increased incidence of aGvHD in humans (Szebeni, et al., 1994) (Sykes, 1993) (Wang, et al., 1995) (Troutt & Kelso, 1992) (Cavet, et al., 2001). INF- γ is suggested to have an important role in anti-tumour responses (Beatty & Paterson, 2001) (Ikeda, et al., 2002) while the presence of a specific IFN- γ haplotype (low-producing haplotype) has been associated with post-transplant viral complications (Bogunia-Kubik, et al., 2006).

Interleukin-1

IL-1 is involved in inflammatory processes. IL-1 α , IL-1 β and interleukin-1 receptor α -agonist (IL-1Ra) are well-characterised mediators of immune and inflammatory responses (Dinarello, 2009).

Interleukin-2 (IL-2)

The importance of IL-2 in the initiation of GvHD has been demonstrated in both experimental and clinical transplantation (Theobald, et al., 1992). For example, IL-2 secretion could be used as a predictive factor for the risk of aGvHD (Theobald, et al., 1992). Soluble IL-2 receptor levels may be a sensitive indicator of impending GvHD onset and correlate with disease severity.

Interleukin-6 (IL-6)

High serum levels of Interleukin 6 (IL-6) are associated with both acute and chronic GvHD (Cavet, et al., 2001), and it has been demonstrated that a (C/G) polymorphism in position -174 of the IL6 gene associated with high levels of production of IL-6 is implicated in the development of higher grades of aGvHD but does not increase the severity (Cavet, et al., 2001). Also IL-6 polymorphisms have been associated with the onset of cGvHD (Symington, et al., 1992) (Abdallah, et al., 1997) (Tanaka, et al., 1995) (Socié, et al., 2001). IL-6 may also play a role in mediating the balance between regulatory and inflammatory responses, diverting CD4 T cells from the Treg pathway to become pro-inflammatory Th17 cells (Kitani & Xu, 2008).

Interleukin-12 (IL-12)

IL-12 is a potent pro-inflammatory cytokine and is involved in the specification of activated naïve CD4 T cells to differentiate into Th1 T cells. It has been shown to have an anti-tumour effect in murine models (Reddy, et al., 2005). When this was tested in humans undergoing HSCT, Reddy et al showed that high levels of IL-12 post-transplant were associated with a lower incidence of relapse and improved disease free survival (DSF) without increasing the risk of GvHD, in comparison with those patients with low levels who showed higher relapse rates (Reddy, et al., 2005). This is consistent with a beneficial anti-tumour effect of high IL-12 levels. Moreover, IL-12 injections in murine models have been shown to induce tumour regression. Therefore, there is a pivotal role of IL-12 in inducing anti-tumour immunity (Nair, et al., 2006).

Interleukin-17 (IL-17)

IL-17 is a cytokine produced by T-cells that stimulates macrophages to secrete pro-inflammatory cytokines such as IL-1 β and TNF- α and induces T-cells to proliferate. It has been suggested that that IL-17 may have a major role in inducing allo-immune reactivity (Antonysamy, et al., 1999).

Anti-inflammatory cytokines

Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is a highly polymorphic immunoregulatory cytokine, which suppresses allo-antigen specific T-cell responses and is strongly anti-inflammatory (Hempel, et al., 1997). IL-10 inhibits the secretion of cytokines such as IL-1, IL-6, IL-8, IL-12 and also TNF- α (Steven, et al., 2000). This inhibitory property of the IL-10 could suggest that it could act as to suppress GvHD and it could induce T cell tolerance in the HSC transplant setting. Several studies have demonstrated that increased IL-10 production is associated with fewer transplant related complications (Remberger & Ringdén, 1997). Holler et al. (Holler, et al., 2000) demonstrated the predictive value of IL-10, showing that increased IL-10 production was associated with better transplant outcome in the absence of GvHD. Also it has been reported that a high level of IL-10 at the time of donor infusion might induce a state of anergy (Willems, et al., 1994)

Exposure to IL-10 also induces regulatory T-cells (Tr1 and Th3) in the periphery, which can be identified by cytokine production (IL-10 and TGF- β); these cytokines act to suppress undesired immune responses (Roncarolo, et al., 2006). Host Tr1 cells correlated with absence of GvHD and long term tolerance in HSCT patients (Bacchetta, et al., 1994) but on the other hand, both IL-10 and TGF- β are associated with inhibition of anti-tumour responses by effector T-cells and induction of tumour recurrence.

Transforming Growing Factor beta (TGF- β)

Transforming growing factor beta (TGF- β) polymorphism has been associated with variation in plasma concentration of TGF- β . The picture with respect to TGF- β and transplant outcome is not clear, and contradictory results have been published. In some studies, neither TGF- β polymorphisms nor plasma levels of the cytokine were found to be associated with transplant complications or transplant outcome (Cavet, et al., 2001) (Grainger, et al., 1999). However, other groups have published that a high expression phenotype of TGF- β was associated with severe and chronic GvHD (Leffell, et al., 2001) (Liem, et al., 1998).

As mentioned in the previous section, TGF- β also has been involved in suppressing anti-tumour immune responses (Seoane, 2008), and it has been implicated as an important factor for tumour progression (Medicherla, et al., 2007) by inducing FoxP3 to promote differentiation of non-regulatory T-cells into regulatory T-cells (Pyzik & Piccirillo, 2007).

This study is focused on the measurement, in the pre-transplant period, of the levels of the cytokines described above and the hypothesis is based on the study described in the previous chapter of this thesis, where higher levels of pre-transplant regulatory T-cells were observed to have a deleterious impact on transplant outcome and increased relapse rate. Here we suggest the effect of Tregs is part of a wider balance of pro-inflammatory cytokines *versus* regulatory cytokines that has an impact on transplant complications such that the overall skewing of the cytokine profile towards immune-regulation has detrimental effect on relapse rates.

Materials and Methods

Patients

The study group consisted of 62 patients undergoing HSCT from an unrelated donor (UD-HSCT) identified through the Anthony Nolan Trust (ANT) donor registry. 16 donors from the registry with the same age range as the patients were analysed as a control group. Both recipients and donors were required to have 4-digit allele typing results at six HLA loci (HLA-A, -B, -C, -DRB1, -DQB1, -DPB1). Because the study was registry based, blood samples and clinical data were provided by the individual transplantation centres (TCs). Clinical data were collected on standard forms, which were collected from data managers at the individual TCs. A basic description of the cohort including patient age, sex, disease and conditioning is presented in Table 6.1. Forty patients (65%) received a standard conditioning regimen; 20 had reduced intensity conditioning (RIC) (32%).

Analysis of Cytokine levels in plasma samples

The cytometric bead array technology (Luminex®) has been applied recently for the simultaneous measurement of a number of different biomarkers including cytokines.

In a multiplex method, the measurement of 9 cytokines (IL-1 β , IL-2, IL-4, IL-10, IL-12p70, IL-15, IL-17, TNF- α and IFN- γ) was quantified simultaneously using a Human cytokine/chemokine panel-9 plex (Lincoplex kit, Linco

Research) in 50 μ l of undiluted plasma. TGF- β requires a pre-treated plasma sample and for that reason TGF- β was measured separately with a singleplex kit also from Linco Research.

Standards and antibodies were also purchased from Linco Research. (Lincoplex kits, Billerica, Ma).

Cytokine quantification was done from plasma samples according with manufacturers instructions and measurements were carried out in duplicate. Standard curves for each cytokine were created ranging from 3.2 to 10,000 pg/ml. The standard curve for TGF- β quantification range from 0.064 to 200 ng/ml.

Table 6.1 Patients and transplant characteristics

<i>Characteristics</i>	<i>N=62 (%)</i>	<i>Controls N=16</i>
Patient age (years)	32.55±2.03	27±2.01
Donor age (years)	33.75±0.79	
Sex Matched	37 (60)	
M patient/F donor	3 (4)	
F patient/M donor	22 (36)	
F/M		9/7
CMV status		
Patient +/Donor +	6 (10)	
Patient -/Donor -	39 (63)	
Patient +/Donor -	8 (13)	
Patient -/Donor +	7 (11)	
Missing	2 (3)	
Disease		
CML	20 (32)	
AML	11 (18)	
ALL	10 (16)	
Other malignant	18 (29)	
Other non-malignant	3 (5)	
Stage at transplant		
Early	38 (61)	
Late	14 (23)	
Relapse	6 (10)	
Missing	4 (6)	
Conditioning regimen		
Standard myeloablative	40 (65)	
Reduce intensity (RIC)	20 (32)	
Missing	2 (3)	
Type of Transplant		
Bone Marrow	51 (82)	
PBMC's	11 (18)	

Statistical analysis

Survival estimation was done by Kaplan-Meier analysis using SPSS software (version 14; Chicago, IL). P values less than 0.05 were regarded as statistically significant, those between 0.05 and 0.1 were considered as indicative of a trend. Disease free survival (DFS) was defined as the time from study entry to a documented progression or death without disease. Overall survival (OS) was defined as the time from study entry to death from any cause. Both survival times were analysed using the Kaplan-Meier method. Univariate analysis of different immunological factors and clinical factors were done using the two-side log-rank test. Patients were segregated based on the mean value of each factor.

Multivariate analysis for DFS, OS and relapse used the stepwise proportional hazards regression method. These models included those cytokines that had shown significant associations in the univariate analyses (at a significance level of $p < 0.05$) along with other clinical variables considered predictors of clinical relevance. The significance of the models was evaluated with the likelihood ratio test.

Results

Cytokine levels in patients and controls

The levels of the panel of cytokines were measured from peripheral blood samples of patients and healthy controls and the values were compared between patients and controls for significant differences in the level of circulating cytokines. The results, presented in Table 6.2, show that only four cytokines, IL-10, TNF- α , IFN- γ and TGF- β , showed significant differences between patients and controls.

The levels of IL-10 were statistically significant higher ($p= 0.0005$) in the patients than in healthy controls (means of 109.13pg/ml and 17.55pg/ml respectively).

The same pattern was observed when IFN- γ was analysed. The healthy controls have lower levels of IFN- γ (mean of 0.34pg/ml) than patients (mean of 2.9pg/ml). The difference was also statistically significant ($p= 0.009$).

The mean level of TNF- α in healthy controls was 1.32 pg/ml (range 0 to 21.1 pg/ml), while the levels in patients were significantly higher, with a mean of 165.16pg/ml (range 0 to 1485.47). The difference was statistically significant ($p<0.0001$, Mann Whitney U test).

The mean levels of TGF- β in healthy controls was 6.62 ng/ml (range 0 to 31.86 ng/ml) and was statistically significant ($p<0.0001$) when it was compared with patients mean levels (258.09pg/ml, range 0-2674.76).

None of the other cytokines analysed (IL-1 β , IL-2, IL-4, IL-12p70, IL-15 and IL-17) showed significant differences between donors and patients.

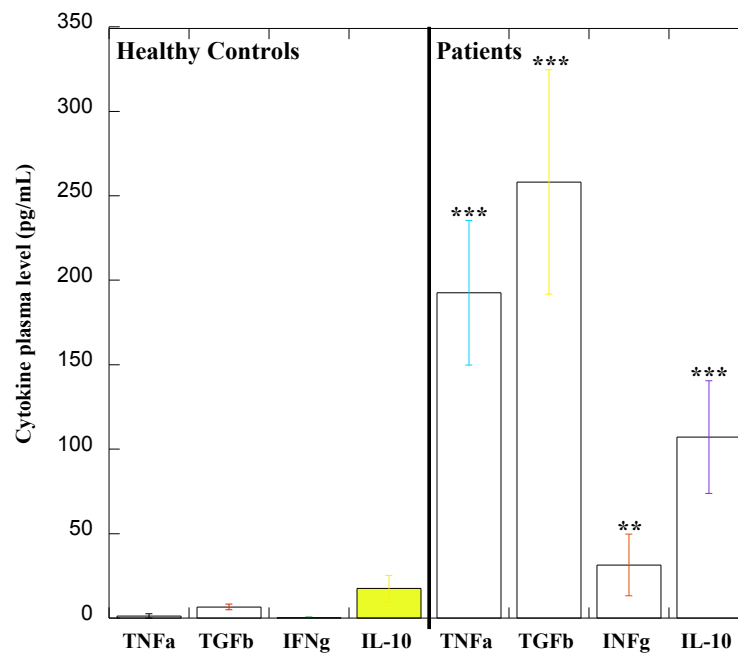
Table 6.2 Cytokine in plasma

Comparison of cytokine plasma levels in patients and healthy controls.

Cytokine	Patients (n= 62)	Controls (n= 16)	
	Mean±SE	Mean±SE	Mann-Whitney Test (<i>p value</i>)
IL-1 β	8.27±2.93pg/ml	2.28±1.40pg/ml	<i>p=NS</i>
IL-2	12.54±6.09pg/ml	7.93±6.17pg/ml	<i>p=NS</i>
IL-4	168.78±53.30pg/ ml	93.59±36.49pg/ml	<i>p=NS</i>
IL-10	109.13±34.09pg/ml	17.55±7.84pg/ml	<i>p=0.0005</i>
IL12p70	41.07±20.06pg/ml	13.33±7.71pg/ml	<i>p=NS</i>
IL-15	313.15±231.10pg/ml	7.81±5.34pg/ml	<i>p=NS</i>
IL-17	21.16±8.22pg/ml	21.20±8.23pg/ml	<i>p=NS</i>
TNF-alpha	165.16±32.93pg/ml	1.32±1.32pg/ml	<i>p<0.0001</i>
IFN-gamma	2.92±0.49pg/ml	0.34±0.34pg/ml	<i>p=0.009</i>
TGF-beta	258.09±66.41ng/ml	6.62±1.74ng/ml	<i>p<0.0001</i>

Figure 6.1 Summary of mean pre-transplant levels of selected cytokines and comparison of the cytokine levels between patients undergoing HSCT (n= 62) and healthy controls (n=16).

In this graph the circulating levels of TNF- α , TGF- β , IFN- γ , and IL-10 are shown. These cytokines showed statistically significant difference in plasma levels between patients and donors (p values of <0.0001, <0.0001, 0.007 and 0.0005 respectively), in the graph the difference are appreciated very clearly that patients undergoing UD-HSCT have higher levels than healthy controls. TGF- β levels are expressed in ng/ml.



Cytokine levels and HSCT outcome

As discussed in the introduction, various authors have suggested correlations between cytokines, their level of expression and the outcome of HSCT. In the previous chapter, an association between high levels of Treg and an increased risk of relapse mortality was identified. To verify whether this was reflected in the profile of cytokines, particularly those characteristic of Treg function, the levels of various cytokines in the pre-transplant samples were measured and, while these showed an increase in TGF- β and IL-10, which would be consistent with increased Treg activity, they also showed an increase in TNF- α and IFN- γ , which are characteristic of inflammatory, type 1 immune responses. Based on the results of the previous chapter, an analysis was therefore carried out to determine whether there was any correlation between the pre-transplant levels of anti-inflammatory and pro-inflammatory cytokines and the outcome for the patients in the cohort.

The patient cohort was described in the methods section above and the patient characteristics are shown in Table 6.1. The median follow-up of the cohort was 1048.73 days and the overall survival (OS) was 40.3%.

A comparison was done between the patients who relapsed and patients who did not relapse, finding that the group that relapsed had lower levels of TNF- α than the group that did not relapse ($p=0.035$, Mann Whitney U test). Subsequently, patients were divided into two groups according to TNF- α plasma levels, the patients with levels of TNF- α higher than the mean, 165.16pg/ml formed the high-TNF- α group ($n=46$), while the patients with

cytokines levels below that level were considered the low-TNF- α group (n=16).

Comparing these two groups, 61.5% of the patients from the low-TNF- α group relapsed, while only the 40% from the high-TNF- α group did (p=0.031, log rank test) as presented in Figure 6.2. These findings are consistent with previously published work of Remberger et al, who also showed a correlation between TNF- α levels and relapse (Remberger, et al., 1995).

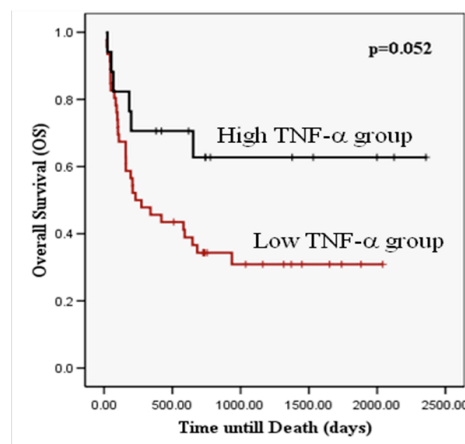
High levels of TNF- α showed also a trend towards better survival than those patients with low levels (64.7% vs. 32.6%; p=0.052). High levels were also correlated with improved DFS (p=0.031).

Accordingly to Holler et al, increases of TNF- α levels before HSCT has been proved to be predictive for development of transplant complications within the first year post-transplant (Holler E. , et al., 1990). However, although variation in TNF- α levels have been strongly associated with the onset of chronic and acute GvHD and with higher incidence of transplant related mortality (TRM), we did not find an association between TNF- α and the onset of either acute or chronic GvHD or TRM.

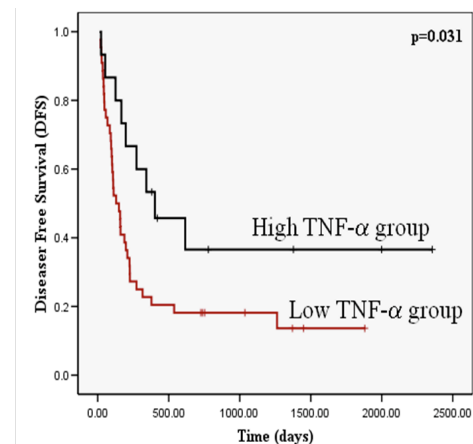
Figure 6.2 TNF- α and transplant outcome.

Survival curves showing the impact of the levels of TNF- α in Overall Survival (OS), Disease Free Survival (DFS) and risk of relapse when patients are stratified into a high-TNF- α group and a low-TNF- α group.

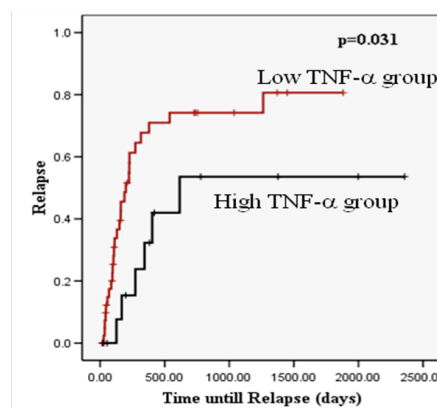
The high level group has better OS with lower rates of relapse and improved DFS.



a) Association of TNF- α and OS



b) Association of TNF- α and DFS



c) Association of TNF- α and Relapse

The cohort was also stratified based on their plasma levels of IL-10. The high-IL-10 group (n=47), when IL-10 plasma levels were above the mean

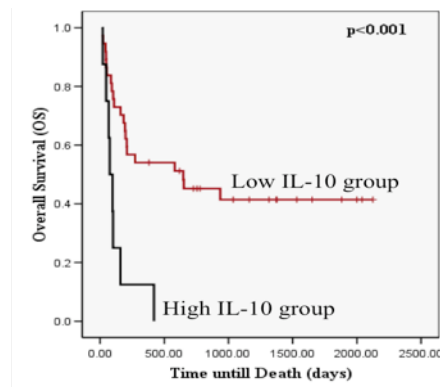
(109.13pg/ml) and the low-IL-10 group (n=15), when plasma levels were below the mean.

The high-IL-10 group did not show any association with the onset of either acute or chronic GvHD. In addition, there was no significant association with TRM.

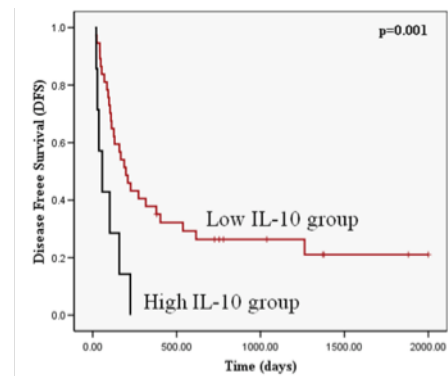
In contrast to published work suggesting that high levels of IL-10 pre-transplant could have a protective effect on transplant outcome; in this cohort the low-IL-10 group had significantly better overall survival than the high-IL-10 group (43.2% versus 0%; $p < 0.001$); all patients in the high group died within the first year following transplantation. Disease free survival (DFS) was impaired in the high-IL-10 group; all patients on this group have died or have relapsed within the first year post-transplant. Relapse rate was also higher in the high-IL-10 group than in the low-IL-10 group, however, the difference is not strong to draw significant conclusions (57.1% versus 51.4%, $p = 0.010$) as is presented in Figure 6.3. The high-IL-10 group was also associated with faster engraftment in comparison with the low-IL-10 group (16.4 days versus 21.5 days; $p = 0.002$). Engraftment after HSCT is define as obtaining an absolute neutrophil count of $>500/\text{ml}$.

Figure 6.3 The impact of IL-10 levels in transplant outcome.

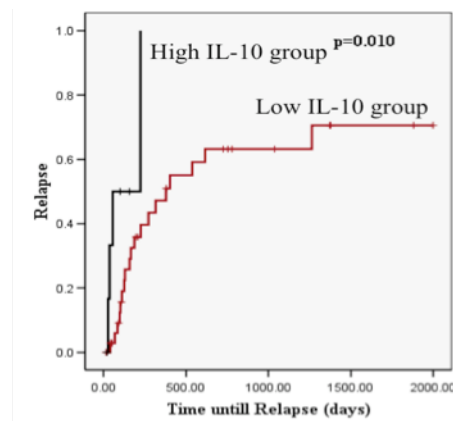
High levels of IL-10 are associated with worse overall survival due to greater relapse rates. Patients with low-IL-10 plasma levels have significantly better overall survival ($p < 0.001$, log rank test), less DFS ($p = 0.001$, log rank test) and lower relapse rates ($p = 0.010$, log rank test). Relapse occurs within the first year post-transplant. Low levels of IL-10 could be associated with better specific allo-responses that contribute to the graft versus leukaemia effect (GvL). High levels of IL-10 also contributes to faster engraftment ($p = 0.002$, log rank test).



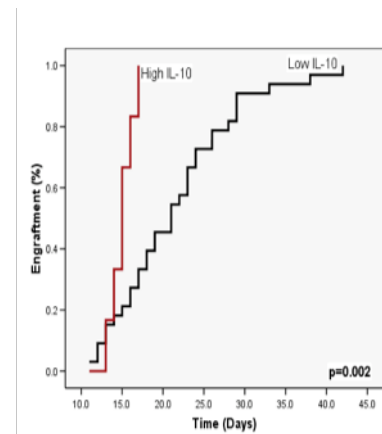
a) Association of IL-10 and OS



b) Association of IL-10 and DFS



c) Association of IL-10 and Relapse



d) Association of IL-10 and engraftment

Transforming growth factor beta (TGF- β) is a regulatory cytokine; it modulates many immune responses, especially inflammatory responses, where it antagonises the functions of TNF- α and other pro-inflammatory cytokines. In the context of HSCT, if TNF- α promotes aGvHD then since TGF- β is a regulatory cytokine, it should inhibit the onset of GvHD; however, in contradiction to that, TGF- β has also been implicated in the onset of aGvHD (Visentainer, et al., 2003).

To analyse the association of TGF- β with transplant outcome, the cohort was stratified into a high TGF- β group with concentrations of TGF- β levels in plasma greater than 258.09ng/ml (the mean value in the cohort) and a low TGF- β group with plasma concentration below the mean.

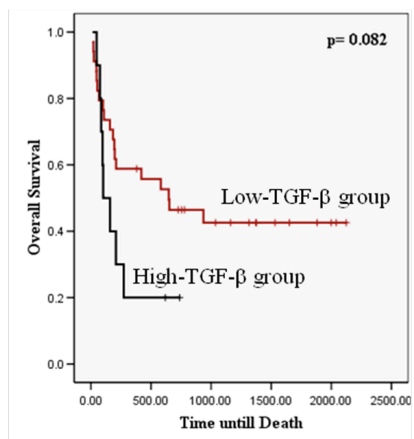
Pre-transplant TGF- β levels did not show a correlation with TRM, neither did they show a correlation to acute nor chronic GvHD, in contrast with what has been published from other studies (Cavet, et al., 2001) (Grainger, et al., 1999).

Low levels of TGF- β showed a trend towards better survival (44.1% versus 20.0%; $p=0.082$). Low levels of TGF- β correlated significantly with better DFS (27.3% versus 10.0%; $p=0.026$); the data is presented in Figure 6.4.

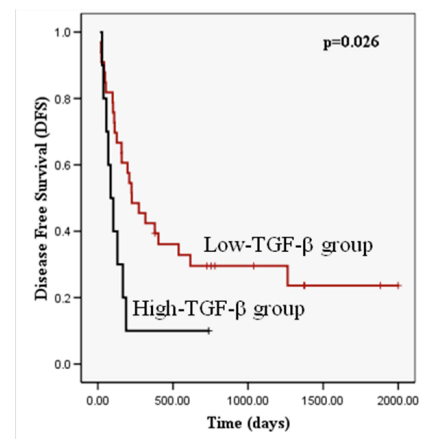
The worse overall and disease free survival in the high TGF- β cohort was correlated with higher rates of relapse, with a strikingly high correlation between high levels of TGF- β and higher risk of relapse (90% versus 42.4%; $p<0.001$), as shown in Figure 6.5.

Figure 6.4 Probability of overall survival and disease free survival (DFS) according to plasma levels of TGF- β levels in patients prior HSCT.

Patients with low-TGF- β levels showed a trend towards better overall survival in comparison of patients with high levels (44.1% versus 20.0%; $p=0.082$) as it is presented in panel a. However, patients with low-TGF- β levels showed a definitively better DFS than patients with high levels (27.3% versus 10.0%; $p=0.026$), presented in panel b.



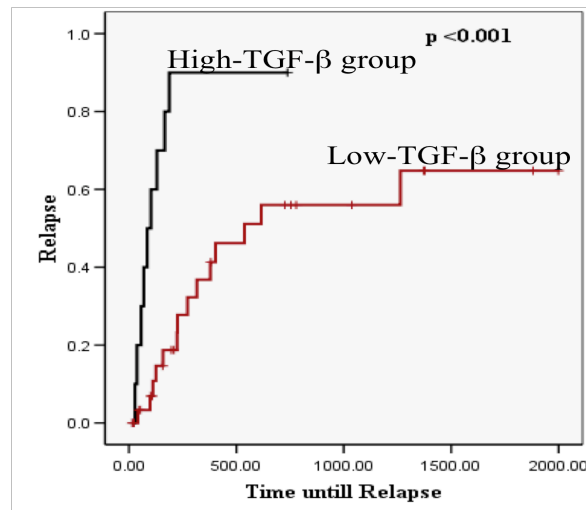
a) Association of TGF- β and OS



b) Association of TGF- β and DFS

Figure 6.5 The levels of TGF- β are associated with relapse risk.

Patients with levels of TGF- β above the mean (258.09ng/ml) in plasma pre-conditioning have higher risk of relapse than patients with levels below the mean $p < 0.001$. The higher risk of relapse could be associated to the immune-regulating function of TGF- β .



As for the cytokines described previously, TNF- α , IL-10 and TGF- β , patients were grouped based on the mean plasma levels of IFN- γ ; a high-IFN- γ group with levels above the mean and a low-IFN- γ group with plasma levels lower than the mean.

Although IFN- γ has been reported to be associated with the onset of acute or chronic GvHD, this was not demonstrated in the analysis of this cohort of patients.

Moreover, no correlation was found with overall survival (OS) or disease free survival (DFS) nor were the rates of relapse different between the high- or low-group for IFN- γ .

The pre-conditioning plasma levels of the rest of the cytokines analysed (IL-1 β , IL-2, IL-4, IL-12p70, IL-15 and IL-17) showed no correlation with transplant outcome. However, IL-4, IL-2 and IL-12p70 had an impact on engraftment. The high-IL-4 group had significantly faster engraftment in comparison with the low-IL-4 group (15.4 days versus 21.5 days; $p=0.002$). The engraftment was also faster in the high IL-2 group than the low-IL-2 group (16.2 days versus 21.2 days; $p=0.029$). The same was found with IL-12p70, the high-IL12p70 group had also a trend towards faster engraftment in comparison with the low-group (16.20 days versus 21.08 days; $p=0.041$).

A summary of the results of the impact of cytokines on transplant outcome are presented in Table 6.3 for those cytokines where a significant association was identified.

Table 6.3 Cytokines and outcome.

	Overall Survival (OS)	Disease Free Survival (DFS)	Relapse	Chronic GvHD (cGvHD)	Engraftment
	<i>p</i> value	<i>P</i> value	<i>p</i> value	<i>P</i> value	<i>p</i> value
TNF- α	0.052	0.031	0.031	NS	NS
TGF- β	0.082	0.026	<0.001	NS	NS
IFN- γ	NS	0.081	NS	NS	NS
IL-10	<0.001	0.001	0.010	NS	0.002
IL-4	NS	NS	NS	NS	0.002
IL-2	NS	NS	NS	NS	0.029
IL-12p70	NS	NS	NS	NS	0.041

To summarise, we present here that high levels of regulatory cytokines (IL-10 and TGF- β) predispose to a decrease in both overall survival and disease free survival after transplant; this impairment is due to higher relapse rates. On the other hand, a high level of pro-inflammatory cytokines such as TNF- α , is associated with better transplant outcome, without increasing the incidence of acute or chronic GvHD. Also, we found that high levels of IL-2, IL-4, IL-10 and IL-12p70 are associated with a faster engraftment.

These data suggests that the analysis of cytokines in the pre-transplant period could be used as a prognostic tool for predicting transplant outcome,

with patients at higher risk of relapse having a regulatory pattern of cytokines, with high TGF- β and/or IL-10 and low TNF- α and IFN- γ while patients at lower risk of relapse showed the opposite pattern, with higher levels of TNF- α and IFN- γ . However, these correlations are not causation, and both the cytokine levels and the transplant outcome could depend upon some other underlying variable. Therefore, a multivariate analysis was performed to calculate the impact of cytokine levels on overall survival (OS), disease free survival (DFS) and relapse simultaneously with other clinical factors, which have previously been shown to be important in transplant outcome. The clinical factors analysed were the age of the patient, underlying disease, stage of disease, conditioning regimen used and stem cell source (Peripheral blood mononuclear cells (PBMC's) vs. bone marrow (BM)). All of these factors were statistically significant in an univariate analysis, as shown in Table 6. 4.

Table 6.4 Factors affecting transplant outcome.

	Overall Survival (OS)	Disease Free Survival (DFS)	Relapse	Chronic GvHD (cGvHD)
	<i>p value</i>	<i>p value</i>	<i>p value</i>	<i>p value</i>
Age of patient	<0.001	0.002	<0.004	NS
Disease	NS	NS	NS	NS
Stage at transplant	0.002	<0.001	<0.001	NS
Conditioning (standard vs. RIC)	0.077	0.053	0.030	NS
Stem cell source (PBMC's vs. BM)	0.011	0.003	<0.001	NS

RIC = reduce intensity conditioning

PBMC's = peripheral blood mononuclear cells

BM = bone marrow

Multivariate analysis

In the multivariate analysis levels of IL-10 were the most significant (RR, 5.48; $p= 0.008$) for predicting OS but also patient age showed to be significant for OS (RR, 1.05; $p= 0.02$), the data is presented in Table 6.5.

Multivariate analysis identified two independent prognostic factors for disease free survival (DFS), in the following order of significance: patient age (RR, 1.06; $P = 0.003$) and conditioning regimen (RR, 0.201; $p= 0.03$). TGF- β levels and stem cell source did not reach statistical significance (RR, 2.60; $p=0.08$ and RR, 2.72; $p=0.07$ respectively). The results of this analysis are presented in Table 6.6.

In the multivariate analysis for the risk of relapse the factors that retained significant prognostic significance were TGF- β (RR, 12.38; $p= 0.0004$) and IL-10 (RR, 50.8; $p= 0.03$), as shown in Table 6.7.

Table 6.5 Multivariate analysis for OS

Multivariate analysis for Overall Survival (OS) including both clinical factors and soluble factors. The most important predictive factor was the level of IL-10.

<i>Variables for OS</i>	<i>p value</i>	<i>Odds ratio</i>	<i>95.0% CI</i>	
			<i>Lower</i>	<i>Upper</i>
Patient age	0.023	1.05	1.00659	1.09743
Stage of disease	NS	1.05	0.87109	1.27188
Conditioning regimen	NS	0.41	0.09156	1.91463
Stem cell source	NS	0.43	0.12363	1.54098
TNF- α	NS	0.72	0.26482	1.97103
TGF- β	NS	2.03	0.65406	6.35291
IL-10	0.008	4.58	1.48179	14.1852

RIC = reduce intensity conditioning

PBMC's = peripheral blood mononuclear cells

BM = bone marrow

Table 6.6. Multivariate analysis for DFS.

Multivariate analysis for disease free survival. Patient age was the most important prognostic factor for DFS, followed by type of conditioning (RIC vs. Standard).

<i>Variables for DFS</i>	<i>p value</i>	<i>RR</i>	<i>95.0% CI</i>	
			<i>Lower</i>	<i>Upper</i>
Patient age	0.003	1.06	1.01914	1.10446
Stage of disease	NS	1.13	0.95282	1.35484
Conditioning regimen	0.039	0.20	0.0439	0.92242
Stem cell source	0.080	2.72	0.88458	8.38451
TNF- α	NS	0.42	0.13753	1.329
TGF- β	0.075	2.60	0.90714	7.48084
IL-10	NS	0.59	0.24471	1.45707
IFN- γ	NS	0.61	0.24202	1.56778

RIC = reduce intensity conditioning

PBMC's = peripheral blood mononuclear cells

BM = bone marrow

Table 6.7 Multivariate analysis for Relapse

Multivariate analysis to assess prognostic factors for the risk of relapse. The most important factors for relapse were the levels of TGF- β and IL-10.

<i>Variables for Relapse</i>	<i>p value</i>	<i>RR</i>	<i>95.0% CI</i>	
			<i>Lower</i>	<i>Upper</i>
Patient age	0.07	1.04	0.99535	1.0987
Stage of disease	NS	1.02	0.82624	1.28259
Conditioning regimen	NS	0.67	0.10052	4.56136
Stem cell source	NS	0.56	0.15021	2.09699
TNF- α	NS	0.46	0.1588	1.39068
TGF- β	0.0004	12.37	3.0898	49.5837
IL-10	0.032	5.07	1.15009	22.4072

RIC = reduce intensity conditioning

PBMC's = peripheral blood mononuclear cells

BM = bone marrow

Discussion

The clinical relevance of cytokine levels in the pre-conditioning period and in the post-transplant period for the onset of GvHD has been established by different groups (Holler, et al., 1993) (Willems, et al., 1994). While some cytokines, such as TNF- α , have been clearly demonstrated to play a role in the onset of GvHD, in transplant related mortality (TRM) and in the induction of anti-tumour T-cell responses, the role of other cytokines, and especially the regulatory cytokines, is not yet clear.

Here 10 different cytokines (TNF- α , IFN- γ , TGF- β , IL-1 β , IL-2, IL-4, IL-10, IL-12p70, IL-15 and IL-17) were measured in pre-conditioning samples from patients undergoing an HSCT. The primary goal was to ask whether the association of high Treg levels with increased relapse mortality would be reflected in a similar association of regulatory cytokines. A secondary question was to ask whether there were any other significant associations between cytokines and outcome measures that might identify useful prognostic markers of HSC transplant outcome. The cytokines were chosen to represent inflammatory, helper and regulatory cytokines and to give some insight into the impact of the major subsets of CD4 T cells on post-transplant outcome.

In the initial study, comparing levels of expression in the patients with controls, it was found that four cytokines had levels that differed significantly in the patient group, in all cases being increased above the levels seen in the controls. Two of these, TGF- β and IL-10 are regulatory cytokines, while the other two, TNF- α and IFN- γ , are inflammatory cytokines. Note that these were not found in the same patients instead some patients

showed increases in both TGF- β and IL-10 while others had increases in TNF- α and IFN- γ . The TGF- β levels were strongly correlated with the levels of Treg measured in the preceding chapter, consistent with these cells being the source of the cytokine. The IL-10 levels did not correlate well with the Treg levels, suggesting that other cell types could be responsible for the production of this cytokine.

When the cytokine levels were compared to the outcomes for the patients, three of these four showed significant correlations, with TGF- β and IL-10 being significantly associated with a worse outcome and an increased relapse risk. Conversely, high levels of TNF- α were associated with lower relapse mortality and a better overall survival, with perhaps a trend for the association of IFN- γ in the same direction. In addition, a number of other cytokines showed associations with some aspects of outcome, IL-2, IL-4 and IL-12 all being associated with better engraftment.

In the multivariate analysis, only IL-10 and TGF- β showed significance as independent risk factors; IL-10 was strongly associated with reduced overall survival, and to a lesser extent with relapse, while TGF- β was the most significant factor associated with relapse.

Comparing these results with others, there was not an association between the plasma concentration of TNF- α and the onset of aGvHD, in accordance with data from Visentainer et al (Visentainer, et al., 2003).

Remember et al. (Remberger, et al., 1995) and Holler et al. (Holler E. , et al., 1990) demonstrated a correlation between higher pre-transplant levels of TNF- α with moderate and severe GvHD and transplant related mortality (TRM). Other groups have associated high TNF- α with the onset of chronic

GvHD (Imamura, et al., 1994). However, in the cohort presented here, in contrast to these reports, there was not an observable association with the onset of either acute or chronic GvHD. The discordance between these results on GvHD with other groups might be the result of T-cell depletion; 60% of the patients included here were T-cell depleted and that has been shown to diminish the incidence of GvHD.

On the other hand, high levels of TNF- α in our cohort were associated with better transplant outcome, with better overall survival, improved disease free survival and consequently less relapse. TNF- α induces T-cell activation and it has been published that TNF- α therapy could enhance anti-tumour responses. Therefore, the lower relapse rate could be associated with antigen-specific T-cell activation to mount an effective response to eliminate remaining tumour cells, inducing the graft versus leukaemia effect (GvL).

Since there was no association of TNF- α with GvHD, these results, together with the converse association of the regulatory cytokines increasing relapse risk while having no association with GvHD, suggest there should be different pathways of specific T-cell activation that can promote either GvL effect or GvHD, and that the pre-transplant cytokine levels influence the GvL pathway, whilst not affecting the GvHD pathway.

Another cytokine closely involved in transplant outcome is IL-10. IL-10 is produced by several cell types (activated T-cells, B-cells and monocytes) and it strongly inhibits antigen-specific T-cell responses.

In the context of HSCT it has been shown that IL-10 inhibits the onset of aGvHD and regulates other transplant complications by the inhibition of pro-inflammatory cytokine production and therefore is associated with better

outcome post-transplant and lower incidence of aGvHD (Ju, et al., 2003). The higher levels of IL-10 production have also been associated with a lower overall occurrence of transplant-related complication and a decreased risk of early death (Baker, et al., 1999). The half-life of IL-10 is quite short (4 hours) and it is thought that the protective effect of IL-10 relies on its continued production by mononuclear cells during the conditioning period.

In the study presented here, the concentration of IL-10 prior to transplant did not show any association with the occurrence of GvHD. In contrast to previous reports there was no protective effect of the higher IL-10 production and consequent better transplant outcome. On the contrary, higher levels of IL-10 in the pre-transplant period were associated with worse outcome due to higher relapse rates. This effect could be explained by the fact that IL-10 is a modulator of immune responses, its functions includes the down-regulation of responses against allo-antigens. In the context of allogeneic-HSCT the allo-responses are associated with the anti-leukemic effect, high levels of IL-10 down-regulate the production of pro-inflammatory cytokines that are necessary for T-cell activation and protective immune responses, thus resulting in an increasing relapse rate.

TGF- β as well plays an important regulatory role in immune responses. Elevated TGF- β plasma levels before HSCT have been shown to be a predictive for a higher incidence of GvHD (Leffell, et al., 2001) (Liem, et al., 1998). In the study presented here, the levels of TGF- β prior to transplant were analysed and correlated with transplant outcome, and showed that high levels of TGF- β in the pre-transplant period have a strong correlation with higher relapse rates, similar to the finding for IL-10. Thus, the relapse

free survival was impaired and also the overall survival showed a trend towards worse survival. The mechanism of this effect is not yet clear, but is likely to depend on pre-existing Treg in the patient surviving the transplant procedure to suppress anti-tumour responses in the post-transplant period. The immune-suppressive effect of anti-inflammatory cytokines, such as IL-10 and TGF- β could modulate specific allo-responses and generate tolerance that could consequently inhibit anti-leukaemic responses. In addition, high production of TGF- β has been demonstrated to induce further Tregs differentiation that also could further regulate allo-antigen specific responses. Furthermore, TGF- β itself has a role in tumour progression and anti-tumour immune response suppression (Seoane, 2008) (Medicherla, et al., 2007).

The associations of the regulatory cytokines reveal a subtle difference, in that TGF- β shows an association with relapse, but not with overall survival, while IL-10 shows an association with survival more than relapse. Perhaps TGF- β is associated with the induction of anti-tumour responses while IL-10 is associated with their efficacy

IL-10 it is thought to be secreted by tumours to evade immune-surveillance, which could also explain the lack of an effective response and the recurrence of the tumour. Likewise it has also been suggested that tumours may be able to induce the differentiation of Treg, for example by the expression of the enzyme indoleamine-2,3-dioxygenase, or IDO (Curti, et al., 2006). IDO functions to deplete tryptophan to produce a metabolite, kynurenine. Either tryptophan depletion, monitored by the enzyme general control nonrepressed 2 (GCN2) kinase, or kynurenine and other tryptophan

metabolites, acting through the aryl hydrocarbon receptor, or a combination of both pathways, are able to act on both dendritic cells and T cells directly, to induce the differentiation of CD4 T cells to Treg (Munn & Mellor, 2007) (Quintana, et al., 2010). In this case, the elevated levels of Treg would be a surrogate marker for those tumours able to express IDO and thus recruit Treg to suppress immune responses against themselves. Measurement of IDO levels in tumour samples and their correlation with the levels of Treg would be required to address this hypothesis, however it may be argued that since IL-10 and Treg levels were not closely correlated, and IL-10 was an independent factor associated with worse survival, then the results presented here cannot simply be explained in terms of recruitment of Treg alone. Instead it appears that it is the overall cytokine environment in the patient that is associated with transplant outcome, either a pro-regulatory environment with elevated TGF- β and/or IL-10 that is associated with worse overall survival, or a pro-inflammatory environment with elevated TNF- α and/or IFN- γ that is associated with better survival.

For the other cytokines that showed associations with transplant outcome the implications are less obvious. IFN- γ has an immunostimulatory function as a mediator of innate immunity. An association between high levels of IFN- γ has also been associated with increased aGvHD and viral infections. However, the data presented here did not show an association either with aGvHD or with viral infections (Sakata, et al., 2001) (Visentainer, et al., 2003). While there was a trend towards an association with improved disease-free survival this did not reach statistical significance.

IL-12 has been shown to have an anti-tumour effect in murine models. Reddy et al. published that high levels of IL-12 were associated with better transplant outcomes and increase relapse free survival (Reddy, et al., 2004). They also showed that high levels of IL-12 pre-transplant have an increased likelihood of higher levels of IL-12 post-transplant. The pre-transplant levels of IL-12p70 were measured but in contrast with Reddy did not show any association between high levels of this cytokine and transplant outcome. However, an association of levels of high IL-12p70 with faster engraftment was observed.

IL-2 was analysed in the cohort and high levels of IL-2 in the pre-transplant period correlated with faster engraftment.

Tanaka and cols. (1997), demonstrated that high levels of IL-4 were elevated in the early engraftment phase while IL-12 was lower. Here, we found that patients with higher levels of IL-4 pre-transplant had faster engraftment. However, in contrast with them we find that also high levels of IL-12 in the pre-transplant period had a trend towards faster engraftment that those patients with lower levels of IL-12.

The mechanisms underlying these latter associations are not clear; IL-12 is a strong inducer of inflammatory, Th1 responses, while IL-4 is associated with helper, Th2 responses and IL-2 drives proliferation of Th0 and Treg. A common mechanism to explain all of the associations is hard to envisage. Given the low p value for the associations with IL-2 and IL-12, it is clear that a larger study is required to clarify their contribution and it is possible that these may in such a wider study lose significance, while the IL-4 association, which has a greater statistical significance might be retained.

Conclusions

In conclusion, from these data it is clear that an imbalance between pro-inflammatory cytokines and immune-regulating cytokines could create a microenvironment that reduces specific allo-responses for anti-leukemic effect, therefore, increasing the rate of relapse. Although the cohort is small the impact of cytokines is very strong, showing a clear pattern of worse overall survival and disease free survival and higher relapse rates in those patients in which an immune-regulatory environment is prevalent. These results are also consistent with previous results of this thesis, where a prevalence of regulatory T-cells is associated with worse transplant outcome and higher relapse rates, while the opposite effect is seen when a pro-inflammatory environment is established. Therefore, cytokine monitoring pre-transplant could be a helpful tool for prediction and early treatment of major transplant related complications, and for monitoring those patients with higher risk of transplant complications. Based on cytokine levels patients could also be stratified in higher or lower risk of relapse.

Further analysis including a larger number of patients would be required to demonstrate the clinical benefits of such stratification.

Chapter 7

Conclusions

Much effort has been expended to improve the outcome of haematopoietic stem transplants, yet nearly 30% of patients die after HSCT due to relapse, graft versus host disease or infections (Gratwohl, et al., 2005). Improvements in supportive care, better prophylaxis and better monitoring of infections have been useful to reduce this mortality. However, the identification prior to the transplant of patients who are at highest risk of adverse outcomes is potentially an important factor to increase the likelihood of success after transplantation.

The recovery of the T cell pool is one of the key elements related to transplant success, therefore, enhancing immune reconstitution is still an area of significant research and many strategies have been used to achieve faster T cell recovery after HSCT, including application of cytokines and growth factors (Seggewiss & Einsele, 2010). Sutherland et al., based on prior experiments in which castration of adult mice gave rise to a rapid re-growth of the thymus and increased thymic function showed previously that patients treated with LHRH agonists (LHRH-A; these act on the hypothalamic-pituitary-gonadal axis to suppress sex steroid production) have a faster recovery of the T cell compartment. However, it is not only the recovery of cell numbers; it is equally important to recover an adequate repertoire of T cells able to recognize an extensive range of antigens and, importantly, also able to establish an anti-leukaemic response to prevent relapse. In the study from Sutherland et al., it was shown that the treatment with LHRH-A not only impacted the T cell recovery, but also wider repertoires were recovered in the CD4 compartment in patients that have been transplanted with allogeneic

grafts. This effect was not seen in the autologous transplant setting, perhaps because of differences in T cell depletion in this patient group and a lesser reliance on thymic function to repopulate the peripheral T cell repertoire. In a separate study, in which patients were treated with LHRH-A 3 weeks prior to HSCT and for 3 months afterwards, patients treated with LHRH-A had better disease free survival (DFS) in the autologous setting ($p=0.04$), although there was no significant difference in overall survival, (Sutherland, et al., 2008). Therefore, therapies to reactivate the thymus, such as LHRH-A treatment, can improve not only the reconstitution of the T cell compartment but also the outcome of the transplant.

Now, we know that immune cells, in particular, from the lymphoid compartment, play an important role in the post-transplant period, in infection control, in GvHD and in GvL responses that can control relapse. Thus it is not unreasonable to hypothesise that the individual differences in the distribution and activity of these cells may influence the post-transplant outcome. Most studies have focused on the post-transplant period, but given that some studies do indicate that T cells survive the conditioning process and contribute to immune responses in the patient after transplant (Chalandon, et al., 2006), I have chosen to ask the question, is there any correlation between the pre-transplant immune state of the patient and the transplant outcome? In other words, can the pre-transplant state either persist into the post-transplant period or condition the responses in the post-transplant period in such a way as to affect the outcome for the patient?

Although the most significant consequence from this thesis would be the identification of parameters that could be modified in the patients to improve their outcome, the identification of pre-transplant immune parameters that do correlate with post-transplant outcome would be of benefit. Rosinski et al.

(Rosinski, et al., 2005) suggested that the immune state in particular of the T cell subsets prior to transplant could be beneficial to identify patients at risk of worse outcome. Thus, one goal of this thesis therefore was an analysis of the immunological state prior to transplant to define biomarkers that might identify patients who would need closer monitoring to prevent undesirable complications. The primary analysis of the pre-transplant state of the cells of the lymphoid compartment was performed using flow cytometry; the cells analysed were the CD3⁺, CD4⁺ subsets, including regulatory T cells (Tregs) and CD8⁺ subsets. In addition, the measurement of the levels of TRECs in the peripheral blood was used as an indicator of thymic function. Finally, the overall immune environment was analysed through the measurement of cytokines prior to transplantation. From each of these types of analysis, the results were correlated with the outcome, overall survival, disease free survival, relapse or GvHD.

As mentioned in Chapter 3, the percentages of CD3⁺ T-cells in the peripheral blood were significantly lower in patients pre-transplant than in healthy controls, due to a decrease in the CD4⁺ population and in particular of the naïve subset, while the percentages of the CD8 compartment were comparable; similar observations were made in the Rosinski study (Rosinski, et al., 2005).

Rosinski and his group also published that the pre-transplant levels of the memory CD4⁺ T-cell (CD4⁺CD45RA⁻CD62L⁻) had a prognostic effect on transplant outcome and progression free survival not only in haematological malignancies but also in breast cancer, independently of other known factors (Rosinski, et al., 2005). However, in this study that effect couldn't be demonstrated. A trend towards better survival was observed in patients who had higher levels of both naïve CD4⁺ and CD8⁺ T cells. High levels of naïve CD4⁺ cells were associated with a decrease in the risk of relapse and with a trend

towards enhanced disease free survival but this didn't reach statistical significance. The mechanisms for such an effect are not clear.

One criticism that could be levelled at the study is that, because of the way the samples were originally collected, only the percentages of each cell subset could be determined and not the absolute counts in the peripheral circulation. Thus a decrease in the percentage of CD3⁺ cells could simply reflect an increase in a CD3⁻ cell subset. In the case of pretransplant samples this could reflect different levels of leukaemic cells, for example. However, if this were the case, one would expect to see the different T cell subsets showing parallel decreases. The fact that only the CD4⁺ T cell subset shows a significant decline in the patient samples while the CD8⁺ subset does not argues that the individual subset changes are real events and not artefactual responses to changes in the representation of other cells.

TREC levels prior to transplant were used by Svaldi et al. as a marker to assess transplant outcome in patients with multiple myeloma (Svaldi, et al., 2003), finding that patients with high TREC levels prior to transplant showed better overall survival and disease free survival. We also analysed TREC level to evaluate the impact of thymic function on the outcome. However, in contrast to the observations of Svaldi, no correlation at all was found between the levels of TRECs and transplant outcome. Svaldi's study was carried out on patients after autologous transplant, while those reported in this thesis received allogeneic transplants. Many factors differ between these two sorts of transplant, including the level of myeloablation in the patient and the T cell depletion of the graft, that may account for the difference in the results obtained.

One other subset of cells analysed was the Treg subset, but the identification of this population of cells was not easy to perform, because the definition of Treg at

the time of the study was not conclusive. Therefore, the first set of markers used to identify Tregs was the expression of CD4 and CD25, with Tregs being defined as a CD4⁺CD25^{hi} T cell. The percentage of CD4⁺ lymphocytes expressing high levels of CD25 was assessed by flow cytometry, as was shown in Figure 5.1 in Chapter 5. An analysis of Treg expression was performed in healthy controls and in two patient cohorts, and when they were compared, low levels of Tregs were found in both patient groups in comparison to the control group. Within the patient groups, the levels of Tregs were particularly low in patients with CML and ALL.

Outcome of the transplant was also analysed with respect to the Treg levels, and according to Zorn et al., an association with the onset of GvHD was expected (Zorn, et al., 2005). However, in contrast to their results, in both cohorts a strong association with relapse was found. In both cohorts, patients who had increased levels of Regulatory T cells (Tregs) pre-transplantation had worse outcome, due to higher rates of relapse.

Since the phenotypic definition of CD4⁺CD25^{hi} was not considered sufficient to identify Treg, to confirm the correlation obtained, another marker to identify Tregs was used. The expression of FOXP3 was assessed by quantitative PCR, and again a correlation with outcome was performed, showing the same results, that high expression of FOXP3 was associated with worse overall survival due higher relapse rates. In the second study cohort, intracellular staining for FoxP3 protein was carried out, and also showed the same association, high levels being associated with higher relapse mortality.

It is likely that the observed association is due to a reduction in the GvL effect mediated by patient Treg. However, this reduction in the GvL response wasn't paralleled, as mentioned before, by any change in GvHD, which might suggest

that both events have independent mechanisms. Numerous questions arise from the analyses described in this thesis, the most important of which is that because the analysis was carried out with samples taken prior to transplant, both myeloablative conditioning of the patient, and infusion of residual T cell depletion agents present in the graft should have eliminated patient immune cells. Thus it is difficult to understand the mechanisms by which pre-transplant cell levels can influence events after the transplant. However, various studies have shown that some T cell depleting agents such as CAMPATH and ATG can spare Tregs (Bloom, et al., 2008) (Watanabe, et al., 2006). Also, it has been shown that Tregs are preferentially expanded under conditions of lymphopenia, such as would exist immediately after the transplant (Cox, et al., 2005). Moreover, a detailed analysis of CMV specific T cell responses in patients receiving T cell depleted allogeneic transplants has shown that patient CMV specific memory T cells can persist through the transplant procedure and establish functional immunity in the patient after the transplant (Chalandon, et al., 2006). Thus there is a precedent for the concept that patient cells might contribute to immune responses in the post-transplant period; in the case of patient Tregs, a greater number of Tregs surviving into the post-transplant period being more able to regulate anti-leukaemic responses.

Similar observations on the relationship between high Treg levels and relapse have been published by Nadal et al., who found that CML patients who had been transplanted and were in remission had significantly fewer Tregs than patients who relapse (Nadal, et al., 2007), leading them to suggest that Treg levels could be used as a surrogate marker for disease relapse. Again, no association was found with GvHD.

Two specific events that may contribute to better or worse transplant outcome are the Graft versus Leukaemia (GvL) effect and the Graft versus Host Disease (GvHD) respectively, both mediated by T cells. It has been widely considered that the two events are linked, and that the GvL response is simply part of a wider GvHD response, yet recent data have shown that they might be separate events, produced by different cell subsets. In the data presented in this thesis, and in the work of Nadal et al (Nadal, et al., 2007), it is clear that the impact of regulatory T cells or the regulatory cytokine milieu affects relapse, and hence presumably the GvL response, whilst having no effect on GvHD.

In the study described in this thesis, an analysis of the microenvironment by analysing the production of cytokines prior the transplant was also performed, and the results showed that an imbalance between pro-inflammatory cytokines and regulatory cytokines had an impact on the outcome of the transplant, suggesting that a immune-regulating microenvironment could lead to higher rates of relapse, may be due to the incapacity of the immune system to mount an effective allo-response against tumour cells while an imbalance towards a pro-inflammatory environment was associated with better overall survival may be because a pro-inflammatory environment may enhance anti-tumour responses, and therefore lower relapse rate could be associated with antigen-specific T-cell activation to mount an effective response to eliminate remaining tumour cells, inducing the desired graft versus leukemic effect (GvL).

Overall, these data, suggest that the immune-regulating environment, created either for the patient's Tregs or tumour induced regulatory cells as a way to evade immune surveillance may reduce allo-responses to produce the anti-leukaemic effect, leading the patients to higher relapse rates to the disease and have worse outcomes after transplant, especially in those patients who have received T cell

depleting agents. Consequently, as mentioned in Chapter 6, there should be different pathways of specific T-cell activation that can promote either GvL effect or GvHD. And understanding the mechanisms by which tumour cells evade immunity to relapse or the delay in immune reconstitution will provide better tools to develop new therapies to improve transplant outcome. Also finding markers to identify a higher risk, will increase the chances to provide adequate treatment and therefore to improve survival.

In recent years, an association between indoleamine 2, 3-dioxygenase (IDO) and Tregs has been found, with both the depletion of tryptophan by IDO and the production of the metabolite kynurenine being implicated in the induction of Treg. The production of IDO by tumours may be a mechanism to escape immune surveillance (Wang, et al., 2009). In this case, the high level of Treg would be a surrogate marker for the immunosuppressive phenotype of the tumour, thus explaining a correlation with relapse post-transplant. Studies performed in melanoma and breast cancer have shown an association between increased IDO expression and higher levels of Tregs with poor outcomes (Prendergast, et al., 2009). Similarly in AML, production of IDO by the tumour is associated with higher Treg levels and increased relapse (Curti, et al., 2009).

In preliminary experiments carried out subsequent to the work in this thesis (P Travers, personal communication) there was no association found between high Treg levels and IDO levels in the cohort described here. However, a more in depth study should be conducted, analysing Treg levels and both FOXP3 and IDO isoform expression in the context of HSCT, to validate the analysis of Tregs as a prognostic factor for relapse. While it may be the case that high Treg levels could provide a useful prognostic marker, even if they were produced in response to the tumour, the question of whether they are a surrogate marker has more

significance with respect to potential therapies to reduce relapse rates and enhance anti-tumour immune responses. Depletion of Treg prior to transplant may have no effect on patient outcome if the underlying mechanism for the elevated levels is their induction by the tumour. A better strategy in this circumstance would be to inhibit the mechanisms used by the tumour to induce Treg, for example, to treat with 1-methyltryptophan to inhibit IDO.

However, one aspect of the data may argue against the possibility that Treg are simply a surrogate marker for an immunosuppressive property of the underlying malignancy, and that is the lack of correlation between Treg and TGF- β levels, which themselves correlate well, and IL-10 levels. If, for example, the primary association was with the induction of Treg by the underlying tumour, with the subsequent production of both TGF- β and IL-10 by the Treg, then all three should be correlated. Treg and TGF- β levels are correlated, suggesting that Treg are the primary source of the TGF- β in the patients. However, IL-10 can be produced from cell types other than Treg, and from this study, this IL-10 makes an independent contribution to the outcome for the patient. It can be argued therefore that there is unlikely to be a single underlying cause for the associations observed and that it is the balance of the immune system in the patients at the time of transplant, either pre-regulatory or pro-inflammatory, which biases the eventual outcome for the patient.

This data suggest that the study of the Regulatory T-cells and the analysis of cytokine production prior transplantation can be used as a predictor of the outcome of the transplant, and can help to stratify patients into different groups, those who are in higher risk and need closer monitoring, and those who are in low risk to relapse.

In this study no functional analysis were performed, due the lack of appropriate material, but for further studies, functional analysis on the regulatory T cells should be performed to confirm their suppressive activity; a complete analysis measuring IDO expression with suppressive function of Tregs should also be performed.

A better study design

The main cohort of samples used in the studies reported in this thesis were collected as part of an ongoing study in which transplant outcome measures are compared against genetic polymorphisms in a variety of genes. For that reason, no particular attention was paid to the number or status of the immune cells in the patient samples and the only attempt to retain viable cells was for the purpose of generating immortalised cell lines by EBV transformation of B cells. In retrospect, the collection and storage criteria of this study are too limited and restrict the conclusions that can be drawn. For any future study, careful consideration would need to be given to the timing and the nature of samples collected, and how they are to be stored.

Ideally a presentation sample would be collected, to allow characteristics of the malignant cells to be determined; this may not be feasible since the decision to proceed to transplant is often made at a later stage in the treatment of the individual patient.

The minimum sample collection would therefore be of a pre-transplant, preconditioning sample. In a prospective study, the analyses would be carried out on this sample without long term storage, giving the most accurate measurements of true pre-transplant cell and cytokine subsets. Irrespective of whether the full analysis is to be made with fresh or frozen samples, a critical measurement that

must be made at this point is that the sample should have a full blood count, giving accurate numbers of CD3⁺ T lymphocytes and preferably numbers of CD4⁺ and CD8⁺ T lymphocytes. Commercial flow cytometry kits such as TruCount (BD Biosciences) exist to provide this information from a small amount (50µl) of fresh blood. This information can also be used as a crosscheck to determine whether there is preferential loss of CD4 or CD8 T cells on freezing and thawing; the percentage of CD4 and CD8 cells in the fresh and thawed samples should be the same.

A separate tube should be collected to provide a sample of serum or plasma to be used for the detection of cytokines. The recommendation from a leading company in the field of multi-analyte profiling, MyriadRBM, is that unless the specific markers of interest are known to differ between plasma and serum (for example, fibrinogen) then it is more important that all samples are of the same type.

The main sample should be collected in citrate or EDTA collection tubes and the peripheral blood mononuclear cell fraction purified by standard procedures. The cell number present should be counted. The cells may be used fresh for analysis of T cell subsets or, if to be stored, should be pelleted in aliquots of known cell number and resuspended in standard heat-inactivated FCS/10% DMSO freezing mix before freezing initially at -80°C and then in liquid nitrogen vapour phase for long term storage. Ideally, in a prospective study, the cells would be analysed fresh *ex vivo* rather than after freezing and thawing.

In general, the tests carried out would be the same as those described within the thesis, though with the advantage that since absolute cell numbers would be known, the potential artefacts arising from variations in the numbers of tumour cells present in the sample can be avoided. Additional phenotypic

characterisation of Treg would be carried out, since the initial study treated only CD4⁺CD25^{hi} cells as Treg; now that phenotype would be extended to include the expression of glucocorticoid-induced TNF receptor (GITR⁺) and low levels of the IL7 receptor α chain (CD127^{lo}). As was carried out for the second cohort described in this thesis, intracellular staining of FoxP3 protein would also be used to define the population of Treg.

Cell surface markers of recent thymic emigrants have been defined, such as for example, CD103 (McFarland, et al., 2000) for CD8 T cells and protein tyrosine kinase 7 (PTK7) for CD4 T cells (Haines, et al., 2009). Measurement of CD103⁺ naïve CD8 T cells and PTK7⁺ naïve CD4 T cells (ie CCR7⁺CD62L⁺CD27⁺CD45RA⁺) can complement TREC measurements to define the level of thymic output.

In the initial cohort, rtPCR was used to estimate the relative level of expression of FoxP3 in the samples; this strategy could also be adapted to estimate the level of other functional CD4 subsets in addition to Treg. TBX21 (the human orthologue of Tbet), GATA3 and ROR γ are the key transcription factors that define Th1, Th2 and Th17 effector cell subpopulations, and quantitation of the levels of transcripts of these can indicate the functional balance of the immune response in the patients. Such measurements would be interpreted together with the measurement of a wider range of cytokines to give a more comprehensive picture of the status of the immune system in the patient prior to transplant.

In the studies reported in this thesis, the levels of 10 cytokines (IL-1 α , IL-2, IL-4, IL-10, IL-12p70, IL-15, IL-17, IFN- γ , TNF- α , and TGF- β) were measured in pre-transplant plasma samples. In a future study, plasma/serum samples would be analysed for a larger panel of cytokines. Although some of these can be made by more than one cell type, they can largely be grouped into the following functional

classes, and the patterns of expression, together with the relative levels of the effector cell specific transcription factors, can give information on the underlying patterns of immune function in the patient at the time of sampling:

Monokines: IL-1, IL6, MIP-1a, MIP-1b, MCAF, IP10, PDGF-BB, G-CSF

Inflammatory responses: IL1ra, bFGF

Th1: IL-2, IL-3, IL-12, IFN- γ , TNF- α

Th2: IL-4, IL-5, IL-6, IL-10, IL-13

Th17: IL-17

Treg: IL-10, TGF- β

These changes will improve the quality of the data. In retrospect, it is clear that for the individual disease entities the patient numbers are not sufficient to allow a statistically significant result to be obtained. Any future study should therefore include greater patient numbers, or perhaps focused on a single disease entity, although the proposed mechanism is likely to apply to all diseases. In theory, this mechanism should apply to autologous transplants as well as to allogeneic ones, although the intrinsic immunogenicity of the tumour in the allogeneic setting may be an essential component; any anti-tumour response in the autologous setting is almost entirely an anti self-response. While there are clear deficiencies in the way the samples were collected and stored, which limit the conclusions from this study, none of these are fatal flaws and the results obtained are self consistent and replicated within the separate cohort of Australian patients. It does therefore make a useful and potentially valuable contribution to the understanding of the factors affecting transplant outcome.

Chapter 8

References

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