THE INFLUENCE OF REGULATORY T CELLS AND MOBILISATION REGIMENS ON GRAFT-VERSUS-MALIGNANCY, GRAFT-VERSUS-HOST-DISEASE AND RELAPSE IN HAEMATOPOIETIC PROGENITOR CELL TRANSPLANTATION

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Abstract

Regulatory T cells (Tregs) are key players in controlling immune responses, limiting autoimmune disease and allergies, and attenuating immune responses to tumours and pathogens. Understanding and harnessing the suppressive effects of Tregs in autologous and allogeneic haematopoietic progenitor cell (HPC) transplantation presents a significant challenge due to lack of consensus over optimal markers to uniquely identify Tregs and variation in centre-specific factors including disease mix, conditioning regimens, graft origin and manipulation and prophylaxis and treatment of graft versus host disease (GVHD). This study aimed to determine if CD3+CD4+CD25^{high}CD127^{low}FoxP3+ Treg quantification, assessed flow cytometrically, in grafts or in the post-transplant peripheral blood of patients who received transplants for malignant disease, could provide a useful predictor for disease relapse in autologous (n=85) and allogeneic patients (n=75) and falling chimerism and/or incidence of GVHD in the latter group. The impact of Treg numbers were quantified in HPC harvests, in transplant grafts and in recipients' peripheral blood during immune reconstitution. Additionally, a simplified Treg enumeration protocol using the marker tumour necrosis factor receptor-2 (TNFR2) with CD3, CD4 and CD25 was assessed.

In autologous donors, significantly higher Tregs relative to CD34 HPCs were noted in harvests mobilised with the more novel regimen, granulocyte-colony stimulating factor (G-CSF) plus Plerixafor than with G-CSF alone or used in combination with cyclophosphamide. In allogeneic harvests Treg numbers following G-CSF mobilisation were significantly lower than in non-mobilised harvests. Lower absolute Treg numbers in donor lymphocyte infusion (DLI) doses were significantly associated with successful outcome in terms of restoration of donor chimerism and resolution of relapse. Cryopreservation of mobilised cells at the time of initial transplant for later use for DLI has thus been incorporated into practice at this Trust as this is expedient in terms of clinical result, convenience and cost. Interestingly although mobilisation regimens influenced Treg levels in harvests, no correlation was apparent between Treg doses transplanted or peripheral blood levels during immune reconstitution post autologous or allogeneic transplantation or with falling chimerism and/or incidence and severity of GVHD in allogeneic patients during the first year post transplant. Extending this follow-up time would be an interesting area of further study as the majority of patients who relapse do so beyond one year.

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Abbreviations

ACD-A	Acid citrate dextrose – solution A
aGVHD	Acute graft versus host disease
auto-GVHD	Autologous graft versus host disease
ALL	Acute lymphocytic leukaemia
AML	Acute myelocytic leukaemia
APC	Antigen presenting cell
APC	Allophycocyanin
BD	Becton Dickinson
BM	Bone marrow
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cGVHD	Chronic graft versus host disease
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CsA	Cyclosporine A
CTL	Cytotoxic lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	Chemokine receptor type 4
DC	Dendritic cell
DL	Donor lymphocytes
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Ca	Cancer
EBMT	European Group for Blood and Marrow Transplantation
ECP	Extracorporeal photopheresis
EDTA	Ethylenediamine tetraacetic acid
FBC	Full blood count
FcR	Fc receptor
FISH	Fluorescence in-situ hybridisation
FITC	Fluorescein-isothiocyanate
FoxP3	Forkhead box protein 3
GARP	Glycoprotein A Repetitions Predominant
G-CSF	Granulocyte-colony stimulating factor
GITR	Glucocorticoid-induced tumour necrosis factor receptor
Gzm B	Granzyme B
GVHD	Graft versus host disease
GVM	Graft versus malignancy
HD	Hodgkin's disease
HLA	Human leukocyte antigen
HPC	Haematopoietic progenitor cell
HPC,A	Haematopoietic progenitor cells - apheresis
HTA	Human Tissue Authority
IFN	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

iTreg	Induced regulatory T cell
JACIE	The Joint Accreditation Committee of the European Group for Blood and Marrow Transplantation (EBMT) and the International Society for Cellular Therapy (ISCT)
LAG-3	Lymphocyte =activation gene 3
MDS	Myelodysplastic syndrome
mHag	Minor histocompatibility antigen
MHC	Major histocompatibility complex
MM	Multiple myeloma
MNC	Mononuclear cell
MRC	Medical research council
MSC	Mesenchymal stem cell
NHL	Non-Hodgkin lymphoma
Nrp-1	Neuropilin-1
NK cell	Natural killer cell
NKT cell	Natural killer T cell
nonMA	Non-myeloablative
nTreg	Natural regulatory T cell
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
ProMISe	Project Management Internet Server
pTreg	Peripheral-induced regulatory T cells
RT-PCR	Reverse transcriptase polymerase chain reaction
RIC	Reduced intensity conditioning
RMH	The Royal Marsden Hospital
SCTL	Stem cell transplant laboratory
SDF-1	Stromal derived factor 1
Sib	Sibling
STR	Short tandem repeat
Tc	Cytotoxic T cell
TCR	T-cell recptor
TGF-β	Transforming growth factor - beta
Th	Helper T cell
TNC	Total nucleated cell
TNF-α	Tumour necrosis factor – alpha
TNFR2	Tumour necrosis factor receptor-2
Treg	Regulatory T cell
tTreg	Thymic derived regulatory T cell
Tx	Transplant
UKAS	United Kingdom Accreditation Service
UVA	Ultraviolet light
VUD	Volunteer unrelated donor
8-MOP	8-methoxypsoralen

1.0 Introduction

Haematopoietic progenitor cell (HPC) transplantation is used to treat a wide range of both haematological and non-haematological disorders. These are costly procedures and in the UK these more complex treatments are only performed in a number of specialist centres able to provide the necessary skills and expertise. Because of the wide range of indications for HPC transplantation, individual centres have tended to specialise in transplantation performed for certain groups of diseases, developing treatment regimens to provide the best outcome for their particular cohort of patients. Although this treatment can offer a cure for some diseases it is not without risk and a percentage of patients will die from or experience long-term transplant-related side effects. Centres therefore constantly evaluate their own and others' practices in order to improve transplant outcomes. A number of researchers have reported interesting associations between regulatory T cells (Tregs) and transplant outcomes, therefore this study was performed to identify if these findings could be used to improve outcomes for the cohort of patients treated at The Royal Marsden Hospital (RMH). This centre is a specialist cancer hospital with the largest HPC transplant programme in the UK, performing over 200 transplants annually for patients with malignant disease. The study only considers adult patients treated for various haematological cancers.

1.1 Haematopoietic Progenitor Cell Transplantation

HPC transplantation is a well-established and increasingly utilised therapeutic procedure in which progenitor cells capable of reconstituting normal bone marrow function are infused into patients following doses of chemotherapy or radiotherapy designed to ablate tumour cells. Unfortunately the anti-tumour treatment is not selective and concurrently impairs normal haematopoietic activity (Fry and Mackall, 2005, Forgáčová and Nečas, 2013, Mackall *et al,* 2009). In autologous transplantation HPCs are sourced from the patient while in the allogeneic setting HPCs are sourced from either a sibling/family member or an unrelated donor (Hoffbrand, 2011). Transplant-based treatment strategies are used for a number of haematological malignancies including the leukaemias, lymphomas, multiple myeloma, and some solid tumours, such as germ cell tumours and Ewing's sarcomas (Appelbaum, 2004, Sirohi *et al,* 2007, Gratwohl and Baldomero, 2009). The number of transplants performed annually in Europe has substantially increased, from 4,200 in 1990 to more than 30,000 in 2010 of which 13,345 were allogeneic and 20,017 were autologous (Gratwohl and Baldomero, 2009, Gratwohl *et al,* 2003, Passweg *et al,* 2013).

In autologous HPC transplantation the patient's own cells are collected, cryopreserved and stored following initial disease treatment. The cells can then be thawed on demand and re-

infused to restore haematopoiesis following high-dose myeloablative chemotherapy with or without radiotherapy, often referred to as 'conditioning'. This autologous haematopoietic rescue allows the patient to be treated with higher doses of chemotherapy than would otherwise be possible, thus increasing the potential to kill malignant cells (Ljungman *et al,* 2010, Gratwohl *et al,* 2003, Kessinger *et al,* 1988).

In allogeneic HPC transplantation cells are collected from a genetically related or unrelated donor and are transplanted following either high dose chemotherapy with or without radiotherapy, or alternatively, using non-myeloablative (nonMA) regimens or reduced intensity conditioning (RIC) regimens (Juliusson *et al,* 2000, Shelburne and Bevans, 2009, Tomblyn *et al,* 2006, Slavin *et al,* 1998). The major histocompatibility complex (MHC) is responsible for immune recognition of self and non-self and is therefore of great importance when selecting allogeneic HPC donors. In humans, MHC proteins are also known as human leucocyte antigens (HLA), and extensive studies have shown that matching of donor and patient HLA-A, C, B, DRB1 and DQB1 alleles is associated with higher survival and lower post-transplant risks compared with mismatching at these alleles (Shaw *et al,* 2010).

1.1.1 Transplant-associated chemotherapy/radiotherapy

High dose chemotherapy and radiotherapy regimens are employed to cause cyto-reduction of malignant cells in both autologous and allogeneic procedures. In the latter, concurrent reduction of recipient immuno-competent cells enables engraftment of the donor haematopoietic cells (Hoffbrand, 2011). However, there is a high incidence of morbidity and mortality associated with these regimens, so they are generally used on patients under 50 years of age with no co-morbidities (Bacigalupo *et al,* 2009, Sorror *et al,* 2011). More recently the use of non-myeloablative and reduced intensity conditioning has extended the possibility of transplantation as a treatment option to older patients (>50 years old) and to those with co-morbidities. For example the Medical Research Council (MRC) AML16 trial aimed at developing treatment for older patients with Acute Myeloid Leukaemia (AML) and high risk Myelodysplastic Syndrome (MDS) offered non-intensive stem cell transplant as one of the treatment options (MRC: AML16 2006). Although RIC and non-MA are defined by the National Institute of Health as involving different conditioning regimens, these terms are typically used interchangeably by transplant centres and researchers (Bacigalupo *et al,* 2009) with RIC being the term of choice at RMH. Regardless of terminology, these less intensive regimens cause myelosuppression which is reversible, usually within a period of 28 days if no stem cell support is given. RIC and non-MA regimens are not as effective as myeloablative conditioning at reducing tumour burden due to their less profound effect on the recipient's haematopoietic system. A significantly lower incidence of severe neutropoenia and resultant infections is associated with this option (Diaconescu *et al,* 2004, Sorror *et al,* 2004). Since these regimens have less of a direct cytotoxic

effect on the malignancy than full intensity regimes these protocols rely to a greater extent on the graft versus malignancy (GVM)effect mediated by donor T cells for their success (Martino *et al,* 2002, Martino *et al,* 2002). After transplant the recipient's immune and haematopoietic cells are replaced by the engrafted donor cells resulting in a chimerism status that is either entirely donor or a mixture of donor and recipient cells (mixed chimerism).

In the 1960s the first allogeneic transplant recipients to receive bone marrow from donors other than an identical twin were found to frequently develop a serious range of conditions which were observed to affect the skin, intestinal tract, liver, lungs and eyes (Greinix *et al,* 2013). This was subsequently termed graft versus host disease (GVHD) and severe manifestation was associated with significant morbidity and mortality. At the same time it was noted that relapse rates in patients receiving transplants from identical twins were significantly higher than in patients with non-syngeneic donors (Vincent, Roy and Perreault, 2011). Additionally, relapse rates were lowest in patients who developed GVHD, suggesting the existence of a GVM effect which is exerted by the transplanted donor T cells (Vincent, Roy and Perreault, 2011, Fowler, 2006).

The immune composition of the donor graft and the recipient's immune reconstitution following transplant have therefore been widely studied in order to identify the cells involved in GVHD and GVM and to improve understanding of these mechanisms (Greinix *et al,* 2013). Tregs, a suppressive subset of T cells, have become of particular interest in the transplant setting due to their ability to suppress auto-immune reactions.

1.1.2 GVM and GVHD

In the early days of allogeneic HPC transplantation, the graft was viewed solely as a way of restoring haematopoietic function after a potentially lethal dose of chemotherapy with or without radiotherapy. However an additional major therapeutic benefit is the ability of allo-reactive donor T lymphocytes and/or natural killer (NK) cells in the graft to recognise differences in minor histocompatibility antigens (mHAGS) expressed on host tissues. The latter are found to mediate a GVM effect against residual disease following anti-tumour conditioning. The use of therapeutic donor lymphocyte infusions (DLI) to induce disease remission in patients who have relapsed following transplant confirms the importance of immuno-competent donor lymphocytes in this context (Bellucci *et al,* 2002, Kolb, 2008, Kolb *et al,* 1995). However, while donor T cells mediate this beneficial GVM effect they are also implicated in GVHD. The observation that T cell depletion of the graft can not only ameliorate GVHD but often leads to a marked increase in relapse rates confirms that there is a balance between the therapeutic and detrimental roles of these cells (Apperley *et al,* 1986, Marmont *et al,* 1991, Booth, Lawson and Veys, 2013).

1.1.3 Pathogenesis and management of GVHD

GVHD is one of the most important, frequent and challenging complications of allogeneic transplantation. The process is mediated by donor T cells that recognise major or minor histocompatibility antigens presented by host APCs and leads to a cascade of events resulting in the destruction of host tissues (Couriel *et al,* 2004, Jaksch and Mattsson, 2005). GVHD can occur despite immunosuppressive therapy, typically with calcineurin inhibitors such as cyclosporine A (CsA), which is given to all patients (Potter and Kerridge, 2004). The development of GVHD is associated with high medical costs caused by long-term hospitalisation and treatment and also non-medical/indirect costs resulting from considerable quality of life impairment for affected patients (Blommestein *et al,* 2012, Dignan *et al,* 2013).

Balancing the inflammatory and destructive effects of GVHD against the desirable outcome of GVM is one of the major challenges of HPC transplantation. The GVM effect appears to be highest in patients with GVHD and disease relapse rates in these patients is significantly lower than in patients with no evidence of GVHD (Stern *et al,* 2014). The caveat, however, is the fact that GVHD can lead to significant morbidity and even mortality. The best scenario in allogeneic transplant recipients is equilibrium between manageable levels of GVHD and an effective GVM. Research is focussing on the immunogenicity of different mHAGs to identify donor/recipient mis-matches in order to predict severity of GVHD, and to identify if the same peptide epitopes are targets for both GVHD and GVM (Recker *et al,* 2011, Spellman *et al,* 2009, Oostvogels *et al,* 2013, Spierings, 2014).

Although many of the mechanisms of GVHD and GVM are thought to be shared, separation of these remains the 'holy grail' of transplantation to enhance tumour elimination while suppressing GVHD. This separation is the subject of intensive research in order to fully understand the mechanisms involved and to identify potential methods to augment the GVM effect (Fowler, 2006, Couriel *et al,* 2004, Zeiser *et al,* 2004, Dürr *et al,* 2012).

The pathophysiology of GVHD was postulated by Billingham who stated that the requirements for acute GVHD are that the graft contains immune-competent cells, the host expresses either minor or major transplant antigens lacking in the donor and that the host is incapable of rejecting the marrow graft (Billingham, 1966). The graft is infused into a recipient where endothelial and epithelial cells have been severely damaged by the effects of not only the conditioning regimen but underlying disease or infection, resulting in pro-inflammatory changes (Figure 1). Damaged recipient tissues secrete pro-inflammatory cytokines which promote the rapid proliferation of donor T cells. The infused cells react in a way that would, in normal circumstances, resolve or control an infection but in the host result in GVHD (Ferrara, Levy and Chao, 1999).

In the initial stage of GVHD an inflammatory milieu results from damage caused by the radiotherapy and/or chemotherapy conditioning regimen used as part of the transplant protocol (Przepiorka *et al,* 1999). The conditioning serves not only to eradicate the tumour but also to eliminate or significantly impair the recipient's haematopoietic system, theoretically allowing space for the engraftment of the donor cells. However, damage to host tissue results in the direct and indirect release of pro-inflammatory cytokines, including IL-1, IL-6 and tumour necrosis factor-alpha (TNF- α). These cytokines increase the expression of adhesion molecules and MHC antigens on host APCs, enhancing the recognition of MHC and mHAGs by donor-derived T cells (Couriel *et al,* 2004, Ferrara, Levy and Chao, 1999). Damage to the gastrointestinal epithelium caused by conditioning allows for translocation of microbial products including lipopolysaccharides across damaged epithelial barriers. This increases activation of host APCs, thus amplifying cytokine release and subsequent tissue destruction (Zeiser *et al,* 2004, Jaksch and Mattsson, 2005). In response to interactions with host MHC antigens presented by APCs, T cells differentiate into T helper type 1 subset (Th1) and produce cytokines such as IL-2 and interferon-gamma (IFN- γ). These cytokines promote further T cell expansion as well as activating NK cells and cytotoxic T lymphocytes (CTLs) which mediate toxicity against target host cells by two different contact dependent pathways: Fas-Fas ligand mediated apoptosis and perforin/granzyme B-mediated cytolysis. Additionally, IFN-γ primes mononuclear phagocytes and macrophages to produce tumour necrosis factor alpha (TNF-α) which also causes direct tissue damage by inducing target cell necrosis and apoptosis (Ferrara, Levy and Chao, 1999).

GVHD can be either acute or chronic and occurs in 30% of patients transplanted with an HLA-matched sibling donor and in 50-60% of those from an unrelated donor (Potter and Kerridge, 2004). The severity ranges from relatively indolent to a debilitating widespread condition, including organ failure and is responsible directly or indirectly for about 50% of allogeneic HPC transplant mortality (Dignan *et al,* 2013). In the majority of cases, the condition is associated with a significant level of morbidity which typically involves the skin, gut, liver and lungs (Appendix 1). GVHD also affects the immune system, resulting in long-term immunodeficiency, which in turn leaves the patient open to life-threatening infections (Przepiorka *et al,* 1999).

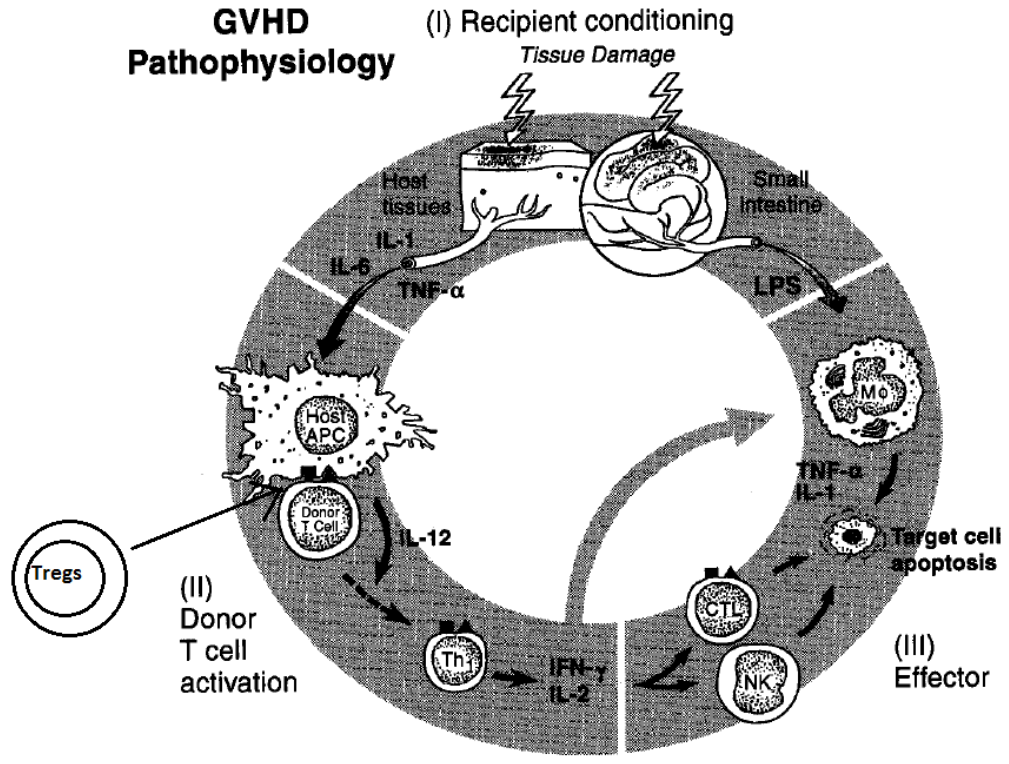


Figure 1: Pathophysiology of acute GVHD shown as a three-phase model. Phase 1- The conditioning regimen causes damage to host tissues causing release of inflammatory cytokines IL6, IL1 and TNF-α. Damage to the intestinal mucosa causes leakage of lipopolysaccharide (LPS) resulting in increased activation of host APCs and subsequent cytokine release. Increased expression of adhesion molecules and MHC antigens on host APCs leads to enhanced recognition of host MHC and mHAGs by donor T cells. Phase 2 - Donor T cells proliferate and differentiate into Th1 type cells secreting IL-2 and IFN-γ. These cytokines promote further T cell expansion as well as inducing NK cells and CTLs. Phase 3 - CTLs and NK cells induce target cell apoptosis. Reprinted from Biology of Blood and Marrow Transplantation : Journal of the American Society for Blood and Marrow Transplantation [online] 5 (6), Ferrara, J.L., Levy, R. and Chao, N.J. Pathophysiologic mechanisms of acute graft-vs.-host disease. pp.347-356. Copyright (1999) with permission from Elsevier.

Historically the definition of acute or chronic GVHD was dependent on time of onset following transplantation, with GVHD occurring within 100 days being defined as acute GVHD (aGVHD) and chronic GVHD (cGVHD) occurring after 100 days. With the introduction of RIC the boundaries have become less distinct. Patients may present with aGVHD after 3 months and others may show symptoms characteristic of cGVHD before 100 days (Greinix *et al,* 2013).

Acute GVHD is the major cause of early transplant related mortality either due to the GVHD itself or due to complications as a result of treatment. Both can cause prolonged neutropoenia leaving the recipient vulnerable to opportunistic infections. Chronic GVHD is the primary cause of late morbidity and non-relapse mortality post-transplant. It may be restricted to a single organ or may be widespread. Chronic GVHD has features which more closely resemble an autoimmune disease and may present as scleroderma, primary biliary cirrhosis, bronchiolitis obliterans, immune cytopoenias and chronic immunodeficiency. The symptoms usually present within the first three years post-transplant but often follow a history of aGVHD. Some risk factors for chronic GVHD have been identified and include a history of aGVHD. Other risk factors for cGVHD besides pre-existing aGVHD include older recipients, female donors used for male recipients, unrelated or HLA-mismatched donors and the use of DLI (Greinix *et al,* 2013).

Treatment of GVHD is a delicate balance as infection and relapse of the original disease can result from the immunosuppressive and anti-inflammatory effects of treatment (Dignan *et al,* 2012). First line treatment of GVHD is steroid therapy and patients may require high doses for prolonged periods of time thus exposing them to the known risks of steroid therapy, such as Cushing's syndrome, diabetes, weight gain, growth retardation and osteoporosis (Dignan *et al,* 2012). Steroid therapy is effective in less than 50% of patients and for those who are steroid refractory there is little consensus on management and treatment options are limited (Dignan *et al,* 2012). More recently extracorporeal photopheresis (ECP) has been introduced as a treatment option (Gatza *et al,* 2008, Bladon and Taylor, 2006, Scarisbrick *et al,* 2008, Marshall, 2006). The process uses a closed system device that centrifuges patient's blood to collect a mononuclear cell fraction which can then be treated with liquid 8-methoxypsoralen (8-MOP) before being exposed to ultraviolet light (UVA) prior to re-infusion into the recipient (Figure 2).

Psoralen or 8-MOP is a small molecule derived from Psoralea plants which can enter cells and their nuclei. The molecule is inert until activated by UVA irradiation when it becomes highly reactive, cross-linking deoxyribonucleic acid (DNA) and causing apoptosis. The 8-MOP returns to its inactivated state as soon as the UVA irradiation stops. Although only 5-10% of circulating mononuclear cells are treated, the procedure causes widespread apoptosis of all T cell subsets over the subsequent 72 hours. Additionally the process of separation of the mononuclear cells during ECP treatment stimulates monocytes to differentiate into activated dendritic cells which

engulf the apoptotic T cells and subsequently regulate immune responses via modulation of the APCs to induce immune-tolerance (Holtick *et al,* 2008, Gatza *et al,* 2008). Proliferation and up-regulation of Tregs after ECP treatment leads to an increase in the anti-inflammatory cytokines interleukin (IL) 10 and transforming growth factor beta (TGF-β). This proliferation of Tregs is detectable in some cases for many months after ECP treatment (Lamioni *et al,* 2005).
Progress in the understanding of the pathophysiology of GVHD has led to attempts to develop therapies directed against various cells and cytokines that play a crucial role in the condition. For example, the central role played by IL-2 in the expansion of allo-reactive T cells and their differentiation into CTLs in acute GVHD is targeted by CsA which inhibits its secretion (Reddy *et al,* 2009). Also mesenchymal (stromal) stem cells (MSCs) have been shown to be effective in modulating the immune and inflammatory response in some patients with steroid-refractory GVHD and are the subject of active research (Kebriaei and Robinson, 2011, Tolar, Villeneuve and Keating, 2011, Reddy *et al,* 2009, Zeiser *et al,* 2004, Baron *et al,* 2014).

Several subtypes of cells of the immune system are implicated in the process of GVHD, however Tregs are key players in this setting and are able to down-regulate responses to auto- and allo-antigens and are therefore important in preventing and controlling auto-immune disease, inflammation and also graft rejection (Schmidt, Oberle and Krammer, 2012, Shalev *et al,* 2011, Sakaguchi *et al,* 2009). Although a number of regulatory T subsets have been identified, cluster of differentiation (CD) 4+ T cells that co-express CD25 (the IL-2 receptor) have provoked the most interest in the field of transplantation as these markers define an immune-regulatory population that have been implicated in the amelioration of the incidence and/or severity of GVHD. Numbers of these CD4+CD25+cells in both the transplanted material and in the patient during immune reconstitution have been studied to evaluate the way the immune system works against malignant cells following autologous and allogeneic transplantation and also their effect on the incidence and severity of GVHD following allogeneic transplantation (Ukena *et al,* 2011b, Stanzani *et al,* 2003, Rezvani *et al,* 2006, Pastore *et al,* 2011, Magenau *et al,* 2010).

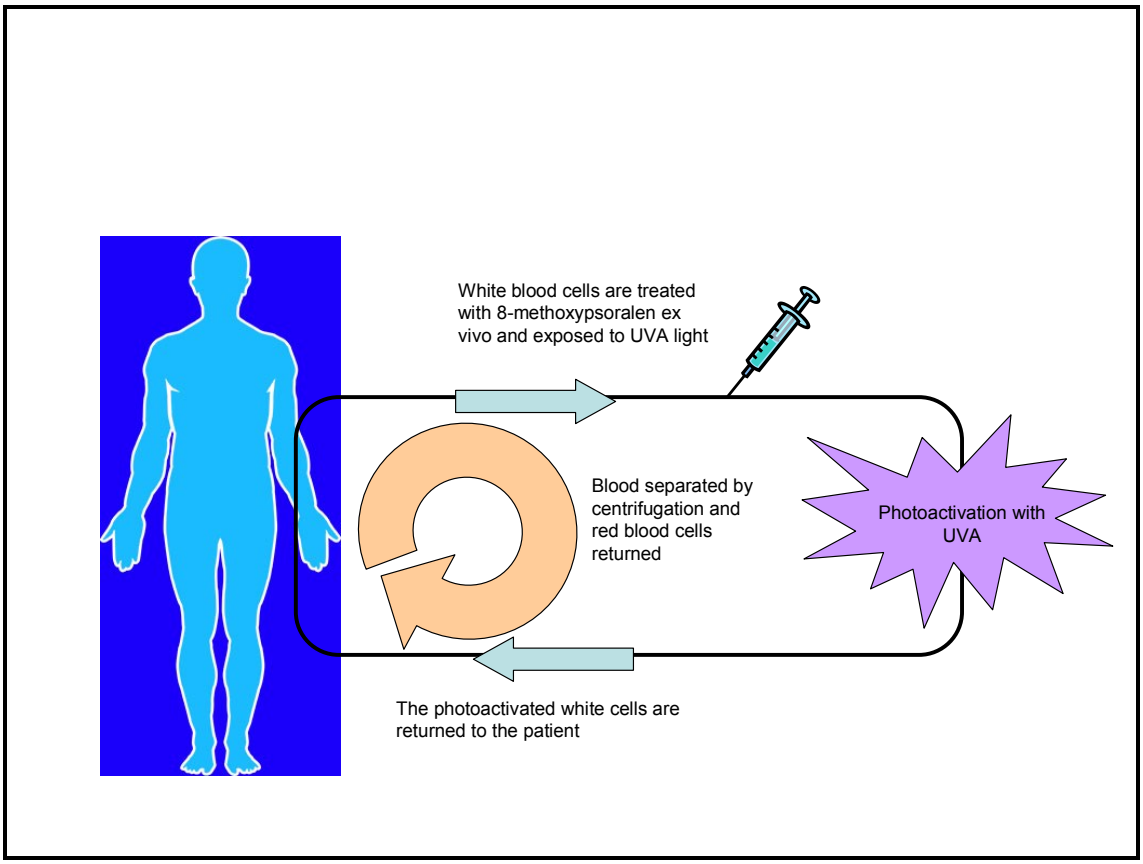


Figure 2: Diagrammatic representation of the ECP procedure. Patient's blood is centrifuged using a closed process that removes mononuclear cells and returns red cells to the patient. Psoralen (8-MOP) is added to the mononuclear cells which are then treated with UVA causing activation of the 8-MOP and subsequent apoptosis of T cells. Treated cells are returned to the patient. The removal of the UVA light returns the 8-MOP to an inert state.

1.2 Regulatory T cells

The existence of a subset of 'suppressor cells' was first postulated in 1970 by Gershon and Kondo, when they successfully employed antigen-experienced cells to transfer antigen-specific tolerance to naïve mice (Gershon and Kondo, 1970). Despite extensive investigation over the next decade investigators were unable to identify the molecular basis of suppression, so the idea of suppressor cells was largely abandoned. In 1995, Sakaguchi and colleagues were able to identify a population of CD4+ cells which expressed high levels of CD25, the high affinity IL-2 receptor, and which were capable of preventing autoimmunity in a murine model (Sakaguchi *et al,* 2009). The identification of CD25 as a phenotypic marker of suppressive CD4+ cells in mice encouraged further research and a similar population of cells, now named regulatory T cells or Tregs, was identified in humans (Baecher-Allan *et al,* 2001, Dieckmann *et al,* 2001).

A number of different Treg subsets are now recognised but they can be divided into two major subsets depending on their source: natural Tregs (nTregs) which originate in the thymus and adaptive or induced Tregs (iTregs) which are induced in the periphery (Figure 3). Following a recent move towards greater clarity in Treg nomenclature, these subsets are now known as thymic derived Tregs (tTregs) and peripheral-induced Tregs (pTregs) respectively (Abbas *et al,* 2013). Depsite the differences in origin, both subsets share common features such as expression of FoxP3 and ability to suppress proliferation of effector T cells. However, there is considerable lineage plasticity (Turka and Li, 2010, Komatsu *et al,* 2009, d'Hennezel *et al,* 2011) and the significance of this plasticity on therapeutic Treg treatments is an area of intense research and debate (Zhou, Chong and Littman, 2009, Komatsu *et al,* 2009, Zhou *et al,* 2009). The best characterised Tregs are CD4+CD25^{high} which are referred to exclusively in this study as Tregs and function by inhibiting effector T cells. As well as maintaining immune tolerance to limit autoimmune responses and regulating homeostatic lymphocyte expansion, they are capable of suppressing antitumour immune activity.

Thymic derived Tregs are selected and matured in the thymus by autoantigens from where they subsequently emigrate to peripheral lymphoid tissues with a mainly autoreactive T-cell receptor (TCR) repertoire to enforce a dominant negative regulation on other cells of the immune system including B cells, NK cells, NKT cells, CD4^{+}, and CD8^{+} T cells, as well as monocytes and dendritic cells (DCs) (Schmidt, Oberle and Krammer, 2012). This autoimmune suppression has led to interest in their role in transplant tolerance (Beres and Drobyski, 2013, Fozza and Dazzi, 2012, Michael, Shimoni and Nagler, 2013).

They constitute approximately 5-10% of peripheral T cells in humans (Shevach *et al,* 2001, Thornton and Shevach, 1998). Differentiation and development of functional Tregs in the

thymus occurs as a number of not yet fully understood interdependent steps as immature CD4+ thymocytes firstly express CD25 and subsequently FoxP3 positivity (Goldstein *et al,* 2013). It is known that development requires the master switch transcription factor fork-head box protein 3 (FoxP3) which inhibits activation-induced IL-2 gene transcription and cytokines and is responsible for maintaining the inhibitory function of pTreg cells (Thornton *et al,* 2004, Sakaguchi *et al,* 2009). Disruption of the tTreg selection and development process that takes place in the thymus, for example by donor T cell destruction of thymic epithelial cells following allogeneic HPC transplantation, can result in an imbalance in tolerance mechanisms (Zorn, 2006). This is likely to contribute significantly to clinical manifestations of graft vs host disease and graft vs malignancy effects observed in transplant patients.

Additionally, pTregs can be generated from naïve T cells in the peripheral lymphoid organs under certain stimulatory conditions, for example TCR activation of naïve CD4+ T cells in the presence of TGFβ (Sakaguchi, 2003). These Tregs demonstrate a TCR repertoire similar to those of the T cells from which they are derived and are therefore more skewed towards foreign antigens. These peripherally induced Tregs demonstrate a more unstable FoxP3 expression than thymically derived Tregs and may revert back to effector T cells. Much work is being carried out to determine to what extent pTregs contribute to the overall Treg pool under various conditions and to determine the extent to which the two subsets, t and pTregs, share functional properties (Curotto and Lafaille, 2009, Michael, Shimoni and Nagler, 2013). Progress has been hampered by the lack of suitable markers that differentiate pTreg and tTregs and to distinguish their functions (Povoleri *et al,* 2013).

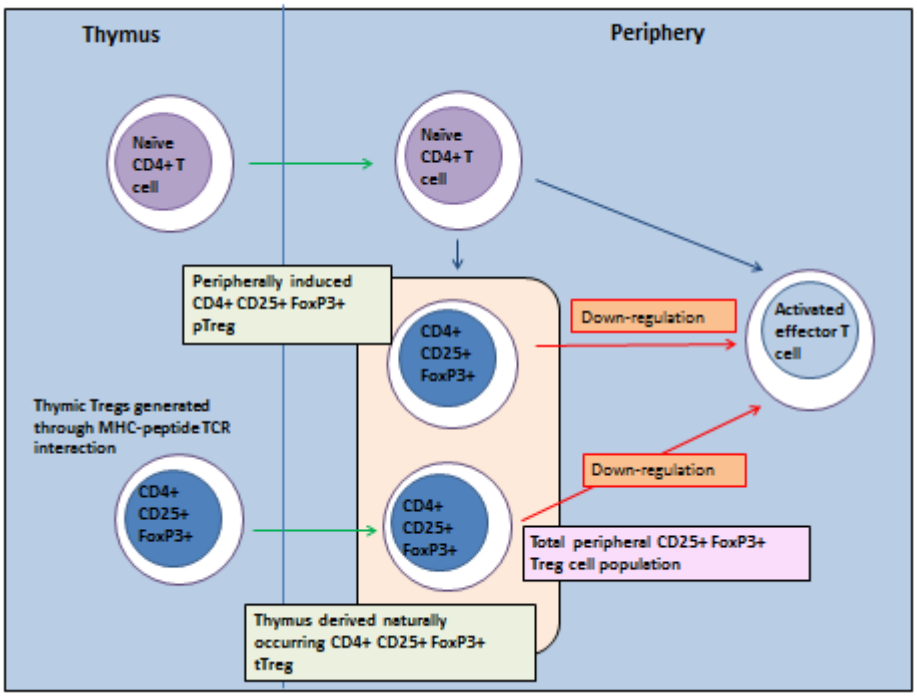


Figure 3: Natural and adaptive Treg generation. Thymic Tregs (tTregs) are generated through MHC-peptide TCR interaction in the thymus from where they migrate to lymphoid tissues in the periphery. As they are selected and matured by autoantigens their TCR repertoire is primarily autoimmune. Peripheral naïve T cells are capable of FoxP3 up-regulation into induced Tregs (pTregs) and these Tregs have a similar TCR repertoire to the effector T cells that they are derived from and are therefore more biased towards foreign antigens. Both tTregs and pTregs contribute to the overall pool of peripheral FoxP3+ Tregs and are capable of suppressing effector T cells. Republished with permission of Cell Press, from Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? Curotto de Lafaille and Lafaille, 30 (5) Copyright 2009; permission conveyed through Copyright Clearance Center, Inc.

It is clear now that Tregs do not use a single mechanism of suppression but a variety of different modes of action to exert a suppressive effect, but some mechanisms are still not fully understood or agreed upon (Vignali, 2012). However, the consensus is that there are three main categories of suppressive mechanism. Firstly, there is a cell to cell contact-mediated inhibition by the inhibitory receptors cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and lymphocyte-activation gene 3 (LAG-3), which modulate the immuno-stimulatory capacity of DCs and the secretion of the cytotoxic molecules granzyme and perforin resulting in T cell apoptosis (Figure 4).

The second mechanism is by metabolic disruption of T cells. CD39 and CD73 on Treg cells are able to generate adenosine which binds with the adenosine receptor of effector T cells, increasing intracellular cyclic adenosine monophosphate (cAMP) which suppresses T cell function (Turka and Li, 2010). Additionally Tregs compete with effector T cells for IL-2 required for T cell proliferation. Tregs do not produce IL-2 but as they require IL-2 for their suppressive function are dependent on their capacity to utilise IL-2 secreted by effector T cells (Brandenburg *et al,* 2008, de *et al,* 2004, Scheffold, Hühn and Höfer, 2005). The high affinity IL-2 receptors of Tregs enable them to consume a large proportion of the IL-2 produced by stimulated CD4 effector cells resulting in the deprivation of IL-2 required for proliferation of effector CD4 cells and results in proliferation arrest and apoptosis (Pandiyan and Lenardo, 2008, Pandiyan, Zheng and Lenardo, 2011). Not only do the Tregs consume growth cytokines essential for effector T cell proliferation, they are also able to cause a failure of the activated cells to produce cytokines later in the activation phase by removal of the essential IL-2 positive feedback.

The third suppressive mechanism is the release of the inhibitory cytokines IL10, IL-35 and TGF-β which exert a suppressive effect by specifically reducing the activation of APCs and by inhibiting effector T cell proliferation (Turka and Li, 2010). In a steady state, Tregs can also be found in non-lymphoid tissues with numbers increasing substantially during an inflammatory state. Recruitment of Tregs to sites of inflammation is through the wide range of chemokine receptors and cell adhesion molecules that they express and they migrate in response to chemo-attractants and ligands expressed during inflammation. This allows on-going suppression of T cells during the different stages of the immune response (Shevach *et al,* 2001).

Because Tregs are involved in the suppression of immune responses their use in controlling auto-immune diseases and tumour immunity is being explored by many researchers (Yakirevich and Resnick, 2007, Curiel, 2008, Ondondo, 2014, Ondondo *et al,* 2013, Burocchi, Colombo and Piconese, 2013, Adeegbe and Nishikawa, 2013). In the context of HPC transplantation, the Treg regulation of both GVHD and GVM after allografting has recently attracted attention (Fozza and Dazzi, 2012, Michael, Shimoni and Nagler, 2013, Beres and Drobyski, 2013). Furthermore there

is interest in their possible role in tumour tolerance in patients undergoing autologous HPC rescue (Kline *et al,* 2008, von Keudell, Rosenbaum and Zimmerman, 2013).

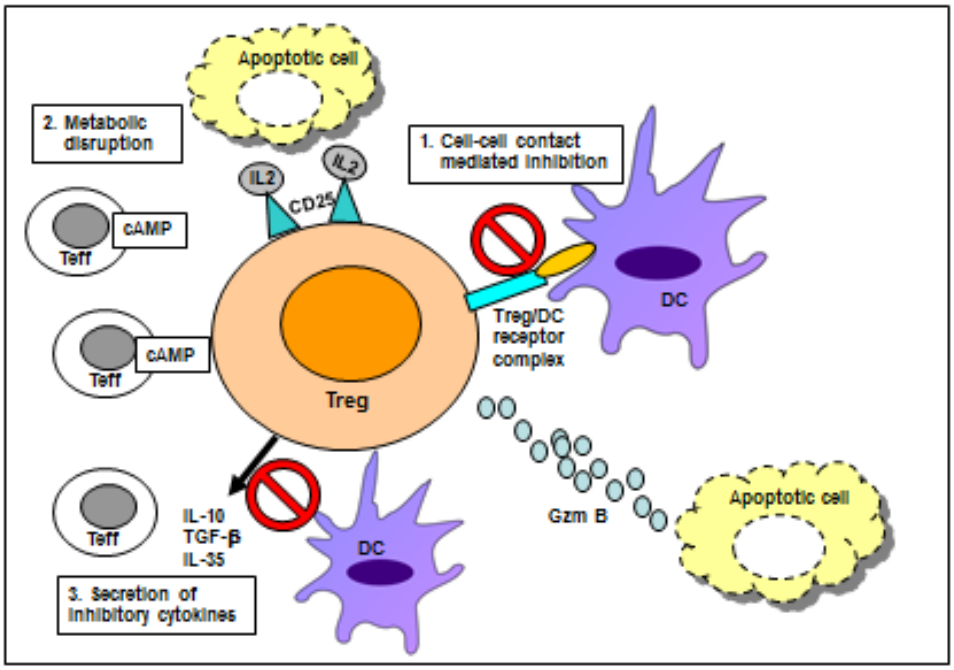


Figure 4: Three categories of Treg suppressive mechanisms. 1) Cell-cell contact-mediated suppression reduces the immuno-stimulatory properties of dendritic cells by Treg inhibitory receptors such as CTLA-4 and LAG-3 interacting with CD80/86 and MHC molecules on the DC (Treg/dendritic cell receptor complex). Treg excretion of Granzyme B (Gzm B) leads to apoptosis of effector T cells. 2) Metabolic disruption of effector T cells by increasing cAMP thus reducing their function and by IL-2 deprivation of effector T cells leading to apoptosis. 3) Secretion of inhibitory cytokines such as IL-10, IL-35 and TGFb1 which inhibit T cells and DCs. Figure adapted from (Schmitt and Williams, 2013). Available under a CC-BY license.

1.2.1 Issues with the identification of Tregs

Despite increasing interest in the biology and function of Tregs, their identification and characterisation have proved controversial (Pini, Ojeda and Portolés, 2007). For future comparison of data emerging from various studies it will be important to reach a consensus about the best way of identifying Tregs.

The expression of CD25 was the original defining marker of Treg cells. However, although $CD25^{high}$ is a good marker for Tregs in mice kept in pathogen-free environments, studies in humans constantly exposed to foreign antigens have shown that CD25 is induced on activated T cells and not just on Tregs. A significant fraction of CD25+ cells are therefore activated effector T cells (Beyer and Schultze, 2006). Earlier workers such as Stanzani and colleagues looked at the incidence of CD4+CD25+ cells in patients post HLA-identical transplant and concluded that the use of the markers CD4 and CD25 was insufficient to identify Tregs in humans (Stanzani *et al,* 2003).

In humans, the loss-of-function mutation of the transcription factor FoxP3 results in the fatal multi-organ auto-immune disease and inflammatory disorder 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX) syndrome (Ziegler *et al,* 2001). A similar syndrome is seen in mice resulting in the mutant strain of scurfy mice and this assisted in the understanding of the mechanisms underlying differentiation and function of Treg cells. In both humans and mice, only males, not heterozygous females, were affected. This confirmed that FoxP3 is an X chromosome transcription factor and the systemic nature of both diseases was consistent with the theory that FoxP3 mutations affected the differentiation and function of Treg cells. The identification of the lineage-specific marker FoxP3 led to its use as a marker to identify Treg cells that have been generated in the thymus or induced by peripheral conversion (Ramsdell, 2003).

Tregs can be distinguished from recently activated T cells by low or absent surface expression of CD127, the α-chain of the IL-7 receptor, which is down-regulated on Tregs and is inversely correlated to the expression of FoxP3 (Liu *et al,* 2006). Used in combination with CD25, CD127 appears to be a better marker than CD25 alone. Recently, commercial Treg identification kits have become available using a range of surface and intracellular markers in combination (including FoxP3, CD25 and CD127) to assist in the accurate identification of specific Treg populations. Although FoxP3 is considered a superior marker for Tregs compared to the cell surface markers CD25 and CD127, it is a nuclear protein and its intracellular nature means that whereas it can be used for detection of Tregs, FoxP3 is not suitable for the isolation of live Treg cells. Additionally, activated T cells have now been shown to transiently express FoxP3, thus reducing its usefulness as a Treg marker (Pillai *et al,* 2007). It is also unable to distinguish tTregs

from pTregs (Povoleri *et al,* 2013). Since the recognition of the two main Treg subsets there has been an emphasis on finding markers able to distinguish between them to enable a greater understanding of their specific functions and to assist in the development of therapeutic options. Recently, an intracellular protein, the Helios transcription factor, has been identified which is important for Treg development. This is a member of the Ikaros family and has been demonstrated to up-regulate the expression of FoxP3, maintain high FoxP3 expression and a stable Treg population (Elkord and Al-Ramadi, 2012). Despite claims by Dhamne and colleagues that Helios is exclusively expressed by tTregs and can therefore be used to differentiate between pTregs and tTregs (Dhamne *et al,* 2013) this has been challenged by other workers who have shown that Helios is expressed during T cell activation and proliferation (Povoleri *et al,* 2013).

The Treg phenotype has also been linked to expression of a number of surface proteins: CD39/CD73 (Dwyer *et al,* 2007), CD45R0 and CD45RA (Miyara and Sakaguchi, 2007), glucocorticoid-induced tumour necrosis factor receptor (GITR) (Yamazaki *et al,* 2002) and CTLA-4 (Su *et al,* 2012, Pini, Ojeda and Portolés, 2007, Wing *et al,* 2008). The results, however, have been disappointing as most of these markers are expressed on activated T cells and are unable to distinguish between tTregs and pTregs so the isolation and characterisation of these cells remains a challenge.

The most promising alternative marker to be described that distinguishes between Treg subgroups is neuropilin-1 (Nrp-1)(Bruder *et al,* 2004). This surface molecule is differentially expressed in murine tTregs and pTregs being poorly expressed in the latter but the findings are yet to be confirmed in humans (Weiss *et al,* 2012, Yadav *et al,* 2012).

As the understanding of Tregs and their markers has evolved, this has resulted in researchers using a variety of markers or combinations of markers in order to best identify these cells. As a consequence, data comparison between studies is difficult as different markers may not identify the same Treg cell populations or may in fact be measuring not only Tregs but activated T cells. It is therefore important to note the markers being used when studying the literature as the variation can lead to confusing and contradictory results.

1.2.2 Tregs in tumour tolerance

The presence of Tregs appears to have a detrimental effect in anti-tumour immune responses. It is well recognised that tumours are able to escape immune surveillance or to suppress immunity (Ghiringhelli *et al,* 2005a). As Tregs are capable of induction and maintenance of tolerance to self-antigens it is not surprising that they can also induce and maintain tolerance to tumour antigens derived from the host (Cao, 2010). Studies in mice have shown that depletion of CD25 Tregs leads to enhanced anti-tumour immune responses (Nagai *et al,* 2004, Ghiringhelli *et al,*

2005b) and that the adoptive transfer of CD25 Tregs can inhibit CD8+ and NK anti-tumour activity (Ghiringhelli *et al,* 2005a, Ko *et al,* 2007). A number of mechanisms are thought to be involved in Treg suppression of anti-tumour immune responses although as yet these are not fully understood (Ondondo, 2014, Adeegbe and Nishikawa, 2013). Treg accumulation in tumour tissues and draining lymph nodes has been reported in individuals with a wide range of solid tumours including ovarian (Curiel *et al,* 2004), breast (Gobert *et al,* 2008), pancreatic adenocarcinoma (Liyanage *et al,* 2006), gastro-intestinal (Ichihara *et al,* 2003, Sasada *et al,* 2003), melanoma (Viguier *et al,* 2004, Piccirillo, 2010) and haematological malignancies (Wolf *et al,* 2003). The accumulation of Tregs may be due to their preferential migration to the tumour environment in response to cytokines secreted by these tumours. Additionally, tumours may have the ability to trigger the production of cytokines that are able to facilitate the conversion of Tregs from conventional T cells (Liu *et al,* 2007) and to increase the proliferation of Tregs within the tumour microenvironment (Ghiringhelli *et al,* 2005b, Curiel *et al,* 2004). The manipulation of the Treg pathway is therefore a strategy being used to improve the efficacy of cancer immunotherapies (Cao, 2010, Curiel, 2008, Fozza and Dazzi, 2012, Wainwright *et al,* 2013, Ko *et al,* 2012).

However it should be noted that although overall most studies showed that accumulation of Tregs is associated with a poor prognosis, not all data demonstrates this. For example, Tzankov and colleagues found that high levels of Tregs positively influenced disease free survival in diffuse large B cell, follicular and Hodgkin's lymphoma although increased Treg numbers had a negative prognostic effect in non-germinal centre diffuse large B cell lymphomas (Tzankov *et al,* 2008). In a study conducted by Salama and colleagues of patients with colo-rectal cancer, high levels of Tregs in tumours were predictive of better survival, however higher levels of Tregs in normal colonic mucosa in the same patients were associated with poor prognosis (Salama *et al,* 2009).

1.2.3 *In-vivo* use of Tregs

Recognition of the importance of Tregs in transplantation stimulated an interest in the *ex vivo* expansion of these cells for possible therapeutic use but the lack of a unique cell surface marker has hindered these studies. Removal of CD4+CD25+ activated effector T cells from the Treg fraction has proved difficult under good manufacturing practice (GMP) conditions (Le and Chao, 2007). In order to obtain the quantities of Tregs required for *in vivo* suppression of GVHD it is also necessary to expand Tregs but obtaining the pure cultures required to conform to regulatory requirements is challenging. Additionally Tregs demonstrate a degree of plasticity in culture and are reported to be able to revert to effector T cells (Turka and Li, 2010). This would be of great concern if adoptive transfer of Tregs was being used for suppression of GVHD

because accidental infusion of effector T cells could enhance GVHD reactions (Miyara and Sakaguchi, 2011, Riley, June and Blazar, 2009). Prevention of Tregs reverting to effector cells is obviously the key to improving safety of this therapy. A number of trials studying the use of adoptive Treg therapy in GVHD are nonetheless in progress (Hippen *et al,* 2011).

1.2.4 The role of Tregs in allogeneic transplantation

Initial studies of the role of Tregs after allogeneic HPC transplant were performed in mice (Cohen *et al,* 2002, Taylor, Noelle and Blazar, 2001). Mice were irradiated or treated with myeloablative conditioning before being transplanted with T cell depleted bone marrow alongside infused peripheral T cells to induce GVHD. It was noted that CD25+ depletion of the peripheral T cells prior to infusion resulted in increased GVHD severity and higher mortality rates thus demonstrating that physiological numbers of Tregs in the graft may offer some protection against GVHD (Taylor, Lees and Blazar, 2002). By transplanting a high ratio of Tregs to conventional T cells, development of GVHD could be prevented even when normally lethal doses of conventional T cells were infused (Hoffmann *et al,* 2002). Hoffmann showed that both the CD4+CD25- and the CD4+CD25+ cells migrate to GVHD target organs such as the skin and gut where the Tregs are able to restrict the local expansion and pro-inflammatory capacity of the effector T cells .

Since mouse models showed that Tregs in the allograft transferred to the recipient could affect GVHD this led to a number of studies in humans (Stanzani *et al,* 2003, Wolf *et al,* 2007, Pastore *et al,* 2011, Rezvani *et al,* 2006). However, results are difficult to compare due to low patient numbers, significant differences in conditioning and immunosuppressive regimens, variation in treatment of the graft and substantial differences in methods and markers used for Treg enumeration (Pini, Ojeda and Portolés, 2007).

One of the first human studies showed unexpected results when Stanzani reported that increased numbers of CD25+ CD4 and CD8 cells in the graft increased the risk of GVHD in recipients (Stanzani *et al,* 2003). More recent studies have not corroborated Stanzani's results, for example, Pastore showed that a low CD3:Treg ratio in the graft resulted in lower incidence and severity of GVHD, as well as a better overall survival, disease free survival and non-relapse mortality (Pastore *et al,* 2011). Pabst investigated the graft composition in unrelated donors and found that higher Treg numbers resulted in a lower incidence of GVHD (Pabst *et al,* 2007). Wolf studied matched sibling donors and recipients undergoing either myeloablative or reduced intensity conditioning (Wolf *et al,* 2007). The study showed that patients receiving low numbers of Tregs in the graft after myeloablative conditioning had a significantly higher incidence of GVHD than those who received higher Tregs, but were unable to demonstrate this after reduced

intensity conditioning. No difference in relapse rates was apparent in either group (Wolf *et al,* 2007).

Some studies have included consideration of Treg numbers in the donors prior to collection. Rezvani and colleagues found that the absolute number of peripheral CD4+FoxP3+ Tregs at 30 days post allogeneic transplant was low in patients suffering from GVHD (Rezvani *et al,* 2006). The same study compared the absolute numbers of CD4+ and CD4+FoxP3+ T cells in the donors of patients who had no GVHD or only mild GVHD, with those of patients who developed marked GVHD. No significant difference was noted in the absolute numbers of CD4+T cells although the number of CD4+FoxP3+ T cells was significantly higher in the donors of recipients who did not develop GVHD (Rezvani *et al,* 2006). Rezvani's team also noted a correlation between the absolute numbers of donor CD4+FoxP3+ cells infused in the graft and a reduced risk of GVHD. However there was no correlation between absolute numbers of CD4+ $CD25^{high}$ T cells infused and the incidence of GVHD which suggests that CD25+ may not be an ideal marker of regulatory activity in these studies and in fact it is now known that the CD25+ T cell subset will include both Tregs and recently activated T cells (Pini, Ojeda and Portolés, 2007).

In contrast, Zhai and colleagues reported that the number of CD4+$CD25^{high}$ T cells in recipients correlated significantly with that of their corresponding donors and that recipients with normal or high CD4+$CD25^{high}$ T cells had no or mild GVHD (Zhai *et al,* 2007). Subsequently, however, Nöel and co-workers (2008) were unable to substantiate this claim. They found no evidence that the protection against GVHD was dependent on the numbers of Tregs transferred from the donor to the recipient and found no correlation between the numbers of circulating CD4+$CD25^{high}$ T cells in the peripheral blood of patients during the early post-transplant period and development of GVHD (Noël *et al,* 2008).

When considering optimal numbers of CD4+FoxP3+ T cells for control of allo-reactivity to prevent GVHD it is important to retain the GVM effect. Studies have shown that Treg frequencies are increased in patients with many types of solid tumours and it has been suggested that increased Treg numbers are a detrimental factor in the generation of host-versus-malignancy immunity by suppression of tumour specific effector T cell responses and development of immune tolerance to tumour cells (Teng *et al,* 2010, Zhou *et al,* 2010, Zou *et al,* 2004).

In mice, ablation of CD4+CD25+ Treg cells by administration of a CD25 monoclonal antibody led to the development of tumour-specific CD8+ effector T cells and NK cells (Shimizu, Yamazaki and Sakaguchi, 1999, Onizuka *et al,* 1999). Furthermore, Nadal and colleagues demonstrated that increased numbers of CD4+$CD25^{high}$ Tregs correlated with increased incidence of molecular disease relapse after stem cell transplant for chronic myeloid leukaemia (Nadal *et al,* 2007).

These findings suggest that the numbers of Tregs post-transplant also adversely affects the degree of GVM. However in Zhai's study all patients with low levels of CD4+CD25^{high} T cells died within a one year period post-transplant. Although all these patients developed severe GVHD, it is not stated whether this was the cause of death. Interestingly, none of the patients with no or mild GVHD died within the study period however levels of relapse in the surviving cohort were not discussed. Taken together, data suggest that in allogeneic transplantation under some circumstances elevated Treg numbers may prevent GVHD. This may decrease the beneficial effects of GVM and conversely removal of Tregs may allow the full effects of GVM but lead to a higher incidence of severe GVHD.

1.2.5 The role of Tregs in Autologous HPC Transplantation

Autologous HPC transplantation is an effective treatment for a number of haematological malignancies. This treatment option is dependent on the patient having achieved remission status and showing no signs of significant tumour resistance to previous chemotherapy. The intensity of the high dose therapy may be limited by bone marrow or other organ tolerance. However, in the majority of autologous transplants, a potentially lethal dose of chemotherapy is administered to eradicate the tumour, with the highly toxic effect on the bone marrow being overcome by re-infusing the patient's own cryopreserved haematopoietic cells. Despite best endeavour, disease relapse remains a significant problem if the residual malignant clone outstrips the normal marrow compartment during the post therapy recovery phase (Ljungman *et al,* 2010, Gratwohl *et al,* 2003, Gratwohl and Baldomero, 2009). Alternatively, re-emergence of the malignant clone following high dose chemotherapy may be due to the ability of residual malignant cells to escape immune surveillance and may be due in part to protection from Tregs which suppress anti-tumour T cell activity (Nadal *et al,* 2007).

This treatment strategy lacks the GVM effects of allogeneic transplantation and contributes to higher relapse rates than are seen after allogeneic transplants. On the other hand, recipients of autologous transplants do not experience the significant morbidity and mortality of acute GVHD. The higher relapse rate may be attributable to the absence of GVM, failure of conditioning treatment to eradicate residual disease or the presence of malignant cells in the harvest being infused (Hoffbrand, 2011). The numbers of Tregs relative to the numbers of effector T cells capable of mediating anti-tumour activity during haematopoietic reconstitution may also impact on relapse rates in recipients of autologous transplants.

The role of Tregs in autologous transplantation has not attracted as much attention as their role in the allogeneic setting. The few studies that have been performed are limited in their scope. Autologous transplant patients have been used a control group against which allogeneic transplant patients can be compared (Magenau *et al,* 2010) or studies have focussed on the

manipulation of the immune system during the post-transplant period to induce 'autologous GVHD' (auto-GVHD) and boost the anti-tumour effect (Kline *et al,* 2008)or most recently *in vivo* and *in vitro* depletion of Tregs in patients undergoing autologous transplants (von Keudell, Rosenbaum and Zimmerman, 2013).

An auto-immune syndrome similar to mild GVHD and affecting mainly the skin has been described following autologous transplantation (Drobyski *et al,* 2009, Fidler *et al,* 2012, Miura *et al,* 2004, Kline *et al,* 2008) and has been termed auto-GVHD or auto-aggression syndrome. This syndrome appears to arise due to a combination of the inhibition of thymic-dependent clonal deletion of auto-reactive T cells and the elimination of the peripheral immuno-regulatory system by the preparative regimen providing a permissive environment for the auto-reactive T cells (Hess, 2010). Auto-GVHD has been noted to occur spontaneously in some patients receiving an autologous transplant following a preparative regimen consisting of total body irradiation and/or cyclophosphamide. Originally it was thought that these were the only situations in which the phenomenon occurred but subsequently it has also been seen in patients treated with a preparative regimen of high dose melphalan who received CsA post-transplant (Baron *et al,* 2000). In experiments with rats and mice auto-GVHD can also be induced by CsA administration in the post-transplant setting (Glazier *et al,* 1983), a finding which has also been demonstrated in humans (Baron *et al,* 2000). Significant anti-tumour activity has been demonstrated following induction auto-GVHD in animal studies and there is some evidence of an anti-tumour effect in humans (Byrne *et al,* 1997). Miura and colleagues found that the use of CD34+ selected grafts also induced auto-GVHD. They postulated that the low incidence of auto–GVHD after non-selected autologous transplants is due to peripheral Tregs transferred with the graft that down-regulate the development of the syndrome and also anti-tumour activity (Miura *et al,* 2004). Auto-GVHD syndrome has been associated with significant anti-tumour activity in animal studies (Bryson *et al,* 1999) and although it was thought that induction of auto-GVHD following autologous HPC transplant could provide a potent treatment for patients with a number of malignancies including breast cancer, myeloma and lymphoma, so far it has shown no definitive benefit in terms of disease free survival or overall survival (Baron *et al,* 2000, van *et al,* 2000, Kline *et al,* 2008).

A recent pilot study to determine the effect of Treg depletion following autologous transplants in myeloma patients showed promising results *in vivo* and *in vitro*, but patient numbers were too small to draw any conclusions (von Keudell, Rosenbaum and Zimmerman, 2013). As Tregs levels both in the graft and in the peripheral blood during recovery from autologous transplant may strongly inhibit anti-tumour immune responses and few large studies have been performed to date, this study proposes to assess the impact of Tregs in grafts and in the peripheral blood of autologous and allogeneic HPC transplant patients on clinical outcome.

1.3 Mobilisation and collection of HPCs

For both allogeneic and autologous transplantation, HPC collection is essential. While it is possible to harvest HPCs directly from the bone marrow space, with the exception of paediatric donors this procedure has been largely superseded by apheresis. Apheresis involves collection of HPCs from the peripheral blood following the use of the haematopoietic growth factor granulocyte-colony stimulating factor (G-CSF) alone, or in combination with chemotherapy such as cyclophosphamide, to mobilise HPCs from the bone marrow. These peripheral blood harvests are associated with a higher incidence of GVHD than bone marrow harvests (Urbano-Ispizua, 2007, Gallardo *et al,* 2009).

In the absence of mobilisation, HPCs are bound to the bone microenvironment marrow niches and this adhesion and proximity plays a large part in regulating their renewal, proliferation and trafficking (Hoffbrand, 2011). Adhesion molecules expressed by sinusoidal endothelial cells and perivascular MSCs in the vascular compartment of the bone marrow anchor the HPCs. A similar role is afforded by osteoblasts in the endosteal niche (To, Levesque and Herbert, 2011, Hopman and Dipersio, 2014). Active HPCs which proliferate to renew the haematopoietic system are located mainly in the perivascular niches and those that are quiescent are preferentially located in the poorly perfused endosteal niches (To, Levesque and Herbert, 2011). The balance of HPC proliferation, differentiation and egress into the peripheral blood requires a high level of control. Only very small numbers (< 0.1% of total white blood cells) of HPCs are normally present in circulating blood of individuals under normal conditions, with this number increasing in times of stress (Hopman and Dipersio, 2014).

The use of the CD34 surface antigen, a trans-membrane glycoprotein present on immature haematopoietic cells, allows identification and enumeration of HPCs. Mobilisation involves the use of an agent or combination of agents in order to cause proliferation of CD34+ HPCs, subsequent severance of their bonds with the marrow microenvironment and egression into the peripheral blood from where they can be collected (Greenbaum and Link, 2010, Zipori *et al,* 2002).

The most commonly used agent for mobilisation of HPCs is the cytokine, G-CSF which controls granulocyte expansion and neutrophil egress from the bone marrow into peripheral blood during non-disease states (Hopman and Dipersio, 2014). Treatment of donors with G-CSF can cause large increases in circulating progenitor cells in the peripheral circulation which can then be harvested using apheresis. The expansion of granulocytes by G-CSF increases the production and secretion of proteolytic enzymes from myeloid precursors which cleave adhesion molecules that anchor HPCs to the stroma and extracellular matrix in the marrow micro-environment (Stroncek *et al,* 1997, Zipori *et al,* 2002, Hopman and Dipersio, 2014).

G-CSF is used for mobilisation of HPCs in both autologous and allogeneic HPC donors. When compared with collection of HPCs directly from the bone marrow, HPCs mobilised into the peripheral blood by G-CSF and collected by apheresis result in harvests with approximately two-fold higher CD34 yields (Favre *et al,* 2003), and reduced time to neutrophil and platelet engraftment post-transplant requiring fewer platelet transfusions, reduced hospital stays and associated costs (Bensinger *et al,* 2001, Blaise *et al,* 2000, Hartmann *et al,* 1997).

G-CSF can be used alone (Table 1) or in combination with chemotherapeutic agents (Table 2) to mobilise HPCs in autologous donors. In the case of allogeneic donors, G-CSF is only ever used alone as it would be ethically unacceptable to do otherwise (Table 3).

Table 1: Autologous HPC mobilisation regimen using G-CSF alone

Day 1	Day 2	Day 3	Day 4	Day 5
G-CSF 10µg/kg	G-CSF 10µg/kg	G-CSF 10µg/kg	G-CSF 10µg/kg	Test peripheral CD34 count. Perform harvest if >10/µl G-CSF 10µg/kg if further harvest required or if peripheral CD34 count <10/µl

Table 2: Autologous HPC mobilisation regimen using cyclophosphamide plus G-CSF (typical example of G-CSF plus chemotherapy)

Day 1	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Cyclophosphamide 1.5g/m^2	G-CSF 5µg/kg	G-CSF 5µg/kg	G-CSF 5µg/kg	G-CSF 5µg/kg	G-CSF 5µg/kg	Test peripheral CD34 count. Perform harvest if >10/µl G-CSF 5µg/kg if further harvest required or if peripheral CD34 count <10/µl

Table 3: Allogeneic HPC mobilisation regimen

Day 1	Day 2	Day 3	Day 4	Day 5
G-CSF 10µg/kg	G-CSF 10µg/kg	G-CSF 10µg/kg	G-CSF 10µg/kg	Test peripheral CD34 count. Perform apheresis if >10/µl or bone marrow harvest if <10/µl

Table 4: Plerixafor plus G-CSF autologous HPC mobilisation regimen

Day 1	Day 2	Day 3	Day 4	Day 5
16:00-18:00 G-CSF 10µg/kg	16:00-18:00 G-CSF 10µg/kg	16:00-18:00 G-CSF 10µg/kg	17:00 G-CSF 10µg/kg Plerixafor 240µg/kg	07:30 G-CSF 10µg/kg 08:30 Take sample for peripheral CD34 count. Start harvesting. Repeat dose of Plerixafor if further harvest required plus 10µg/kg G-CSF on morning of 2nd harvest.

In autologous donors only, chemotherapy is often combined with G-CSF in the mobilisation regimen as an anti-cancer treatment modality but it also results in a higher yield of HPCs which can be harvested during the haematopoietic recovery phase (Lickliter *et al,* 1994). Allogeneic donors only ever receive G-CSF alone. Typically, effective mobilisation results in the release of adequate HPCs into the peripheral blood to enable the collection of a sufficient cell yield over one to three harvests to perform a transplant either in the autologous or allogeneic setting (Hoffbrand, 2011).

In patients undergoing autologous transplantation, previous treatment of disease with chemotherapeutic agents or radiation may impair CD34 cell mobilisation (Boccadoro *et al,* 2002, Kumar *et al,* 2007, Mazumder *et al,* 2008, Tournilhac *et al,* 2003). In some heavily pre-treated patients it is not possible to collect adequate HPCs to perform a transplant using G-CSF alone or even in combination with chemotherapy (To, Levesque and Herbert, 2011, Hopman and Dipersio, 2014).

The advent of a novel mobilisation agent, Plerixafor, has yielded encouraging results in patients who are poor mobilisers (Cashen, Nervi and DiPersio, 2007, Flomenberg *et al,* 2005, Tanhehco *et al,* 2013, Hopman and Dipersio, 2014, Lemoli and D'Addio, 2008). Plerixafor works synergistically with G-CSF (Figure 4) resulting in successful mobilisation in the majority of heavily treated patients who often fail to mobilise sufficient HPCs using other mobilisation regimens (table 4).

The chemokine receptor type 4 (CXCR4) on early HPC progenitor cells and its ligand stromal derived factor 1 (SDF-1) on marrow stromal cells are involved in homing and mobilisation of HPCs (Hopman and Dipersio, 2014, To, Levesque and Herbert, 2011). This is a potent adhesive relationship between developing progenitor cells and the bone marrow microenvironment. Disruption of the CXCR4/SDF-1 axis by Plerixafor leads to enhanced mobilisation of HPCs. The mechanism by which Plerixafor functions is different from that of G-CSF and it has been shown that the CD34+ cell sub-populations that are mobilised by each agent differs (Donahue *et al,* 2009). In a rhesus macaque model, genes which were up-regulated in Plerixafor mobilisation and G-CSF mobilisation as single agents were different (Donahue *et al,* 2009) and these also differed from those which were up-regulated when G-CSF and Plerixafor were used in combination. Cells mobilised using Plerixafor alone included more B-cell, T-cell and mast cell precursors than cells mobilised by G-CSF alone which instead contained more neutrophil and mononuclear phagocyte precursors. Plerixafor and G-CSF in combination resulted in higher numbers of B-cell and T-cell precursors.

1.4 HPC and donor lymphocyte harvesting

1.4.1 Allogeneic harvests

In healthy allogeneic donors, collection or harvesting of HPCs takes place after administration of 10µg/kg/day of G-CSF for four days (Akizuki *et al,* 2000). On the fifth day, a peripheral CD34+ cell count is used to determine if sufficient CD34+ cells have mobilised into the peripheral circulation to perform a cell harvest. At RMH this target peripheral count is > 10/µl (for mobilisation regimen see Table 3). A small proportion of healthy donors will fail to mobilise using G-CSF and, because of the ethical implications of giving alternative mobilising agents, the collection in these cases is performed by bone marrow harvesting. The usual target CD34+ dose for allogeneic transplants is between 4 x 10^6/kg and 8 x 10^6/kg based on the recipient's weight (Hoffbrand, 2011). A dose which is too low may result in delayed or failed engraftment and too high a dose may increase the risk of severe GVHD in the recipient (Mehta, 2009). Above a threshold of approximately 2 x10^6 CD34+cells/kg, no correlation between dose transplanted and time to neutrophil engraftment can be shown in the Royal Marsden data (data not shown). This is corroborated by the studies of Pelus and Cottler-Fox and colleagues (Pelus, 2008, Cottler-Fox *et al,* 2003).

1.4.2 Autologous harvests

As described above, patients having HPCs collected for autologous transplant are mobilised using G-CSF either alone or in combination with chemotherapy (usually cyclophosphamide) or Plerixafor in combination with G-CSF (Tables 1, 2 and 4). Patients are tested at an appropriate point in the mobilisation regimen to determine whether adequate CD34+ cells have mobilised into the peripheral blood to enable harvesting to take place. In RMH, a white blood cell count of > 2.0x10^6/ml and a CD34+ cell count of >10/µl is used to indicate effective mobilisation and patients will then undergo apheresis to collect cells. If adequate mobilisation has not been achieved after five days, patients are given a further dose(s) of G-CSF and tested again over the subsequent days. If the patient fails to achieve an adequate peripheral CD34 count after a number of doses of G-CSF the mobilisation attempt is stopped. The number of doses is dictated by centre-specific mobilisation data and logistics surrounding apheresis slot availability. A clinical decision is then made about suitable regimens for further mobilisation attempts. In these circumstances a failed attempt with G-CSF alone is usually followed by at least one further G-CSF plus chemotherapy mobilisation. Failure of these attempts can be followed by a request for funding of a Plerixafor plus G-CSF mobilisation.

In patients treated with Plerixafor in combination G-CSF, apheresis takes place on a pre-set day in the regimen. It has been shown that adequate numbers of cells can be harvested despite very low peripheral CD34 counts in patients being mobilised using this regimen (Wells, Shaw and

Smith, 2009). Harvests performed on a cohort of patients with peripheral CD34 counts of >7/µl and < 10/µl were shown to yield adequate doses of CD34+ cells for transplantation in contrast to those patients mobilised using other regimens with similarly low peripheral counts.

1.4.3 Mechanism of HPC harvesting

Harvesting of peripheral blood HPC and donor lymphocytes is performed by apheresis using machines designed for the purpose. RMH uses Caridian Optia apheresis machines in common with most other units across Europe and the USA. Typically, a mononuclear cell (MNC) collection programme is used to collect the fraction of the peripheral blood which contains CD34+ HPCs and CD3+ donor lymphocytes (DLs). The process uses closed system centrifugation of approximately two total blood volumes in sequential 200ml volumes. Cells are distributed along a density gradient and the composition of the final harvested product depends on the layer that is collected. Within the white cell layer density gradient separation results in layering of the different cellular components with the lighter, lymphocyte-rich cells at one end of the gradient and the denser granulocyte-rich cells at the other. The Optia uses optical detection of the cellular interface (Figure 5) thus enabling the operator to visualise the layers after density gradient separation and select the desired component for collection (Caridian BCT Optia Operator Manual).

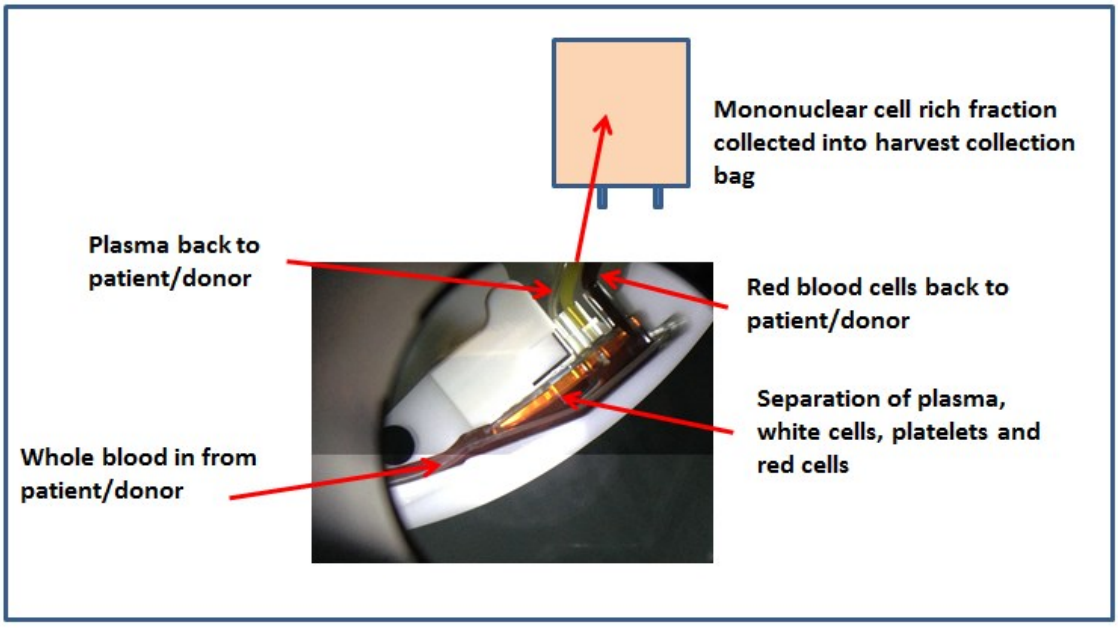


Figure 5: Apheresis using the Caridian Optia. The Caridian Optia separates plasma, white cells, platelets and red cells according to density and using its automated interface management (AIM) system can harvest the desired layer. Mononuclear cells are collected into the the harvest collection bag of the closed collection system while red cells and plasma are returned to the donor. Photograph taken by author.

1.4.4 The Impact of mobilisation regimens on Tregs in the graft

The different mobilisation kinetics are attributable to the various regimens used and result in variation in the CD34+ cell populations which are mobilised (Condomines *et al,* 2006). It is therefore probable that there are differences in numbers of Tregs harvested using these different mobilisation regimens. Vela-Ojeda and colleagues demonstrated a 2.4-fold increase in CD4+CD25+ T cells following mobilisation using a higher than normal dose of 16μg/kg G-CSF (Filgrastim) in patients with myeloma and non-Hodgkin's lymphoma, although the author cautions that, because of the markers used for Treg identification, these cells could be activated instead of regulatory lymphocytes (Vela-Ojeda *et al,* 2006). There is growing evidence that G-CSF is capable of altering T cell reactivity and modifying APC activity (Rutella, 2007, Rutella *et al,* 2005, Buzzeo *et al,* 2007).

The relative numbers of Tregs in conventional G-CSF or G-CSF plus chemotherapy compared with Plerixafor plus G-CSF mobilised cell collections is a growing area of interest as the role of Tregs in the graft becomes better defined. Kean and colleagues noted significant mobilisation of both T cells and Tregs when Plerixafor was used on its own to mobilise cells in rhesus macaque monkeys (Kean *et al,* 2011). When used in combination with G-CSF, Plerixafor resulted in significant mobilisation of T cells, B cells and NK populations into peripheral blood although, overall T cell levels were lower than when Plerixafor was used on its own. Devine and colleagues (2008) considered a series of 20 normal donors mobilised using Plerixafor alone. When re-mobilised with G-CSF starting seven days following the Plerixafor mobilised harvest collection, lower CD34 doses were noted than were achieved after mobilisation with Plerixafor alone. An analysis of the T cell subsets in four of these harvests collected six hours after the injection of Plerixafor found no change in Tregs as a percentage of CD4+ cells (Devine *et al,* 2008). In a more recent study by Gaugler *et al* (2013), autologous apheresis products were analysed after G-CSF or G-CSF plus Plerixafor mobilised collection (Gaugler *et al,* 2013). Although this particular study examined an autologous cohort and testing was undertaken 10 to 11 hours after administration of Plerixafor, the group confirmed Devine's findings that Plerixafor given with G-CSF mobilised greater numbers of CD3+ cells than after G-CSF alone. They also reported that Tregs as a percentage of CD4+ cells did not increase although the absolute numbers were higher. The study showed that the Tregs collected after mobilisation with G-CSF and Plerixafor displayed a significantly higher expression of CD127 than those harvested after G-CSF alone and they postulated that this could be due to a difference in the Tregs mobilised by each mobilisation method and that these Tregs may exhibit different functional properties.

The influence of Tregs in the graft on GVHD in patients receiving allogeneic transplants and on anti-tumour activity in both autologous and allogeneic transplants is of obvious importance. Differences in mobilisation protocols between apheresis centres mean that although results of

studies in the literature are of interest it is important to take into consideration the fact that dose timings and testing intervals vary considerably between centres. Furthermore many studies are based on small cohorts of patients. Even large transplant units vary in their approach to mobilisation regimens so this lack of consensus of treatment between centres makes interpretation of the literature difficult. Given that the RMH is currently the busiest Haematopoietic Stem Cell Transplant unit in the UK, transplanting over 200 patients per year and that the role of Tregs in transplantation is of significant clinical relevance, a study on the effects of different mobilisation regimens on Treg numbers and clinical outcome within the RMH setting is merited.

1.4.5 Reconstitution of haematopoiesis following HPC transplant

Following myeloablative conditioning, rather than RIC based therapeutic options, patients typically develop profound pancytopoenia. The duration of the pancytopoenic phase is partly dependent on the donor source with HPC apheresis grafts typically demonstrating the quickest neutrophil engraftment at about 2 weeks, and bone marrow and cord grafts taking 3 and 4 weeks respectively. In RIC transplants the duration and severity of pancytopoenia varies depending on the regimen used but is significantly shorter than following myeloablative conditioning (Mackall *et al,* 2009).

With RIC and full intensity conditioning, myeloid reconstitution occurs quite rapidly. For both types of transplant, the lymphoid compartment recovery is slower than the myeloid (Baron *et al,* 2004). In most cases, NK cell numbers return to normal within a month and B cells recover more quickly than T cells but B cells are still markedly reduced in the first three months post-transplant. Full B cell reconstitution covering all immunoglobulin isotypes can take up to two years following transplantation to return to normal levels. Reconstitution of T cells is a lengthy process and may be prolonged by immunosuppressive therapy and GVHD. CD4+ T cells take longer to recover than CD8+ T cells, the latter taking between three and 18 months to return to normal levels. CD4+ T cells remain low for over a year but this is dependent on the age of the patient and probably reflects thymic function. T cell regeneration is predominantly by means of a thymic independent pathway or homeostatic peripheral expansion, as thymic function is adversely affected by chemotherapy and radiotherapy. Other factors that can adversely affect thymic function are age (Fry and Mackall, 2005) and GVHD (Mackall *et al,* 2009). Regenerating T cells can originate from a number of sources: they may be recipient cells that have survived high dose therapy, T cells contained in the graft, donor stem cell progenitors that develop into T cells in the recipient or residual recipient stem cells which develop into T cells (Porrata, Litzow and Markovic, 2001). There appears to be consensus that a number of recipient T cells including Tregs are able to survive the preparative conditioning with memory T cells showing better

survival than naïve T cells (Isaacs and Thiel, 2004, Bayer *et al,* 2009). The recipient derived Tregs have been found to undergo peripheral expansion before the emergence of donor Tregs (Bayer *et al,* 2009). Ultimately these comprise the predominant component of the Treg compartment at approximately two months post-transplant. Recipient Tregs are thought to suppress recipient T cell alloimmune responses to incoming donor cells and may therefore facilitate engraftment (Bayer *et al,* 2009).

Reconstitution following autologous transplant shows a similar pattern to that of allogeneic transplants but is not complicated by immunosuppressive therapy or, in most cases, by GVHD. Tregs surviving the conditioning therapy rapidly expand in the immediate post-transplant period and are thought to be capable of suppressing anti-tumour immune responses (Bayer *et al,* 2009, Porrata, Litzow and Markovic, 2001).

1.5 Donor lymphocyte therapy in allogeneic transplant patients

The beneficial association of tolerable GVHD with lower incidence of relapse in leukaemic patients receiving allogeneic transplants is well documented (Sullivan *et al,* 1989, Weiden *et al,* 1979, Weiden *et al,* 1981). Depletion of donor T cells from the graft successfully reduces the incidence of GVHD but leads to higher rates of disease relapse particularly in patients with chronic myeloid leukaemia (CML), but also in AML and acute lymphocytic leukaemia (ALL) (Goldman *et al,* 1988, Marmont *et al,* 1991). Syngeneic transplants from identical twins also results in higher rates of disease relapse than allogeneic transplants and identical twins are therefore not now routinely used as donors. These data confirm the potent immunotherapeutic GVM role played by donor T cells.

Engraftment of donor cells and anti-tumour activity in RIC transplants may be enhanced by the use of donor lymphocyte infusions (Kahl *et al,* 2007, Bacigalupo, 2004, M, M and S, 2013). Often a mixed chimerism of recipient and donor haematopoietic cells exists in the early stages post-transplant reverting to full donor chimerism on withdrawal of immunosuppression, although mixed chimerism may be persistent (Mielcarek *et al,* 2002). In all allogeneic recipients post-transplant immune suppression provides an additional mechanism for regulating recipient responses to donor cells. Withdrawal of immunosuppressive therapy post-transplant must be undertaken carefully to maintain stable graft function (Woolfrey and Anasetti, 1999).

Early experiments using canine models examined time intervals post-transplant when infusion of donor T cells could be administered that were able to convert a mixed haematopoietic chimerism of both host and donor origin to complete donor chimerism. As a falling percentage of donor chimerism may be indicative of potential disease relapse this discovery was an important step in being able to treat relapse post-transplant (Kolb *et al,* 1997). This concept was

extended to humans resulting in a successful infusion of DL into a patient with relapsed CML and a lasting remission (Kolb *et al,* 1990). The observation led to the use of DLI to treat relapse in a range of haematological malignancies (Dey *et al,* 2003, Krishnamurthy *et al,* 2013, Liga *et al,* 2012, Deol and Lum, 2010). The ability to control residual disease and relapse through the use of DLI has greatly assisted the introduction of RIC and non-MA transplants and has thus widened the age range and eligibility of patients suitable for transplant to older patients and those with co-morbidities (Roddie and Peggs, 2011). The level of immunosuppression resulting from conditioning in these patients is sufficient to facilitate engraftment but does not cause the degree of conditioning-related damage and inflammation experienced by patients receiving full intensity transplants. The anti-tumour effect in this setting is not attributable to the reduced intensity conditioning per se but is mediated by the GVM effect of the donor immuno-competent cells in the graft. DLI thus offer a valuable means to manage residual disease and relapse in all allogeneic patients but particularly those receiving RIC transplants. The exact mechanism by which DLI induce GVM reactions is still unclear but it is thought that host APC stimulate donor T cells by presenting target antigens which may be differentially expressed on haematopoietic lineage cells (Kolb, 2008). Interestingly DLI appears to be more effective when used in patients with myeloid malignancies than those of lymphoid origin (Roddie and Peggs, 2011, Deol and Lum, 2010, Kolb, 2008).

Despite the benefits of DLI therapy, GVHD is a potentially life threatening complication occurring in about 30% of DLI recipients, however, the development of GVHD can be an indicator of alloreactivity and therefore GVM. Although severe GVHD can be life threatening, mild GVHD is associated with better prognosis in terms of disease relapse. The incidence of GVHD appears to be influenced by the interval between transplant and DLI with highest prevalence seen when DLI are given less than 30 days from transplant (Roddie and Peggs, 2011). Other factors influencing the incidence of GVHD are the number of T cells infused and the composition of the T cells. Additionally, recipients of T cell depleted initial transplant grafts have a higher risk of developing GVHD after DLI than those receiving T cell replete initial transplant grafts (Roddie and Peggs, 2011).

1.5.1 Tregs in DLI

The role of Tregs in the DLI setting has been studied using two different approaches: depletion of Tregs in the patient prior to infusion of DLI and Treg depletion of the DL product which is infused. Patients treated with the Treg depleting agents fludarabine or cyclophosphamide before DLI show significantly higher levels of GVHD suggesting a preventative role for Tregs in this setting (Deol and Lum, 2010). In a study conducted by Maury, seventeen patients with relapsed disease following allogeneic transplant, all of whom had failed to respond to at least

one dose of donor lymphocytes, were further treated with CD4+CD25+ depleted DLI. Two patients developed GVHD and, following fludarabine plus cyclophosphamide treatment prior to further DLI, four of the remaining patients developed GVHD. The study demonstrated that development of GVHD following DLI was associated with complete or partial disease remission and survival (Maury *et al,* 2010). Maury's group postulated that improved allo-reactivity and therefore GVM could be achieved by Treg depletion of the DLI. In patients resistant to treatment with donor lymphocytes, allo-reactivity could be improved by depletion of Tregs in the recipient allowing homeostatic expansion or activation of infused T cells. A recent Phase 1 study conducted by Nikiforow and colleagues (2013) also showed increased incidence of GVHD and GVM following CD25+ depletion of donor lymphocytes (Nikiforow *et al,* 2013). Observation of falling chimerism levels in patients post-transplant can indicate the return of host-derived haematopoietic cells, graft failure and subsequent relapse. In myeloablative transplants full donor chimerism is expected post-transplant, however, patients receiving RIC or non-MA transplants may show mixed chimerism which gradually converts to full chimerism over many months. A falling or persistent mixed chimerism is suggestive of potential returning disease and can be used alongside residual disease monitoring to indicate the need for DLI although in some high risk diseases pre-emptive DL therapy is used (Roddie and Peggs, 2011). The timings and doses of DLI have been the focus of many studies in order to obtain GVM without unacceptable GVHD (Fozza *et al,* 2007, Raiola *et al,* 2003, Simula *et al,* 2007, Shaw *et al,* 2007, Shaw and Russell, 2008). To reduce the risk of GVHD incremental doses of T cells are usually given, starting with smaller doses in patients with falling chimerism ($5x10^5$/kg CD3+ cells in patients with unrelated donors, $1x10^6$/kg in patients with sibling donors) or larger doses ($1x10^7$/kg CD3+ cells) in cases of disease relapse (Shaw *et al,* 2007). GVM reactions may not be observed until about eight weeks after DLI with molecular remission taking longer. Monitoring of chimerism and residual disease must therefore be undertaken carefully and further doses of DLI withheld until it is clear that the previous dose has not elicited the desired response (Shaw and Russell, 2008).

With increasing use of DLI as a treatment option and pressure to reduce costs, many centres including RMH aim to cryopreserve donor lymphocytes taken from the initial allogeneic peripheral blood stem cell donation especially where patients are at high risk of disease relapse. This allows rapid intervention with 'off the shelf' DLI without the need to recall the volunteer donor for specific donor lymphocyte collection. The practice also negates any problems with the donor no longer being able or willing to donate more cells. Recent changes to the funding of transplants mean that there are considerable cost-savings to be made by proactive preparation and cryopreservation of DL at the time of initial transplant donation.

Enumeration of CD3+ cells present in the original collection enables preparation of specified doses that can be processed and cryopreserved as five escalating doses of CD3+ donor lymphocytes, typically $5x10^5$/kg, $1x10^6$/kg, $5 x10^6$/kg, $1x10^7$/kg and $5x10^7$/kg. Alternatively, DL can be harvested from the original donor at a later date after the initial peripheral blood stem cell donation. As sufficient CD3+ cell numbers can be collected from an un-stimulated peripheral collection, the donor does not need to receive a mobilising agent for DL specific collections.

Although the practice of cryopreserving donor lymphocytes at the time of initial G-CSF mobilised collections is common in many Stem Cell Transplant Laboratories it is unknown whether the difference in collection protocols i.e. G-CSF mobilised or not, affects the immune phenotype, in particular CD4+CD25+FoxP3+ Tregs, and what effect this may have on outcome of the DL infusion. In a study performed by Hicheri and colleagues they found that lower CD4+CD127$^{low/neg}$ content of DL correlated significantly with favourable haematological response (Hicheri *et al,* 2008). However, small numbers of patients treated in any one centre, poor outcomes and heterogeneous patient groups make it difficult to conduct clinical trials of patients receiving DLI.

The role of NK cells, CD4 and CD8 cells are well characterised in the allogeneic transplant setting (Lowdell, 2003, Mackall *et al,* 2009), however, it now appears that both donor derived Tregs in the recipient and in the infused DL product may play an important role (Roddie and Peggs, 2011, Hicheri *et al,* 2008, Kolb, 2008). DLI based treatment varies from centre to centre, but nonetheless, numbers of Tregs are likely to be an important predictor of outcome or at least shed some valuable light on clinical scenarios following donor lymphocyte infusions.

1.6. Studies on Tregs in the graft and post-transplant

In both human studies and animal models, there has been a significant lack of consensus regarding the effect of Tregs in autologous and allogeneic transplantation. Although extrapolation from animal-based findings to human application must be undertaken with caution, the observation that donor Tregs could prevent GVHD in allogeneic murine studies prompted many clinical investigations. In ensuing human studies, some researchers concentrated on the levels of Tregs in the graft and some on Treg levels during the period post-transplant, in particular during the onset of GVHD. Furthermore it has been postulated that in autologous transplants Tregs levels in both the graft and during reconstitution may be implicated in suppression of anti-tumour immunity (Hess, 2010). Factors such as mobilisation regimen which may affect Treg numbers in the graft are therefore of interest. Additionally, depletion of Tregs in the graft and in autologous transplant recipients is being studied as a possible therapeutic option to reduce relapse rates (von Keudell, Rosenbaum and Zimmerman, 2013).

1.6.1 The impact of Tregs in the graft

Stanzani's team found that higher Tregs in the sibling allogeneic grafts they studied increased the risk of acute GVHD (Stanzani *et al,* 2003). As a FoxP3 antibody was not available at the time of this research, CD25 was used as a marker for Tregs. Because CD25 identifies activated effector T cells as well as Tregs, results of studies using this marker should be interpreted with caution. In contrast Wolf and colleagues noted that increased Tregs in the graft decreased the incidence of acute GVHD in the recipients of myeloablative allogeneic transplants (Wolf *et al,* 2007). In their study, peripheral blood stem cell (PBSC) donations were from sibling donors and CD4+CD25+FoxP3+ Treg enumeration was performed using flow cytometry. Research groups led by Pastore (Pastore *et al,* 2011), Rezvani (Rezvani *et al,* 2006), and Pabst (Pabst *et al,* 2007) all agreed with Wolf's findings that an increase in Tregs in the graft was associated with a decreased incidence of acute GVHD.

Although their findings were similar, these groups differed in their experimental approach (Table 5). Pastore's group only studied the grafts of patients with AML, ALL and CML receiving myeloablative allogeneic transplants conditioned using busulphan/cyclophosphamide. CD3+ CD4+ CD25+ FoxP3+ Tregs were identified using flow cytometry. Pabst also studied the grafts of patients receiving full intensity conditioning but myeloablative regimens included TBI plus cyclophosphamide, TBI plus fludarabine and busulphan/fludarabine. Treg identification was by quantitative polymerase chain reaction (PCR) rather than flow cytometry. In the Rezvani study, the diagnosis of patients was AML, ALL, CML or chronic lymphocytic leukaemia (CLL), and they all received full intensity conditioning followed by a sibling donor PBSC transplant. The harvested products in this protocol were subjected to *ex vivo* CD34 selection and T-cell depletion with a known number of T-cells added back to the graft. This level of graft manipulation is not standard practice in most centres. As donor Treg numbers were measured prior to any mobilisation or harvesting regimen in this particular study, it did not address the direct impact of Tregs on the recipient.

Because sibling donors tend to have fewer mHag mis-matches with their recipients than unrelated donors, the patients in Rezvani's and Wolf's studies were less likely to develop GVHD than a cohort of patients that included significant numbers of unrelated donor transplants. Noel's study considered only recipients receiving RIC transplants. Both pre and post G-CSF mobilisation CD4+CD25^{high}CD127^{low}FoxP3+Treg numbers in allogeneic donors were measured using flow cytometry (Noël *et al,* 2008). No correlation was found between the numbers of Tregs infused and tolerance to acute GVHD.

Table 5: Summary of studies examining Treg numbers in the graft and incidence of GVHD

Reference	Patient population	Treg markers	Conclusions
Stanzani *et al* 2003	Sibling allografts (n=60). Full intensity, non-MA and RIC NHL, AML, CML, ALL, CLL, MM, HD, Ca breast	Testing on cryopreserved samples CD25+	↑Tregs ↑GVHD
Rezvani *et al* 2006	AML, ALL, CML, CLL Full intensity (n=32) Siblings *ex vivo* CD34 selection and T-cell depletion with a known number of T-cells added back to the graft. Tested at 30, 45, 60, 90, and 120 days post-transplant	CD4, CD25, CD45R0, CD27, CTLA-4, FoxP3 Quantitative PCR for FoxP3 gene expression	↑Tregs ↓GVHD
Pabst *et al* 2007	VUDs (n=63) AML/MDS, ALL, lymphoma Full intensity: TBI+cyclophosphamide, TBI+fludarabine and Busulphan/fludarabine Patients followed-up for 0.6-62 months post-transplant	Testing on cryopreserved samples CD3+, CD4+, CD25+ Quantitative PCR for FoxP3 gene expression	↑Tregs ↓aGVHD No correlation between Treg numbers and cGVHD
Wolf *et al* 2007	Sibs (n=58) Full intensity (n=34), RIC (n=24) Haematological malignancies	CD4+CD25+FoxP3+	↑Tregs ↓GVHD but not after RIC. Relapse rate independent of Tregs
Noel *et al* 2008	RIC Sibs and VUDs (n=32) Donors mobilised with 7 days of G-CSF Tested at 30 to 60 days post-transplant	$CD4+CD25^{high}CD127^{low}FoxP3+$	No correlation between Treg numbers and aGVHD
Pastore *et al* 2011	Sibs (n=45) and VUDS (n=20). ALL, AML, CML Full intensity: Busulphan/cyclophosphamide Tested at 1,2,3,6 months post-transplant	CD3+CD4+CD25+FoxP3+	↑Tregs ↓GVHD Relapse rate independent of Tregs

NHL, non-Hodgkin lymphoma; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; ALL, acute lymphocytic leukaemia; CLL, chronic lymphatic leukaemia; MM, multiple myeloma; HD, Hodgin's lymphoma; Ca breast , breast cancer; non-MA, non-myeloablative; RIC, reduced intensity conditioning; Sib, sibling donor; VUD, volunteer unrelated donor; ↑, increased numbers or incidence;↓, decreased numbers or incidence.

Over all these studies suggest that levels of Tregs in the graft may affect the incidence of GVHD but it is difficult to draw definitive conclusions due to the large variation in mobilisation protocols, transplant procedures, patient cohorts and methods of Treg enumeration in the study groups.

With regard to allogeneic transplantation, the current study considered the range of full intensity and RIC, unrelated and sibling transplants to reflect the patient population at RMH. Testing of CD3+CD4+CD25^{high}CD127^{low}FoxP3+ Tregs was performed using flow cytometry in peripheral blood and harvests from sibling donors, and in harvests of unrelated donors.

Donor lymphocytes at RMH are cryopreserved using standard non-mobilised collections but increasingly are also processed from initial transplant mobilised collections. Since levels of Tregs are implicated in GVM and GVHD it is important to observe the effect of mobilisation agents on numbers of Tregs in the collection.

Autologous grafts are also considered in the current study. Elevated Treg levels in patients suffering from haematological malignancies have been described (Michael, Shimoni and Nagler, 2013) and harvests from these patients would be expected to contain higher Treg levels than those collected from healthy donors. These increased Treg numbers can inhibit anti-tumour immune responses and negatively impact on the desired GVM effect following transplant.

Studies examining the effect of mobilisation regimens on Tregs are also difficult to interpret due to collection centre variation between mobilisation strategies, differences in peripheral CD34 count that determine when a donor is considered to have mobilised and the timing of mobilisation agents prior to harvest. In particular the timings of injection of Plerixafor prior to harvesting are noted to vary between centres and this may affect the mobilisation kinetics of Tregs. Studies range from administering Plerixafor 6 hours before the start of harvesting (Devine *et al,* 2008), 10-11 hours pre-harvest (Gaugler *et al,* 2013) and the current study where the time interval before harvesting is 16 hours.

1.6.2 Tregs in the peripheral blood following transplant

On the important question of whether circulating Tregs in the peripheral blood affects GVHD, studies have yielded variable data. The occurrence of Tregs in the peripheral blood of patients following autologous and allogeneic transplantation is likely to be intrinsically linked to their effect. Miura demonstrated a correlation between circulating Treg numbers detected by quantitative PCR and incidence of GVHD although they were unable to state whether this was cause or effect (Miura *et al,* 2004).The patient cohort for this study included recipients of autologous transplants for breast cancer as well as those receiving HLA-matched or haplo-

identical allogeneic transplants.The source of the graft in all cases was bone marrow. Peripheral blood levels of Tregs in the allogeneic setting were decreased in patients who developed acute GVHD. In the autologous patients auto-GVHD was intentionally induced using CsA to enhance anti-tumour immune responses (Byrne *et al,* 1997). These studies which link a low number of circulating Tregs with development of GVHD, whether autologous or allogeneic were unable to demonstrate if Tregs were reduced as a result of GVHD or if low Tregs contributed to GVHD development.

Magenau's study is one of the largest and most comprehensive studies to have been undertaken, with a patient cohort receiving either un-manipulated allogeneic or autologous bone marrow, PBSC or cord blood transplants from related or unrelated matched donors. Patients received no T cell depleting antibodies as part of their conditioning unlike some of the RMH patients. Blood samples were collected within 24 hours of clinical signs of GVHD and prior to initiation of any steroid therapy. Magenau's group concluded, as above, that Treg levels were decreased at the onset of GVHD and also low levels were observed in the preceding two weeks. Additionally, they noted that Treg levels indicated severity and eventual grading of GVHD. Tregs were determined by flow cytometry using the CD4+CD25^{hi}FoxP3+ phenotype (Magenau *et al,* 2010).

A series of further studies have also confirmed these findings. Groups led by Ukena, Zhai and Bremm all demonstrated that GVHD incidence was higher in patients with reduced peripheral blood Treg numbers than in patients with normal or high Treg levels as defined by each group. Ukena measured CD4+CD25^{high}CD127^{low} Tregs by flow cytometry in the six months post-transplant (Ukena *et al,* 2011b). Zhai's group used CD4+CD25+ as markers of Tregs and showed that the majority of these cells expressed FoxP3 (Zhai *et al,* 2007). The patients in Bremm's investigation were transplanted with either bone marrow or PBSC - some of which were T cell depleted – and donors were either HLA-matched or haplo-identical (Bremm *et al,* 2011). Reduced expression of CD127 was observed in the first two months following transplant, which the authors suggest may be as a result of the cytokine milieu immediately post-transplant, and then increased over the first year. These investigators measured CD4+CD25^{high}CD127^{low} Tregs and they comment that as they were only able to gate on the CD4+CD25^{high} population during the early post-transplant period their results may not be accurate due to inclusion of activated effector T cells that can also be identified using these markers. They also noted the highest proportions of Tregs in patients at the point of disease relapse. In CML patients more than 18 months post-transplant Nadal's group also demonstrated that high levels of Tregs were more commonly associated with disease relapse (Nadal *et al,* 2007). Nadal's study was restrictive in its cohort selection as only patients being transplanted for CML were considered as these patients were easier to monitor for molecular remission. All the patients studied were more than 100

days post-transplant so the study only examined the incidence of chronic GVHD which, while interesting, does not address the fact that 30-50% of patients experience acute GVHD.

By contrast, other researchers reported no difference in Treg levels in patients with and without GVHD (Lord *et al,* 2011, Arimoto *et al,* 2007). Lord and colleagues studied Treg levels in the blood and gastric biopsies of patients suffering from gastrointestinal GVHD and those without and demonstrated no significant differences between the two groups (Lord *et al,* 2011). Arimoto's group studied full intensity and RIC conditioned patients and found no correlation between Treg numbers and the incidence of either acute of chronic GVHD (Arimoto *et al,* 2007).

In patients with chronic GVHD researchers also failed to reach consensus about the influence of Treg numbers. Studies by Zorn (Zorn *et al,* 2005) and Matsuoka's (Matsuoka *et al,* 2010) groups reported reduced levels of Tregs in patients developing chronic GVHD but Ukena and Clark's teams demonstrated increased numbers in patients with chronic GVHD (Ukena *et al,* 2011a, Clark *et al,* 2003, Sanchez *et al,* 2004). Although many of these studies offer interesting hypotheses, there is a lack of consensus in the literature (Table 6).

Table 6: Summary of studies examining Treg numbers in peripheral blood post-transplant and incidence of GVHD

Reference	Patient population	Treg markers and testing performed	Conclusions
Clark *et al.* 2003	Allos (n=40) Sibs (n=34) VUDs (n=6) Full intensity (n=34), RIC (n=6) Source: bone marrow (n=18), PBSC (n=22) Tested at day +100 onwards post-transplant	CD4+CD25+ absolute number Functional activity	↑CD4+CD25+ in cGVHD Tregs show normal activity in cGVHD
Sanchez *et al.* 2004	Allos (n=35) Sib (n=29) Matched VUD (n=4) Mismatched VUD (n=2) Source: bone marrow T cell depletion of 12 bone marrow harvests	$CD4+CD25^{+}$	↑Tregs ↑active cGVHD
Miura *et al.* 2004	Allos (n=34) HLA matched (n=17) and haplo-identical (n=4) Autos with Auto-GVHD induction (n=39) Source: bone marrow Samples at onset of GVHD, 5 patients received DLI	FoxP3 expression in peripheral blood	↓FoxP3 ↑ aGVHD and cGVHD severity
Zorn *et al.* 2005	Allos (n=57) Sibs(n=32) VUDS (n=25) Full intensity (n=51), RIC (n=6) Source: bone marrow (n=36), PBSC (n=21) Patients with chronic GVHD	CD4+CD25+ FoxP3 in total lymphocytes not just CD4+CD25+ cells Functional activity	↓ Tregs with active cGVHD ↑ratio of activated CD4+:Tregs ↑cGVHD
Arimoto *et al.* 2007	Allos (39) Full intensity (24) and RIC (15) Tested monthly up to 1 year post-transplant	CD4+CD25+ FoxP3mRNA	No correlation between Tregs and aGVHD or cGVHD
Nadal *et al.* 2007	Allos (n=76) Source: bone marrow (n=57), PBSC (n=19) Full intensity and RIC CML patients only Day +100 onwards,33 patients received DLI	$CD4+CD25^{+}$	↑Tregs ↑disease relapse but not a reduction in GVHD Tregs post Tx more suppressive than in normal controls
Zhai *et al.* 2007	Allos (n=12) VUD (n=1) and Sibs (n=11) Full intensity Source: bone marrow, PBSC and bone marrow+PBSC Samples taken up to 15months post transplant	$CD4+CD25^{+}$	↓Tregs ↑aGVHD
Magenau *et al.* 2010	Allos (n=125) autos (n=90) Source: unmanipulated PBSC, bone marrow or cords Samples at onset of GVHD and D20,30,60 and 100 post-transplant	CD4+CD25+CD127+FoxP3+	↓Tregs at time of onset of GVHD ↑Tregs ↓severity of GVHD

Matsuoka *et al.* *2*010	Full intensity (n=33) Source: PBSC Samples tested up to 1 year post- transplant	CD4+CD25$^{med-high}$CD127^{low}	↓Tregs ↑cGVHD Inadequate CD4+ reconstitution ↑cGVHD
Ukena *et al.* 2011	Allos (n=29) VUD (n=11), Sibs(n=11) mismatched VUDs (n=7) Samples up to 6 months post-transplant	CD4+CD25^{high}CD127^{low}	↓Tregs ↑GVHD
Bremm *et al.* 2011	HLA-matched or haplo-identical (n=16) (n=14 paediatric) RIC and full intensity Acute leukaemia or neuroblastoma Source: Unmanipulated bone marrow (n=8) Unmanipulated bone marrow (n=8) Tcell depleted PBSC (n=6) Up to 2 years post-transplant, 6 patients received DLI	CD4+CD25^{high}CD127^{low}	↓Tregs ↑GVHD Highest Tregs at point of disease relapse

Allos, allogeneic transplant; Sib, sibling donor; VUD, volunteer unrelated donors; RIC, reduced intensity conditioning; PBSC, peripheral blood stem cells; CML, chronic myeloid leukaemia; cGVHD, chronic graft versus host disease; ↑, increased numbers or incidence;↓, decreased numbers or incidence.

1.6.3 Tregs in donor lymphocyte products and the peripheral blood following infusion

Unlike Maury's study of patients receiving Treg depleted DL following relapse after HPC transplant, the current study will include all patients who receive Treg replete donor lymphocyte infusions for falling chimerism or relapse (Maury *et al,* 2010). Hicheri's study examined Tregs in DLI products but all patients in the study showed actual disease relapse and therefore received higher CD3+ doses of DLI than would have been administered for falling chimerism (Hicheri *et al,* 2008). Additionally over half the patients had received an initial transplant with bone marrow as the stem cell source.

DLI protocols also vary from transplant centre to centre. There is a degree of consensus about DLI CD3+ doses but the intervals between infusions and the point at which a falling chimerism is treated with DLI remain individual to centres and even clinicians within a centre. The use of prophylactic DLI in some centres further complicates interpretation of results from different research groups. Methods for assessing chimerism differ between different centres with some centres performing the more sensitive myeloid and T cell lineage testing in addition to whole blood chimerism on peripheral blood and others carrying out whole blood chimerism on bone marrow samples.

1.7 Reasons for undertaking this study

Tregs are clearly important and have an impact on clinical outcome in autologous and allogeneic transplantation. Although significant progress has been made in delineating their biology, careful analysis of the literature reveals conflicting results in the transplant setting. Attention has recently focussed on understanding the impact of Tregs *in vivo* but variations between clinical centres in their conditioning regimens, graft source, GVHD prophylaxis, patient age and underlying disease, together with the different parameters used to assess Tregs, make comparison of data challenging. Extrapolation of optimal Treg numbers from one centre and application to another is impossible, thus requiring centres to undertake centre-specific studies to determine the influence of Treg numbers on clinical outcome of patients treated with protocols specific to that centre.

The RMH has the largest haematopoietic stem cell transplant practice in the UK and operates according to unified and well-defined clinical protocols. This study aims to build a comprehensive and cohesive database of information which will clarify the impact of Tregs on clinical outcome in the transplant setting. In the future, testing of Treg levels at critical points in harvested products and in patients undergoing autologous and allogeneic transplantation and donor lymphocyte infusions may be valuable in predicting the occurrence and severity of relapse or GVHD.

1.8 Aims of the current study

The aims of this study are to determine the influence of CD4+ $CD25^{high}$ $CD127^{low}$ FoxP3+ Treg numbers in the graft and in the recipient on incidence and severity of GVHD and evidence of GVM, and to determine the effect of different mobilisation regimens and collection methods on CD4+$CD25^{high}CD127^{low}$FoxP3+ Treg numbers harvested. In order to achieve these aims, the objectives of this study were as follows:

1. To determine the effect of HPC mobilisation regimens on CD4+$CD25^{high}CD127^{low}$FoxP3+ Treg numbers in both autologous and allogeneic harvests.
2. To measure CD4+$CD25^{high}CD127^{low}$FoxP3+ Treg numbers in autologous grafts and in peripheral blood of patients during the first year following autologous transplant using flow cytometry and correlate levels with the clinical outcome measured by disease relapse for up to a year post-transplant.
3. To measure CD4+$CD25^{high}CD127^{low}$FoxP3+ Treg numbers in allogeneic donor grafts and in the peripheral blood of patients during the first year following allogeneic transplant using flow cytometry and correlate levels with clinical outcome determined by clinical assessment of GVHD, disease relapse and donor chimerism for up to a year post-transplant.
4. To correlate CD4+$CD25^{high}CD127^{low}$FoxP3+ Treg numbers in DLI, with the clinical outcome determined by incidence of GVHD and disease remission or restoration of donor chimerism at relevant clinical intervals.

The study aims to undertake a large and comprehensive study of the impact of Tregs in transplants patients at RMH. The study includes autologous harvests and transplants, volunteer unrelated donor (VUD) and sibling allogeneic harvests and transplants, and DLI. The aim is to collect data from 40 autologous and 40 allogeneic transplants, with additional data from 12 patients receiving DLI. Based on a relapse rate of 10-20% by the end of the first year post-transplant, at least eight patients would be expected to relapse. Of the 40 allograft patients, two (5%) would be expected to develop severe GVHD by the end of the first year post-transplant and 13 (33%) mild GVHD. In setting up this study it was recognised that numbers would be dependent on clinical and sample availability constraints.

1.8 Hypotheses

1. Mobilisation regimens will impact on numbers of Tregs collected in the DL or PBSC harvest and non-mobilised DL collections may result in lower CD4+CD25^{high}CD127^{low}FoxP3+ Treg numbers in the harvest than in mobilised collections.

2. In autologous transplants high CD4+CD25^{high}CD127^{low}FoxP3+ Treg numbers in the graft and in the peripheral blood following transplant will be associated with a higher incidence of disease relapse.

3. In allogeneic transplants high CD4+CD25^{high}CD127^{low}FoxP3+ Treg numbers in the graft and in the peripheral blood following transplant will result in mild or no GVHD but poorer GVM.

4. High CD4+CD25^{high}CD127^{low}FoxP3+ Treg numbers in donor lymphocyte infusions will correlate with a poorer response to DL infusion as measured by disease remission or donor chimerism status but will correlate with a lower incidence of GVHD.

2.0 Materials and methods

2.1 Patient and donor selection

This study included patients and donors who underwent collection of cells or who received transplants during the period October 2011 to July 2013. Full patient characteristics are shown in Appendix 4.

2.1.1 Patient cohorts

The study examined 109 harvests collected from patients mobilised for autologous harvest collection by G-CSF alone (n=11), G-CSF plus cyclophosphamide (n=82) or G-CSF plus Plerixafor (n=16).

Paired pre-harvest peripheral blood and harvest sample pairs were examined from 45 autologous and 11 related allogeneic donations.

Mobilised harvests from 59 allogeneic donors including VUDs (n=39) and siblings (n=20) were compared with 13 harvests from VUD (n=3) and sibling (n=10) donors who received no mobilisation regimen.

The grafts of 82 autologous and 53 allogeneic transplants recipients and transplant outcome in terms of disease status were examined over a period of one year post-transplant.

Thirteen patients who received a total of 18 donor lymphocyte infusions were included in the study.

A cross-sectional study was performed on 197 samples from 75 patients who received allogeneic transplants from either a sibling (n=25) or an unrelated donor (n=50) and 180 samples from 82 patients who underwent autologous transplant. Patients were followed for a year post-transplant.

2.2 Consent and ethical considerations

Ethical (09/H0801/95) and research and development (CCR3312) approval was obtained for this study from the Royal Marsden Hospital's review board and all testing was performed in accordance with the approved protocol. The study was non-interventional in design as all patient blood samples were taken for routine testing with only material surplus to requirement being used in this study.

Testing was performed only where donors or patients had provided written, informed consent for surplus material taken for routine testing to be used in research. This consent was obtained as part of the cell harvesting or transplant consent procedure and was performed by clinicians trained in the consenting process in accordance with Human Tissue Authority (HTA) and Trust requirements. The investigator was not directly involved in the consenting process but wrote an

information leaflet approved by the Trust Ethics Committee which was provided to patients and donors to inform them about the study.

2.3 Factors affecting testing

As the study was non-interventional and because the harvesting and treatments were pre-determined, it was not possible to pre-allocate patients to different arms of the study. Therefore there was no control over patient and donor numbers in each group. Due to sample limitations it was not possible to test post-transplant samples at all desired time points. Additionally samples were not available from any patients transferred to intensive care following transplant, from patients referred back to their original hospital, or from those who died during the course of the study.

The timings of patient samples obtained were based around those already being taken for routine testing pre- and post-transplant. Peripheral blood study samples and were sourced from surplus material remaining after routine full blood count analysis. Apheresis product samples were taken from surplus material following routine total nucleated cell counts undertaken on harvested products. Sample volumes and cell counts were dependent entirely on residual material available.

2.4 Pre-apheresis patient and donor samples

Peripheral blood samples were taken from donors on the ward in the morning prior to commencing apheresis collection. This included both autologous and allogeneic sibling donors. Surplus sample material was collected from the haematology laboratory after full blood count (FBC) testing was completed and validated. No unrelated donor harvests are collected at RMH so peripheral blood samples were not available for these donors.

2.5 Harvest samples

All harvests received by the RMH Stem Cell Transplant Laboratory (SCTL) are sampled aseptically by laboratory personnel according to standard operating procedures. Samples for total nucleated cell (TNC) counting, CD34 ± CD3 enumeration and viability testing were taken. The surplus material remaining after the TNC count had been performed was used by the investigator for this study. TNC analysis was undertaken using a bench-top ABX Pentra 60 analyser (Horiba Medical) in the SCTL according to standard procedures. The TNC results obtained were used in the study. Enumeration of CD34/CD3 positivity undertaken by the Trust's Immunophenotyping laboratory was utilised in the study. Data from these tests were obtained from the SCTL paper and electronic patient records and the Trust's patient clinical database where appropriate.

2.6 Post-transplant samples

Samples for FBC are typically taken at regular intervals from patients post-transplant. During the initial in-patient phase these are routinely performed daily until the patient is discharged. Thereafter testing is usually performed at a minimum of 28 days, three months, six months and a year post-transplant with many patients being tested more regularly. These follow-up blood tests were collected as part of out-patient clinic appointments. Surplus samples from the above tests were collected from the haematology laboratory after scrutiny of clinic appointment lists and haematology laboratory patient sample databases on a daily basis.

2.7 Clinical data collation

2.7.1 Assessment of patient outcome - GVHD

Patients were assessed clinically for GVHD on a daily basis during the engraftment period immediately post-transplant and, after discharge from the ward, at each out-patient visit. Clinical GVHD was assessed and graded according to standard criteria described by Glucksberg and data entered into the patient clinical database (see Appendix 1). These widely-used grading criteria assessed extent, severity and pattern of organ involvement.

2.7.2 Assessment of patient outcome - Disease relapse

Transplant patients are continually monitored for clinical outcome by employing a range of tests. Clinical status is determined by microscopic examination of bone marrow aspirate samples taken at 28 days, 3 months and one year post-transplant. Additionally samples are analysed for residual disease/relapse using multicolour flow cytometry undertaken by the Trust's Immunophenotyping laboratory to detect malignant cell markers. Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) technology is used by the Trust's Molecular Diagnostics Laboratory as a highly sensitive method for detection of residual disease or relapse before signs of actual clinical relapse. If appropriate, fluorescence in-situ hybridisation (FISH) is performed by the Trust's Cytogenetics Laboratory to detect persistence or re-appearance of chromosomal aberrations associated with the original malignant clone post-transplant. The results of all these tests were obtained from the patient clinical database.

2.7.3 Assessment of patient outcome - Chimerism

Relative levels of donor and recipient cells (chimerism) were tested on samples taken at out-patient clinic attendances at 28 days, three months, six months and one year post-transplant with testing at additional time-points if clinically indicated. The Trust's Molecular Diagnostic Laboratory chimerism testing uses a panel of polymorphic short-tandem repeat (STR) markers with allele sizes that differ between individuals. Repeat regions of 6 STR loci that differ between donor and recipient allow for relative numbers of donor and recipient cells to be measured. The analysis is performed on DNA extracted from both whole blood and bone marrow and considers

chimerism status in both T cell (CD3) and myeloid (CD15) lineages. Analysis of sorted cell lineages increases sensitivity of the test and allows mixed chimerism to be detected more readily than using analysis of whole blood alone. Specifically, evidence of falling T cell chimerism is used clinically to indicate loss of graft or sign of early relapse. Results of chimerism testing were available from the patient clinical database and reported as percentages of total lymphoid or myeloid counts.

2.8 Sample preparation

In all cases, patient and donor peripheral blood samples were collected into BD (Becton Dickinson) Vacutainer™ sample tubes containing the anticoagulant ethylene-diamine tetra-acetic acid (EDTA). Samples were prepared for flow cytometry by isolation of mononuclear cells by density gradient centrifugation using Ficoll-Hypaque with a specific gravity of 1.007 (GE Healthcare, Sigma- Aldrich). Samples were layered carefully over an equal volume of Ficoll-Hypaque and centrifuged at 300g for 20 minutes ensuring that the centrifuge brake was not used. The mononuclear layer was collected and the cells washed in saline for 5 minutes at 400g. The pellet was either re-suspended in an equal volume of saline and cryopreserved using the method in 2.8.1 or re-suspended in 100µl of BD Pharmingen™ stain buffer (BD Biosciences) ready for immediate staining.

Autologous and allogeneic apheresis collections and donor lymphocyte harvests are anti-coagulated with acid citrate dextrose - solution A (ACD-A) (Baxter Healthcare) as part of the apheresis process therefore no further anti-coagulation was required. Furthermore, no density gradient separation was required for apheresis product samples as the harvests are always depleted of red cells and granulocytes during the apheresis procedure. Samples were either tested fresh within 48 hours of collection or following cryopreservation and subsequent thawing.

2.9 Cryopreservation, storage and thawing of cell samples

2.9.1 Preparation for cryopreservation

Cryopreservation of cells was performed according to validated standard operating procedures that are used within the SCTL for freezing cells for therapeutic use or as viability reference samples. The methodology used to freeze samples employed a scaled-down version of the methodology validated and used in the laboratory to cryopreserve HPCs for therapeutic use. The freeze mix comprised clinical grade cryoprotectant CryoPur dimethyl sulphoxide (DMSO) (Quest Biomedical) and normal saline (9.0%) for injection (B.Braun Medical). DMSO was diluted in saline to produce a total concentration of 12% DMSO in the freeze mix which was then added to an equal volume of cells to give a final DMSO concentration of 6%. Cells were frozen at a

concentration of less than 2.0 x10^8/m in aliquots of 1ml in Nunc cryotubes (Fisher Scientific). The DMSO/saline cryoprotectant mix was cooled in the fridge before adding to the cells immediately prior to cryopreservation. The time from addition of DMSO to the cells until start of freezing was carefully monitored in accordance with standard operating procedures as delays to freezing can affect cell viability, and was not allowed to exceed 20 minutes.

2.9.2 Cryopreservation process

Cells were frozen in a Kryo 560 controlled rate freezer (Planer PLC) according to SCTL standard operating procedures using a pre-programmed protocol validated to ensure optimised viability of cells (Appendix 2). The freeze profile used is identical to that used for cryopreservation of transplant material. Viability and engraftment data are consistently checked and are well within acceptable limits as recognised and approved by the Joint Accreditation Committee of the European Group for Blood and Marrow Transplantation (EBMT) and the International Society for Cellular Therapy (ISCT), (JACIE).

2.9.3 Storage

At the end of the freeze profile when the cells had reached a final temperature of -130°C, cryotubes were transferred to storage in temperature monitored vapour phase liquid nitrogen large capacity MVE vessels (Chart Biomedical), maintained at temperatures of less than -140°C. The vessels are all validated by RMH as suitable for long-term storage of cells for transplant. They are continually monitored according to regulatory requirements for temperature stability.

2.9.4 Thawing

Thawing of samples was performed according to the validated standard operating procedures used for thawing reference samples, also stored in Nunc cryotubes, for viability assessment of stored transplant material.

Frozen cryotubes were thawed using a Clifton NE1-4 water-bath (Jencons Scientific) which was filled with a measured amount of water, allowed to heat to and stabilise for at least 15 minutes at 39-40°C as measured by two independent temperature loggers (United Kingdom Accreditation Service (UKAS) calibrated and certified). Once the temperature was stabilised and acceptable, cryotubes were quickly removed from storage, thawed and the cells immediately washed in sterile saline for 5 minutes at 400G to ensure rapid removal of DMSO. Cells were re-suspended in 100µl of BD Pharmingen™ stain buffer (BD Biosciences) and staining for flow cytometry was performed immediately.

There is lack of consensus between researchers concerning the success of Treg staining following cryopreservation and the differences in methodology used may explain this. Elkord (Elkord, 2009) found that the frequency of Treg cells in peripheral blood was significantly

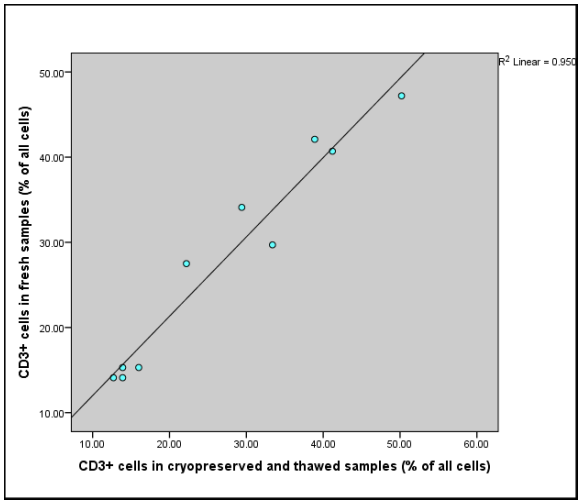
diminished after cryopreservation but this was disputed by Van Hemelen and colleagues who did not observe this in their testing (Van Hemelen *et al,* 2010). However it should be noted that the findings of both these papers were based on very small sample numbers of 6 and 3 samples respectively. Law and colleagues (Law *et al,* 2009) tested different FoxP3 antibody clones and found that the different clones behaved differently with fresh and cryopreserved and thawed samples. In tests on 10 frozen peripheral blood samples the 259D/C7 clone showed the highest levels of staining of Tregs due to better separation of FoxP3+ and FoxP3- populations than observed with the other clones tested. Furthermore, in a comparison of 4 donor samples tested both fresh and after freezing there was no difference in Treg frequencies detected.

For the purposes of the current study, cryopreserved and thawed samples were tested to ensure that comparable results were obtained. Due to the difficulty in obtaining sufficient surplus material from post-transplant peripheral blood samples this comparison (Table 7), was undertaken on surplus harvest samples as sample volumes were typically greater. Correlation was performed on percentages of Tregs, CD4+ cells and CD3+ cells obtained after testing fresh and frozen/thawed samples to ensure that there were no significant differences between the results. As strong correlation was noted between fresh and cryopreserved sample results for all cell type percentages, samples were cryopreserved for future testing if it was not possible to test them fresh.

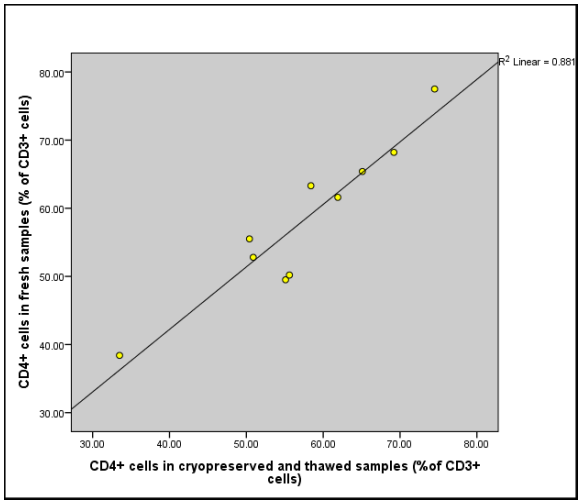
Table 7: Comparison of fresh and cryopreserved/thawed harvest samples stained and analysed by flow cytometry. Fresh and frozen/thawed samples were tested for Tregs as a percentage of CD4+ cells, CD3+ cells as a percentage of all cells and CD4+ cells as a percentage of CD3+ cells. Spearman rank correlation (ρ) and significance is shown.

	FoxP3+ Tregs as percentage of CD4+ cells	CD3+ cells as percentage of all cells	CD4+ cells as percentage of CD3+ cells
Correlation (ρ) and significance (p) for fresh and cryopreserved/thawed samples (n=10)	ρ = 0.985 p = <0.0001	ρ = 0.960 p = <0.0001	ρ = 0.879 p = 0.001

a)



b)



c)

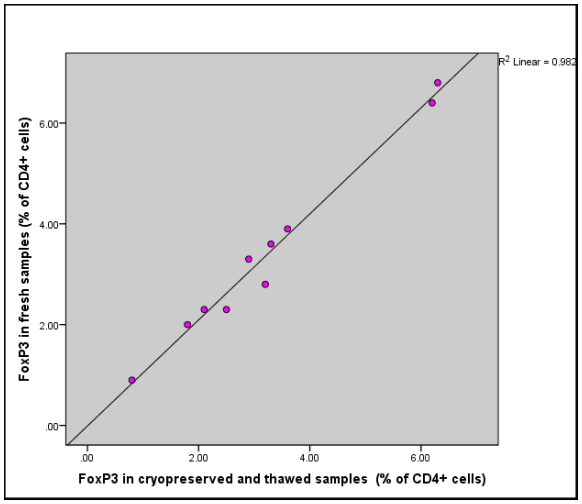


Figure 6: Comparison of fresh and cryopreserved/thawed samples. (a) Comparison of CD3+ cells as a percentage of all cells (b) Comparison of CD4+ cells as a percentage of CD3+ cells and c) FoxP3+ cells as a percentage of CD4+ cells. Samples of harvests (n=10) were either used fresh or cryopreserved and thawed using protocols identical to those used for transplant material. Samples were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry.

2.10 Flow cytometry

2.10.1 Initial Treg testing

Treg enumeration was performed using multicolour flow cytometry and both fresh and thawed samples stained using the same methodology. Although a number of FoxP3 antibodies were available commercially, the use of a Treg detection kit was favoured as it provided flurochrome conjugated anti-human antibodies and critical buffers with manufacturer optimised protocols. Initially the Miltenyi Biotec Treg detection kit (Miltenyi Biotec GmbH, Germany) was selected. The kit comprised CD4 and CD25 antibodies conjugated to fluorescein-isothiocyanate (FITC) and phycoerythrin (PE) respectively. Fixation/permeabilisation solution and buffer were included in the kit together with FcR blocking reagent. Following staining with the Anti-CD4 and Anti-CD25 antibodies, the cells were fixed and permeabilised according to manufacturer's instructions to allow subsequent intracellular FoxP3 staining using FoxP3 antibody conjugated to allophycocyanin. Repeated attempts to validate this Treg enumeration method failed and a search of the literature failed to identify any researchers using this kit.

The majority of studies appear to have used either the FoxP3 antibody clone PCH101 (produced by ebioscience) or clone 259D (produced by both Biolegend and BD Biosciences). In a study by Grant (Grant *et al,* 2009) cited in Lanza's review 'Toward standardisation of FoxP3 regulatory T-cell measurement in clinical settings' (Lanza, 2009) clone PCH101 showed the greatest percent positive staining of the clones tested, but it also exhibited a degree of non-specific staining. This concurred with the findings in a study by Tran (Tran and Shevach, 2007). There has been considerable correspondence published in the literature in response to these studies. Pillai and Karandikar (Pillai and Karandikar, 2007) found that clone 259D showed lower specificity than PCH101, 236A/E7 and 206D. Fox and colleagues defended clone 259D and stated that clones 259D, 236A/E7 and 206D were originally generated from the same fusion which conflicts with Pillai and Karandikar's findings (Fox *et al,* 2008). Further correspondence continues (Tran and Shevach, 2007). In a study by Law and colleagues (Law *et al,* 2009) a clone 3G3 Imgenex reagent showed the lowest levels of FoxP3 staining of all the clones tested.

Discussions with product specialists at Miltenyi were unsuccessful in finding a solution to the staining problem.

2.10.2 Definitive Treg testing

A group of researchers at the Anthony Nolan Donor Registry performing Treg enumeration in umbilical cord blood donations kindly met to discuss kits, methods and gating strategies. As a result of this, the BD Pharmingen™ Human Regulatory T Cell cocktail (BD Biosciences) was chosen comprising FITC Anti-CD4 , a tandem conjugate system that combines Allophycocyanin and a cyanine dye (PE-Cy7) Anti-CD25 and Alexa Fluor ®Anti-CD127. This was used in conjunction with BD Pharmingen™ PE Mouse anti-Human FoxP3 clone 259D/C7 (BD Biosciences) and with BD Pharmingen™ Vioblue Anti-CD3 (BD Biosciences). The BD Pharmingen™ Human FoxP3 Buffer Set (BD Biosciences) comprising Buffer A and Buffer B was used for fixation and permeabilisation of the cells. The additional surface marker, CD127, assists in identifying and enumerating Tregs and its inclusion was recommended by the researchers at the Anthony Nolan Registry. The addition of Anti-CD3 enabled absolute Treg counts to be calculated.

Briefly, the cells suspended in BD Pharmingen™ stain buffer (BD Biosciences) were stained using BD Horizon™ V450 Mouse anti-human Anti-CD3 (BD Biosciences) and human regulatory T cell cocktail at room temperature. After washing in stain buffer the cells were fixed using a freshly made working solution of 1 x FoxP3 Buffer A. The fixative was removed and the cells permeabilised by adding a 1 x working solution of Buffer C (FoxP3 Buffer B diluted in FoxP3 Buffer A). Following removal of the permeabilisation solution the cells were washed and divided between two tubes. The cells in one tube were incubated with Anti-FoxP3 and those in the second tube incubated with BD Pharmingen™ PE Mouse immunoglobulin (Ig)G1, κ isotype control. The cells were analysed immediately following staining (full method shown in Appendix 3). A minimum of 50,000 events were counted for each analysis.

2.10.3 Principles of flow cytometric data acquisition and analysis

Following sample staining, testing was performed on a BD LSR11 flow cytometer (Becton Dickinson) fitted with four lasers, to allow multicolour analysis of cells labelled with different fluorochromes. Acquisition data was analysed using BD FACSDiva software following laser interrogation of the labelled cells. Cellular populations were discriminated on the basis of size and granularity using measurements of light forward and side scatter respectively as shown in Figure 7.

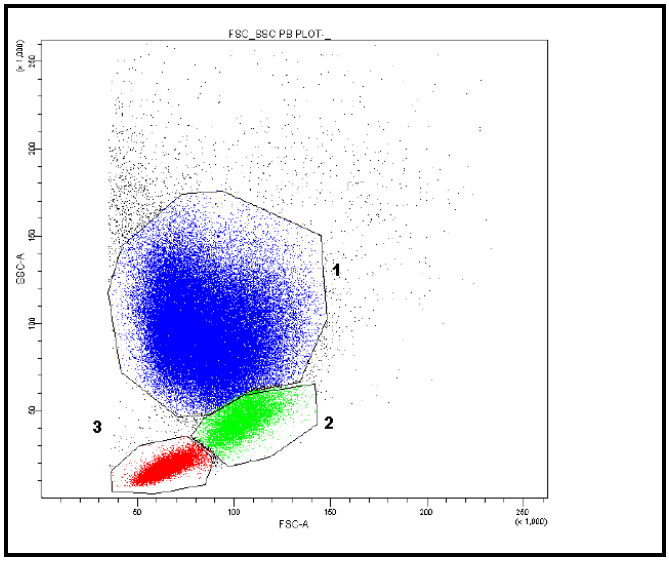


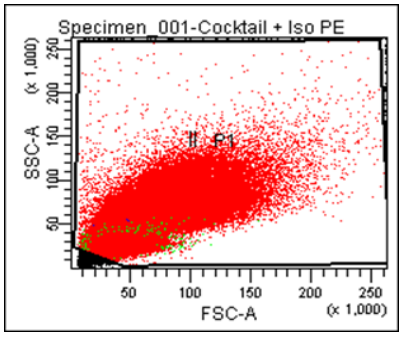
Figure 7: Blood sample flow cytometry plot. Plot of forward scatter (FSC-H) versus side scatter (SSC-H) using flow cytometry, showing expected areas of separation of granulocytes (1), monocytes (2) and lymphocytes (3) in a peripheral blood sample. Region 2 is the expected location of haematopoietic progenitor cells collected by apheresis (HPC,A).

2.10.4 Gating

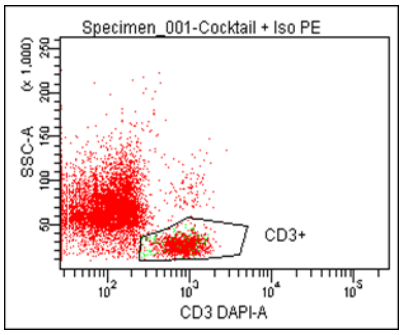
Gating was performed according to protocols and advice from a BD Biosciences technical advisor. Firstly cells stained with Anti-CD3, Treg cocktail and PE isotype control were analysed and a sequential gating method employed as shown in Figure 8 (a-h). Using the same gates, cells stained with Anti-CD3, Treg cocktail and AntiFoxP3 and were then analysed as shown in Figure 9 a-h).

Using an initial forward versus side scatter dot plot of cells stained with Anti-CD3, Treg cocktail and PE isotype control, a gate was applied to all cells excluding debris and dead cells as shown in Figure 8(a). The gated cells were then displayed on a dot plot and CD3+ cells (lymphocytes) gated as shown in Figure 8(b). Within the lymphocyte gate, using a side scatter versus CD4 plot, the CD4+ cells were gated as shown in Figure 8(c). Three independent plots were then created of the CD4+ cells, CD127 versus CD25 as shown in Figure 8(d), FoxP3 versus CD25 as shown in Figure 8(e) and CD127 versus FoxP3 as shown in Figure 8(f). In each plot cells were gated – $CD127^{low}$ $CD25^{high}$, FoxP3+ $CD25^{high}$ and $CD127^{low}$ FoxP3+ populations respectively. A combined gate was created in the analysis hierarchy. The PE isotype control ensured that the FoxP3 gates were in the correct position (Figures 8 (e) and (f)).

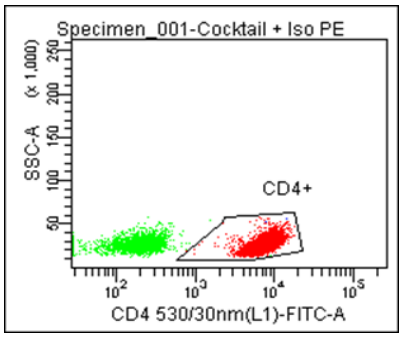
Having established optimal gating for the cells stained with Anti-CD3, Treg cocktail and PE isotype control, the gates remained in the same position during the analysis of the cells stained with Anti-CD3, Treg cocktail and AntiFoxP3 and were analysed as shown in Figure 9 (a-h).
A global template was used to provide approximate gating positions based on previous samples but for each new sample tested the gating positions were adjusted using the isotype control to take account of variations between samples. Using the combined gating in Figure 10 (a-c) only the cells that appear in all three gates are CD3+CD4+$CD125^{high}$$CD127^{low}$FoxP3+ cells (coloured purple).



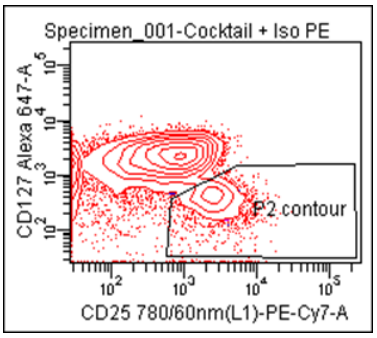
a)



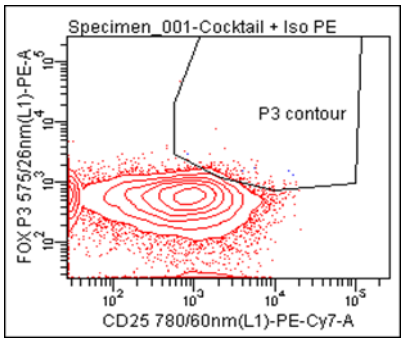
b)



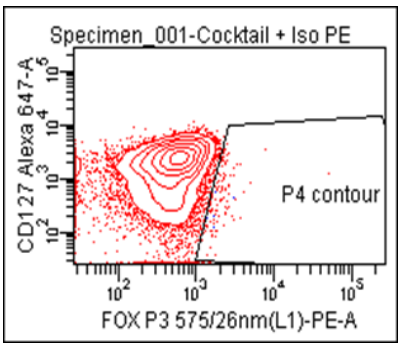
c)



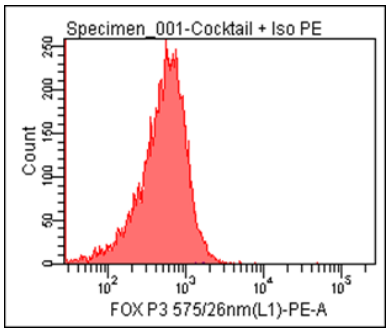
d)



e)



f)



g)

Figure 8: Representative flow cytometry analysis showing staining with anti-CD3, a cocktail of anti-CD4, anti-CD25 and antiCD127 with PE isotype control: Plots show cells stained with Anti-CD3, Treg cocktail and PE isotype control. (a) Dot plot with gate P1 to exclude dead cells and debris. (b) Dot plot restricted to gate P1. CD3+ cells gated. (c) Dot plot restricted to CD3+ with gate applied to CD4+ cells. (d) Contour with outliers plot with $CD25^{high}$ $CD127^{low}$ cells gated in P2 contour. (e) Contour with outliers plot with gate P3 contour placed to detect $CD25^{high}$ FoxP3+ cells. Isotype control used to set gate position. (f) Contour with outliers plot with P4 contour gate placed to detect $CD127^{low}$FoxP3+ cells. Isotype control used to set gate position. (g) Histogram shows cells falling outside combined gate P2 contour and P3 contour and P4 contour. Cells falling within the combined gate are too low in number to be seen.

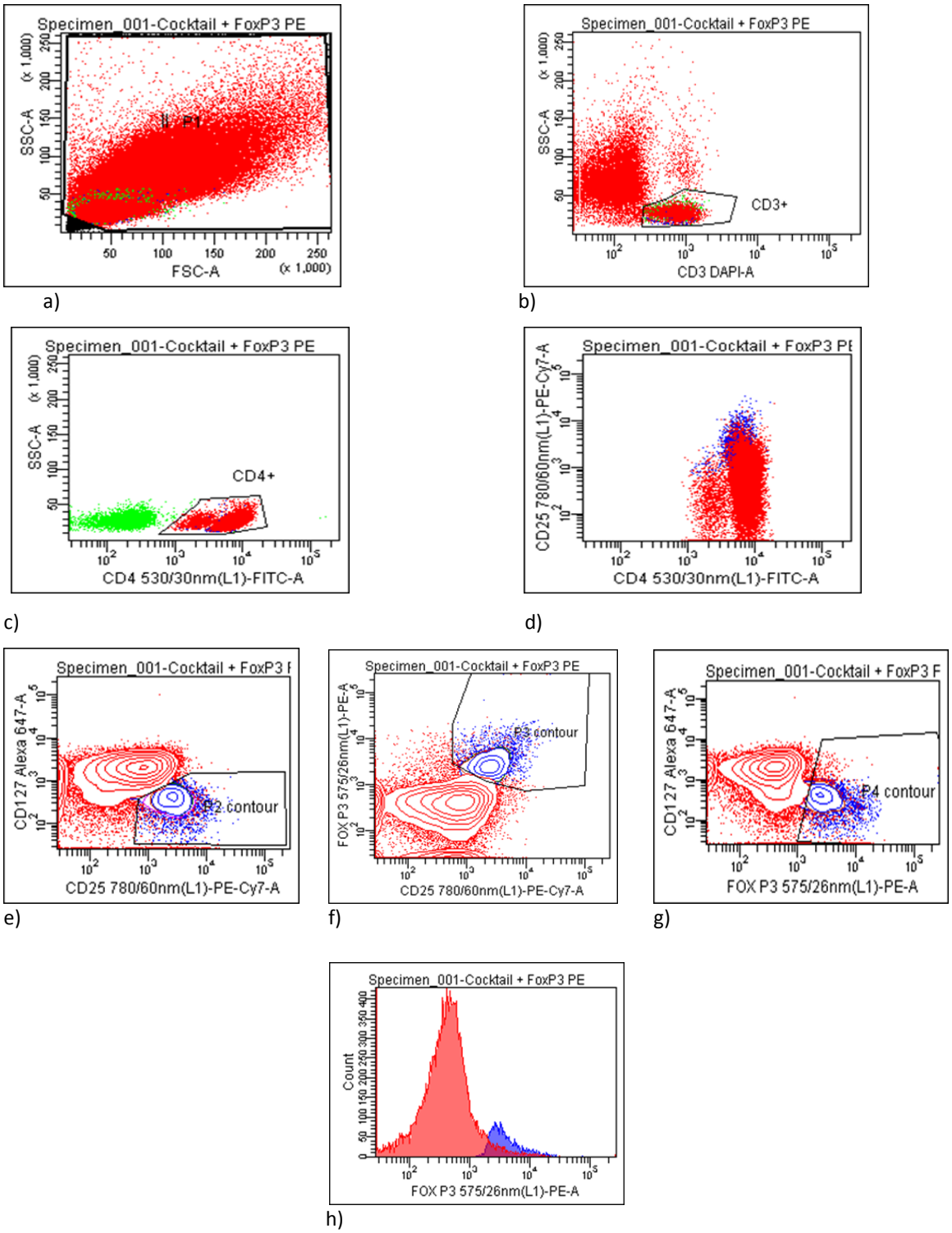


Figure 9: Representative flow cytometry analysis showing staining with anti-CD3, a cocktail of anti-CD4, anti-CD25 and antiCD127 with PE Anti FoxP3: (a) Dot plot with gate P1 to exclude dead cells and debris. (b) Dot plot restricted to gate P1. CD3+ cells gated. (c) Dot plot restricted to CD3+. CD4+ cells gated. (d) Dot plot shows CD3+CD4+cells with $CD25^{high}$ cells in purple. (e) Contour with outliers plot with $CD25^{high}$ $CD127^{low}$ cells gated in P2 contour. (f) Contour with outliers plot with $CD25^{high}$ FoxP3+ cells gated in P3 contour. (g) Contour with outliers plot with P4 contour gate on $CD127^{low}$ FoxP3+ cells. (h) Histogram shows cells falling outside combined gate P2 contour and P3 contour and P4 contour in red and Treg cells falling within the combined gate in purple.

2.10.5 Presentation of flow cytometry data

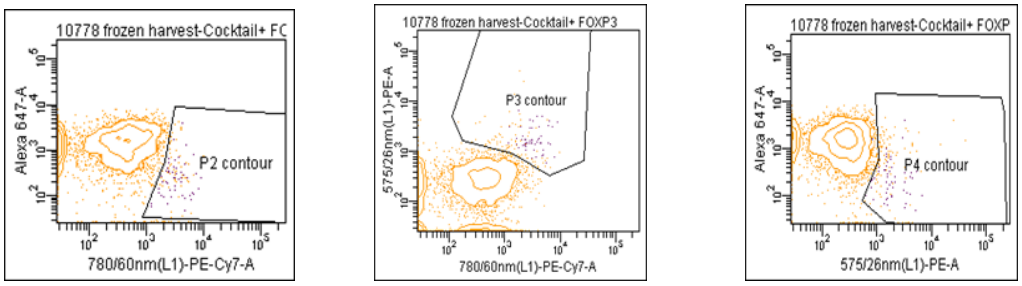
Initially gating was performed using flow cytometer data displayed as a dot plot and also as contour plus outliers as shown in Figure 11 (a-b). When high numbers of events were displayed the dot plots became saturated and it was difficult to separate the populations easily (Figure 11a). Contour plus outlier plots preserved the different populations regardless of the numbers of events and this enabled more accurate gating (Figure 11 b). As a result of these tests contour plus outlier plots were used to display data for gating.

2.10.6 Anti-CD3

The inclusion of Anti-CD3 in the staining panel not only provided a means of calculating absolute values of Tregs but also helped by 'cleaning' the dot plot enabling more accurate gating of CD4+ cells as shown in Figure 12 (a-d).

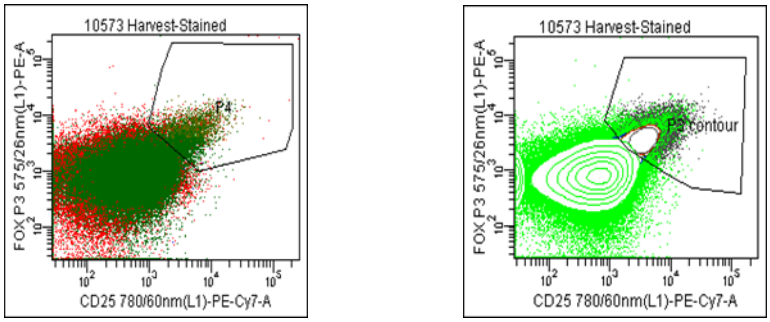
2.10.7 Analysis of flow cytometric data

Data from the flow cytometer hierarchy tables shown in Figure 13 (a) and (b) were used to calculate the number of Tregs present in the sample. The value obtained for the percentage of cells within the isotype combined gate was subtracted from the value obtained within the fully stained sample combined gate. The number of CD4+ cells was given as a percentage of CD3+ cells. White counts or TNC counts were available on all samples tested meaning that absolute Treg numbers could be calculated and either expressed as a value per millilitre for peripheral samples or a total Treg cell count for harvests.



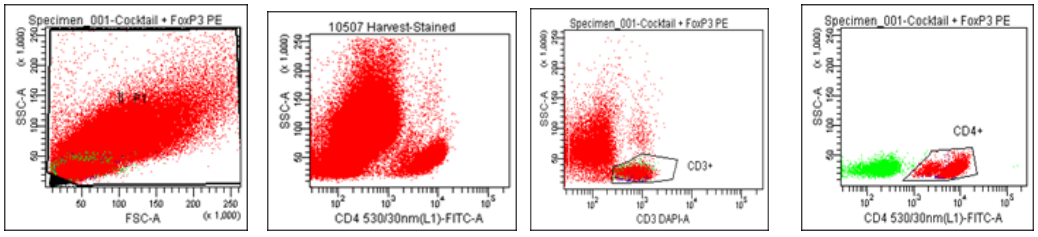
a) b) c)

Figure 10: Combined gate for Treg detection. Figure shows gates (a) P2 contour (b) P3 contour and (c) P4 contour. Cells that appear in all three gates are CD3+CD4+CD25^{high}CD127^{low}FoxP3 positive.



a) b)

Figure 11: Gating. Figure shows (a) gating using dot plot showing difficulty in separating and therefore gating different cell populations and (b) gating using contours with outliers to identify different cell populations in order to assist gating.



a) b) c) d)

Figure 12: Use of CD3 to assist gating of CD4+ cells. Figure shows (a) forward versus side scatter dot plot gated to exclude debris (b) side scatter versus CD4 on all cells minus debris (c) side scatter versus CD3 with CD3+ cells gated (d) dot plot of CD3+ cells side scatter versus CD4 with CD4+ cells gated.

a)

Tube: Cocktail + Iso PE

Population	#Events	%Parent	%Total
All Events	153,814	####	100.0
P1	101,407	65.9	65.9
CD3+	14,067	13.9	9.1
CD4+	9,800	69.7	6.4
P2 contour	1,428	14.6	0.9
P3 contour	151	1.5	0.1
P4 contour	124	1.3	0.1
P2 contour AND P3 contour AND P4 contour	5	0.1	0.0
NOT(P2 contour AND P3 contour AND P4 contour)	9,795	99.9	6.4

b)

Tube: Cocktail + FoxP3 PE

Population	#Events	%Parent	%Total
All Events	358,067	####	100.0
P1	255,963	71.5	71.5
CD3+	33,022	12.9	9.2
CD4+	23,126	70.0	6.5
P2 contour	3,411	14.7	1.0
P3 contour	3,151	13.6	0.9
P4 contour	3,383	14.6	0.9
P2 contour AND P3 contour AND P4 contour	2,570	11.1	0.7
NOT(P2 contour AND P3 contour AND P4 contour)	20,556	88.9	5.7

Figure 13: Data from analysis. Figure shows (a) hierarchy analysis data from cells stained with Anti-CD3, Treg cocktail and PE isotype control and (b) hierarchy analysis data from cells stained with Anti-CD3, Treg cocktail and Anti-FoxP3.

2.10.8 Controls

Unstained controls were originally run for each test on all sample types but no auto-fluorescence was detected in any of the samples tested (n=10) (data not shown). However, limited sample volumes of surplus material and low white counts post-transplant meant that testing of unstained controls was not practical and therefore was not performed on samples thereafter.

Controls using a BD Pharmingen™ V450 Mouse IgG1,κ isotype control (BD biosciences) and cells stained with only Anti-CD3 were also initially run for each test sample to detect any non-specific staining, however none was seen (n=10) (data not shown). Based on this data a decision was made to discontinue the use of the V450 isotype control because for many samples there were insufficient cell numbers. Priority was given to ensuring that a control using Anti-CD3, Treg cocktail and isotype FoxP3 stained cells was always used in addition to the fully stained test sample.

2.11 Surface marker staining for Tregs – Tumour Necrosis Factor Receptor 2 (TNFR2)

As the method for FoxP3 intracellular staining requires time to fix and permeabilise the cells, this is not an ideal staining method to be used as a predictive test to be performed on high sample numbers. More importantly, an intracellular marker does not allow for identification, selection and expansion of Tregs in the laboratory for therapeutic use and this has fuelled the search for an ideal surface marker for this purpose. From the range of potential surface markers for Tregs identified in the past few years, one of these was selected for the purposes of this study to be performed in parallel with intracellular FoxP3 staining. The aim was to ascertain if the same population of cells was being identified and if this method might provide a more suitable and less time-consuming method for measurement of Treg numbers.

Chen and co-workers demonstrated that co-expression of TNFR2 (CD120b) and CD25 identified the most suppressive sub-set of Tregs in mice and that these findings could be extrapolated to humans (Chen *et al,* 2010). Their studies revealed that more than 90% of human CD4+ CD25+ TNFR2+ cells were also FoxP3+. TNF working with IL-2 expands Tregs and up-regulates expression of CD25 and FoxP3, resulting in enhanced immunosuppressive function.

Invitrogen mouse monoclonal antibody to human TNFR2 conjugated to phycoerythrin (PE) was chosen. Staining with Anti TNFR2 was performed at room temperature in conjunction with BD Horizon™ V450 Mouse anti-human Anti CD3 (BD Biosciences) and BD Pharmingen™ Human Regulatory T Cell cocktail (BD Biosciences) comprising FITC Anti-CD4 and (PE-Cy7) Anti-CD25. The cocktail also contained Alexa Fluor ®Anti-CD127 but was not required for this staining method. The Anti-CD3 and human regulatory T cell cocktail were the same reagents as were

used for the FoxP3 staining to eliminate differences in staining with the use of different antibody clones and fluorochromes.

2.11.1 Gating for CD25+TNFR2+ cells

Using an initial forward versus side scatter dot plot of cells stained with Anti-CD3, Treg cocktail and PE isotype control, a gate was applied to all cells excluding debris and dead cells as shown in Figure 14. Gated cells were then displayed on a dot plot and CD3+ cells (lymphocytes) gated as shown in Figure 14(b). Within the lymphocyte gate, using a side scatter versus CD4 plot, the CD4+ cells were gated as shown in Figure 14(d). Sequential gating of CD3+ and CD4+ cells was performed. Using a contour with outliers plot of TNFR2 vs CD25 gating was performed to identify $TNFR2^{negative}$ cells.

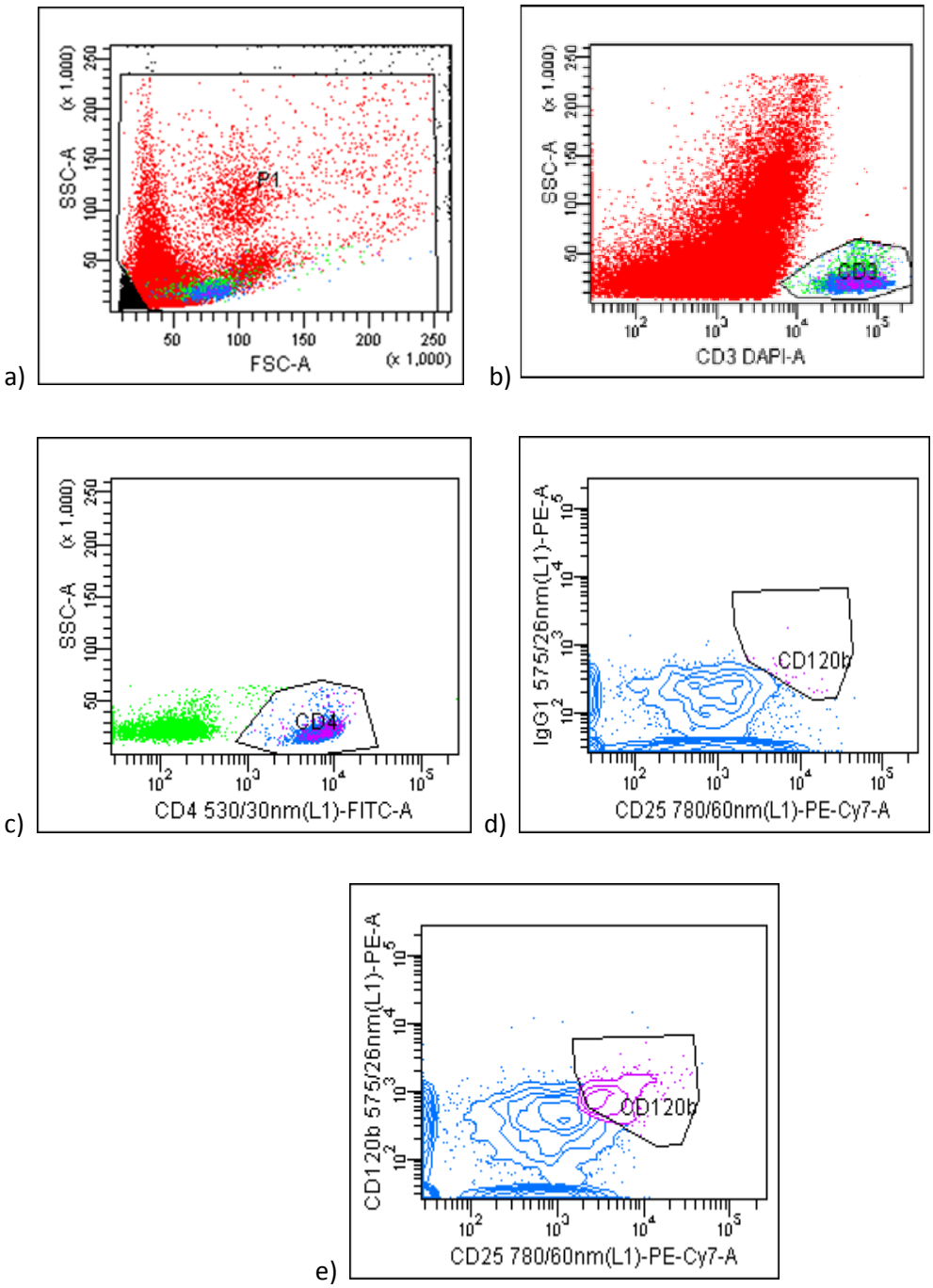


Figure 14: Gating to identify CD4+ CD25+ TNFR2+ Tregs. Representative flow cytometry analysis of cells stained with Anti-CD3, Treg cocktail and either PE isotype control or PE anti-TNFR2. (a) Dot plot with gate P1 to exclude dead cells and debris. (b) Dot plot restricted to gate P1. CD3+ cells gated. (c) Dot plot restricted to CD3+ with gate applied to CD4+ cells. (d) Contour with outliers plot of CD25 vs PE isotype control used to set gate position. (e) Contour with outliers plot of CD25 vs TNFR2 with pre-placed gate TNFR2 used to detect CD25+ TNFR2+cells.

12.12 Statistics

Statistical analyses were performed using SPSS version 20 software (SPSS Inc, Chicago, Il, USA) Summary statistics were used to describe harvest and patient characteristics (mean, median, standard deviation, standard error).

All harvest data was analysed using analysis of variance and Kruskal-Wallis tests. Patient and graft characteristics were compared either using Mann-Whitney U tests (two populations) or Kruskal-Wallis tests (three or more populations) for continuous variables. Peripheral and harvest Tregs were compared using a paired two-tailed t-test and correlation performed using Spearman rank correlation (ρ). All p values were two-tailed and were considered significant at <0.05.

Correlation was performed using Spearman rank correlation. Values of ±0.5 to 1.0 were considered to show high correlation, values of ±0.3 to 0.5 were considered to show medium correlation.

Incomplete data sets were inevitable as patients were transferred to other hospitals, returned home to other countries, relapsed or died. Additionally some surplus samples were inadequate in size for testing.

3.0 Results – Mobilisation and harvesting

The effect of HPC mobilisation regimens, pre-harvest peripheral blood counts and day of harvest on CD4+CD25^{high}CD127^{low}FoxP3+ Treg numbers in both autologous and allogeneic harvests.

3.1 Introduction

Mobilisation of HPCs in autologous patients and allogeneic donors is a multifactorial process. Various regimens used could potentially affect the levels of Tregs present in the HPC collections from both autologous patients and allogeneic donors. Furthermore, as HPC mobilisation is a dynamic process in terms of CD34 cell production, with variable levels observed across sequential harvest episodes, it is logical to presume that Treg levels may be similarly affected.

Treg occurrence in pre-harvest peripheral blood in autologous patients and allogeneic donors may correlate with levels in the harvested material and thus enable tailoring of harvesting to influence Treg content.

This chapter considers the effect of factors that may affect CD3+CD4+CD25^{high}CD127^{low}FoxP3+ Treg levels in harvested HPCs from autologous patients and allogeneic donors and the influence of these cells in pre-apheresis peripheral blood on collection levels.

Since the mobilisation regimen used to treat autologous donors prior to apheresis has been demonstrated to mobilise different subsets of CD34 cells (Donahue *et al,* 2009) this study set out to determine if Treg numbers are also affected by mobilisation regimen. As the numbers of Tregs in the graft may influence anti-tumour activity following transplant it is important to establish if mobilisation regimen can influence this.

In recent times, RMH and many other transplant centres have introduced the practice of proactively cryopreserving surplus G-CSF mobilised allogeneic material for possible use as donor lymphocyte infusions should they be required. Originally, donor lymphocyte infusions successfully used products from non-mobilised collections. However, the practice of using surplus G-CSF mobilised apheresis HPC (HPC,A) material, may result in donor lymphocyte products which differ in terms of the Treg numbers and in turn may impact on the effectiveness of the donor lymphocyte based treatment.

This study aimed to determine if Treg numbers differ between mobilised and non-mobilised products.

If Treg numbers in the graft are able to influence anti-tumour activity following transplant, it might be beneficial to predict expected Treg numbers in harvests. This study therefore looked at

Treg levels in autologous and allogeneic donor peripheral blood to identify any correlation with Tregs collected in donor harvests.

During the early stages of the study it appeared that Treg numbers might differ between first and second days of harvesting. If anti-tumour activity is influenced by Treg levels in the graft it would be possible to exploit any differences due to day of harvesting by extending or reducing apheresis time on the first day of harvesting. This tailored harvesting is already employed to optimise CD34 collection and therefore could realistically also be used to optimise Treg levels.

3.2 Data expression

3.2.1 Mobilisation regimens for autologous harvests

Treg numbers were compared in autologous apheresis products (n=109) following mobilisation with G-CSF (n=11), G-CSF plus cyclophosphamide (n=82), and G-CSF plus Plerixafor (n=16).

3.2.2 Donor lymphocytes

Treg levels were measured in non-mobilised harvests (n=13) and compared with G-CSF mobilised sibling and unrelated allogeneic harvests (n= 59). In addition to Tregs/ml, Tregs as a percentage of harvested CD4+ cells and theTreg:CD3+ cell ratio in harvests was examined.

3.2.3 Pre-harvest

Treg numbers in the peripheral blood samples of 45 autologous and 11 allogeneic donors, taken prior to apheresis for assessment of CD34, were compared with Treg numbers in the associated harvests.

3.2.4 Day of harvest

The study analysed autologous harvests collected on a single day (n=79) with those collected over two (n=28) or three (n=2) consecutive days.

3.2.5 Autologous, allogeneic and DL harvests

Tregs measured by flow cytometry were expressed as Tregs x10^6ml, Tregs as a ratio of CD34+ or CD3+ cells and Tregs as a percentage of CD4+ cells. These measures of Tregs were chosen for both autologous and allogeneic harvests to allow meaningful comparisons.

Measuring Tregs/ml enables a direct comparison of the Treg levels in autologous and allogeneic harvests regardless of the volume of product harvested. This is important as RMH does not run apheresis collections for standard times so collection volumes vary.

Examining Tregs as a proportion of CD4+ cells in autologous, allogeneic and DL harvests shows if mobilisation regimens cause a skewed increase or decrease in this subset. Analysis of the data using the Treg: CD34 ratio in autologous and allogeneic harvests shows the relative proportions of Tregs that the patient will receive in their transplant.The amount of product transplanted into the patient is based on the CD34 dose/kg with a minimum dose being $1.5x10^6$/kg. Thus the Treg: CD34 ratio indicates the number of Tregs relative to the dose of CD34+ cells. Similarly in DL harvests the Treg: CD3 ratio can be used to indicate the relative Treg levels in DLI where the calculated dose is based on CD3+ cells/kg.

3.3 Results

3.3.1 Effect of mobilisation regimens on Treg levels

No significant differences were observed between numbers of Tregs/ml in autologous harvests mobilised by G-CSF plus cyclophosphamide (0.28 x10^6/ml ±0.03) compared with G-CSF alone (0.18 x10^6/ml ±0.05) (p=0.234) or G-CSF plus Plerixafor (0.42 x10^6/ml ±0.09) (p=0.148). Furthermore, comparison of harvests mobilised by G-CSF plus Plerixafor with those mobilised with G-CSF as a single agent, was not significant in this series (p=0.068) although consistently higher levels of Tregs were observed with the combined regimen (Figure 15(a)).

No significant difference was noted between the Treg: CD34+ cell ratio for autologous harvests mobilised with G-CSF alone (0.35±0.11) and G-CSF plus cyclophosphamide (0.28±0.05) (p=0.686) or G-CSF plus Plerixafor (0.66±0.15) (p=0.152). However, harvests mobilised with G-CSF + Plerixafor showed a significantly higher Treg: CD34+ cell ratio than those mobilised with G-CSF plus cyclophosphamide (p=0.002) (Figure 15(b)).

When Treg levels were expressed as a percentage of CD4+ cells (Figure 15(c)) no significant difference was apparent between autologous harvests mobilised with G-CSF alone (1.24±0.31) and either G-CSF plus cyclophosphamide (1.58±0.14) or G-CSF plus Plerixafor (2.03±0.38), (p=0.446 and p=0.138 respectively). There was also no significant difference between harvests mobilised by G-CSF plus cyclophosphamide and G-CSF plus Plerixafor (p=0.235).

3.3.2 Comparison of Treg levels present in G-CSF mobilised and non-mobilised allogeneic harvests

Products for use as DL, whether derived from conventional non-mobilised allogeneic DL collections or from surplus G-CSF mobilised donations were compared. Mobilised harvests included sibling (n=20) and unrelated (n=39) donors. Non-mobilised harvests included sibling (n=10) and unrelated (n=3) donors. No significant difference was observed (Figure 16(a)) between Tregs x10^6/ml in allogeneic harvests mobilised with G-CSF (0.43 x10^6/ml ±0.09) and non-mobilised allogeneic harvests (0.61 x10^6/ml ±0.15) (p=0.162). Expressing data as Tregs: CD3+ cells ratio highlighted a significant difference between numbers of Tregs in mobilised (0.006 ±0.0009) and non-mobilised (0.131 ±0.003) harvests (p=0.022) (Figure 16(b)). Treg precentages of CD4+ cells in DL harvests collected after no mobilisation agent (2.21±0.43) and those collected after mobilisation with G-CSF (1.24 ±0.15) verged on significance (p=0.058) (Figure 16(c)).

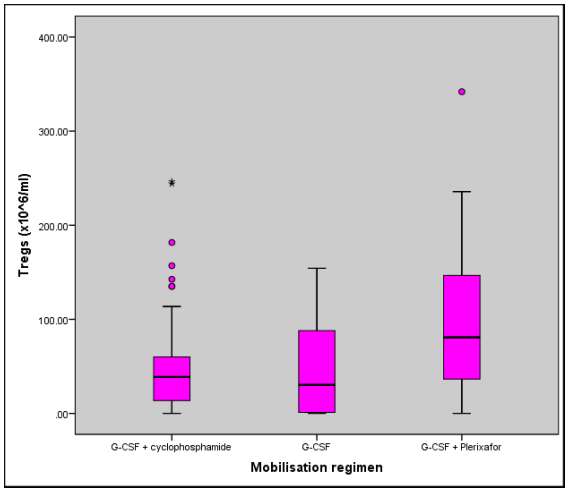
3.3.3 Treg levels in autologous and allogeneic donor pre-harvest peripheral blood samples and associated harvests

Treg levels in the peripheral blood of autologous patients measured prior to apheresis showed no correlation with Treg levels collected in the harvests from those patients (Spearman rank correlation (ρ) = -0.090, p=0.558) (Figure 17(a)). Likewise, peripheral blood Treg levels in allogeneic patients also showed no relationship to harvested Treg levels (ρ= 0.144, p=0.569) (Figure 17 (b)). Harvested cells contain 10-20% higher concentrations of Tregs than peripheral blood since only the mononuclear cell layer is collected during apheresis.

3.3.4 Treg levels in harvests by day of collection

The levels of Tregs (cells $x10^6$/ml) in autologous harvests were not found to differ significantly across sequential days (day 1 and 2) of harvesting (0.31 $x10^6$/ml±0.03 and 0.25 $x10^6$/ml±0.05 respectively) (p=0.178). As insufficient data points for the third day of harvesting were collected this data was not included in the analysis (Figure 18(a)). The data also showed no significant differences between levels of Tregs harvested on days 1 and 2 when analysed by mobilisation regimen (Figure 18(b)), G-CSF plus cyclophosphamide (p=0.473), G-CSF plus Plerixafor (p=0.545) and G-CSF (p=0.538).

a)



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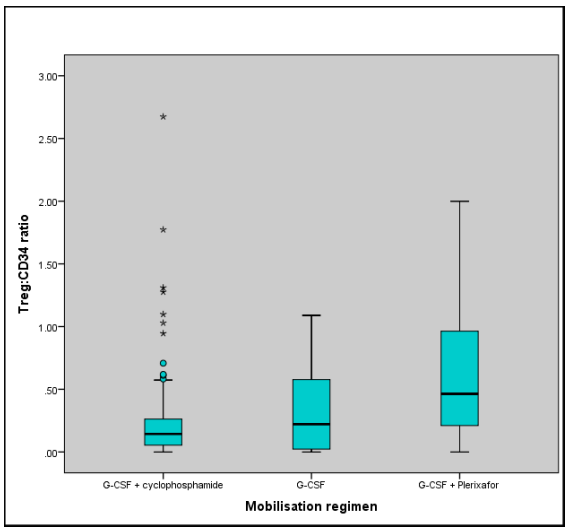


c)

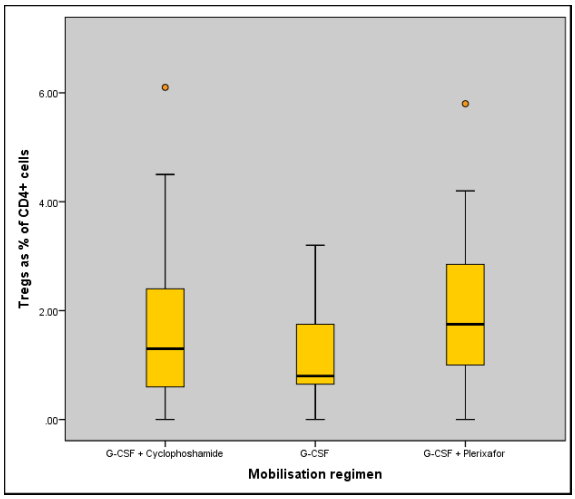
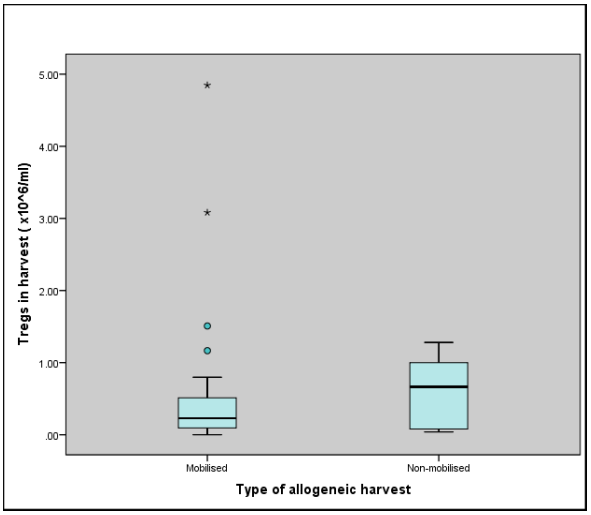


Figure 15: The influence of mobilisation regime on Tregs in autologous harvests: (a) Treg levels x10^6/ml (b) Treg:CD34 ratio (c) Treg as a percentage of CD4 cells. Samples of autologous harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. Mobilisation regimens: G-CSF plus cyclophosphamide (n=82), G-CSF (n=11) and G-CSF plus Plerixafor (n=16).

a)



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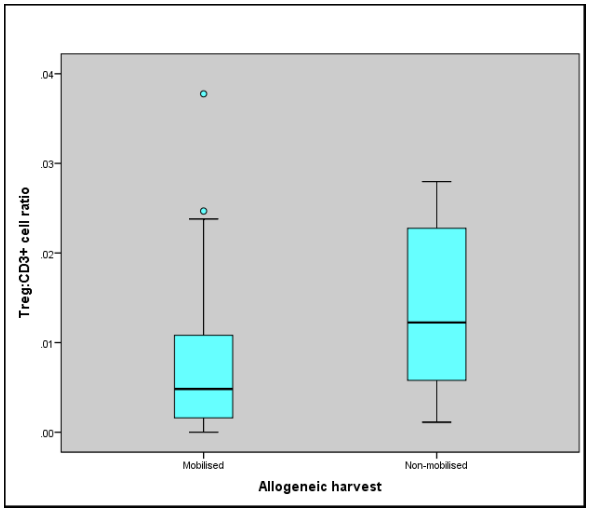

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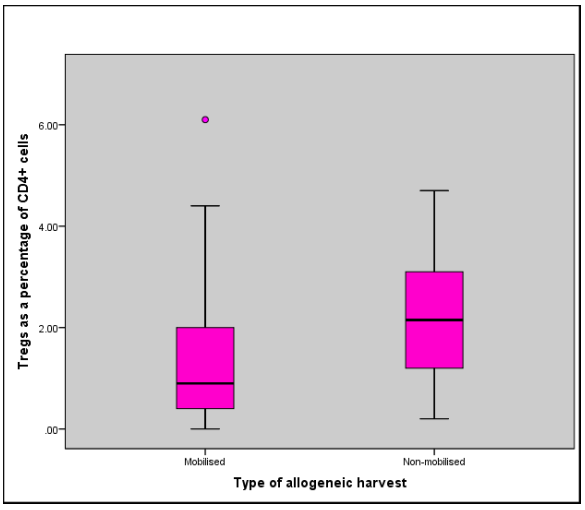
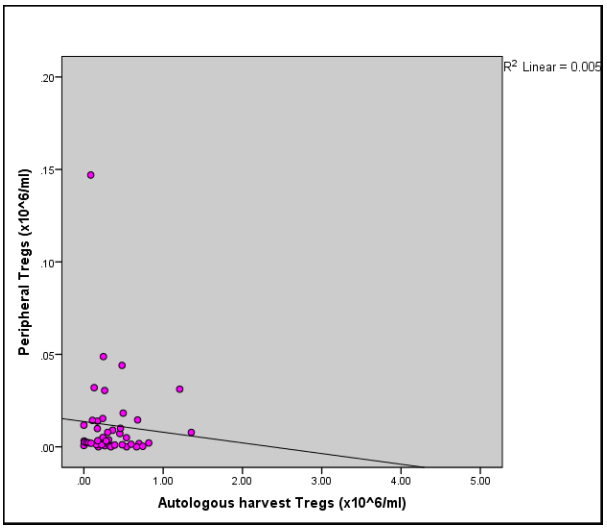


Figure 16: Levels of Tregs in mobilised and non-mobilised allogeneic harvests: (a) Treg levels x10^6/ml (b) Treg:CD34 ratio (c) Treg as a percentage of CD4 cells. Samples of autologous harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. Harvests were mobilised with G-CSF (n=59) compared with harvests where no mobilising agent was used (n=13).

a)



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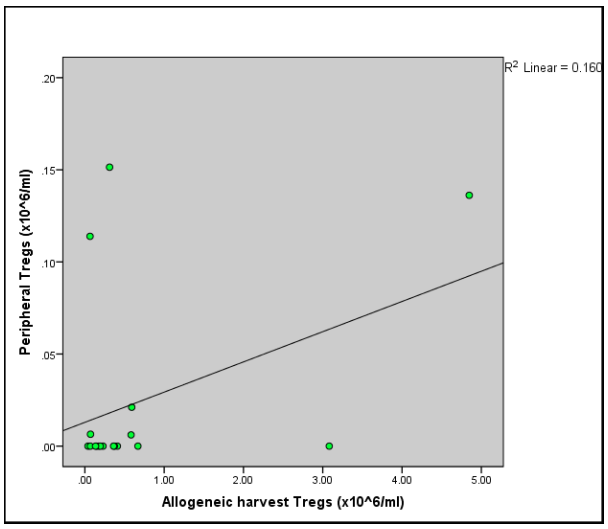
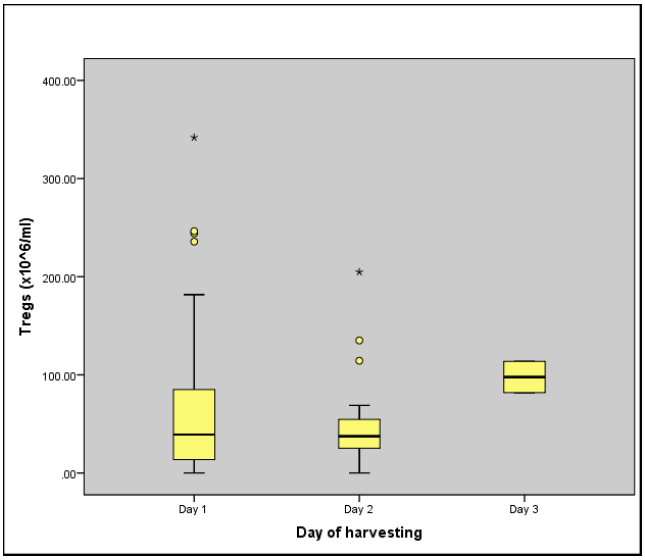


Figure 17: Scatter plots to show relationship between Treg levels in peripheral blood and associated harvests: (a) Autologous donor peripheral blood and harvests (b) Allogeneic donor peipheral blood and harvests. Samples of peripheral blood and harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry.

a)



b)

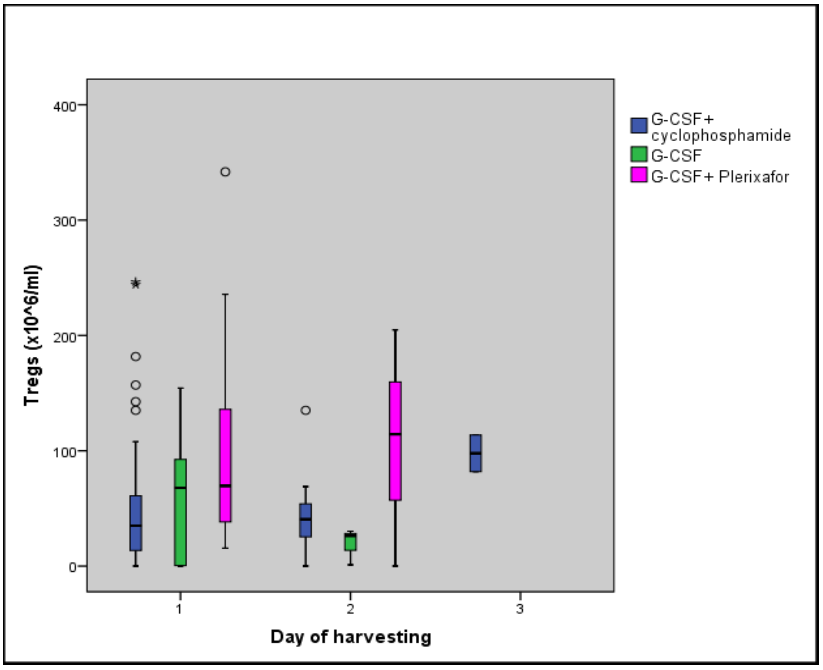


Figure 18: Levels of Tregs in harvests collected on sequential days of apheresis: (a) Tregs x10^6/ml by days 1-3 of harvesting. (b) Tregs x10^6/ml by mobilisation regimen across days 1-3 of harvesting. Samples of autologous harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. Tregs were measured in harvests collected on day 1 (n=79): G-CSF + cyclophosphamide (n=58), G-CSF + Plerixafor (n=13) and G-CSF (n=8), day 2 (n=28): G-CSF + cyclophosphamide (n=22), G-CSF + Plerixafor (n=3) and G-CSF (n=3) and day 3 (n=2): G-CSF + cyclophosphamide (n=2).

3.4 Discussion

3.4.1 Mobilisation regimen

The composition of PBSC grafts used for transplants is accepted as being important in terms of CD34+ cell dose, but Treg numbers may also have an effect on the success of the transplant with higher levels of Tregs inhibiting desirable anti-tumour responses in both autologous and allogeneic patients. In allogeneic recipients Treg numbers infused in the graft may also be expected to have a protective effect on the occurrence and severity of GVHD. Any potential relationship between conventional mobilisation and Treg levels is therefore of clinical interest. The introduction of the novel mobilisation agent Plerixafor has enabled mobilisation of functional CD34+ cells into the peripheral blood where other mobilisation regimens have failed in a significant number of cases (Lemoli and D'Addio, 2008, DiPersio *et al,* 2009). However, only a few studies have reported the effect of Plerixafor on the mobilisation of Tregs (Devine *et al,* 2008, Gaugler *et al,* 2013). Because of the considerable variation in the use of Plerixafor, in particular the timing of administration between centres, it is particularly important to study the effect of this agent and other more typical regimens in individual centres on a typical patient cohort.

Results from this study showed no significant differences between the absolute levels of Tregs ($x10^6$/ml) in G-CSF and G-CSF plus cyclophosphamide mobilised harvests (Figure 15(a)). Harvests mobilised with G-CSF plus Plerixafor showed no significant difference in Treg levels to those mobilised by GCSF alone although a small increase was noted (p=0.068) (Figure 15). These findings agree with data published by Kean and colleagues who found in simian models that increased numbers of Tregs were mobilised and harvested with both Plerixafor alone and after G-CSF plus Plerixafor (Kean *et al,* 2011). These small scale studies on the peripheral blood of macaque monkeys demonstrated a significant increase in Tregs at two and four hours after administration of Plerixafor, but the increase lacked significance after six hours and no further time points were tested. However, as only three monkeys were studied in each mobilisation group these results may not be representative. RMH apheresis is initiated 16 hours post administration of Plerixafor, so the results obtained suggest that increased Treg levels may be maintained in the peripheral blood for somewhat longer than noted by Kean. Higher Treg frequency was also observed by Kao and colleagues in harvests mobilised by G-CSF and Plerixafor compared with G-CSF and cyclophosphamide (Kao *et al,* 2010).

The lack of significance in the RMH data may be due to the time interval between the administration of Plerixafor and start of harvesting which has been adopted at RMH due to logistical and financial constraints. However, the local timing may benefit patients as a

consequence of lower Treg levels in the product than might be harvested with a shorter interval between Plerixafor administration and collection. This is speculation however as there are no comparative studies which considered different time frames and Treg yield.

Previous studies examining subsets of harvested cells have shown a significant increase in mobilisation of B and T lymphocytes into peripheral blood with Plerixafor alone (DiPersio *et al,* 2009, Tanhehco *et al,* 2010). For this reason, Tregs as a percentage of CD4+ cells were considered in this study but no significant differences between mobilisation regimens were apparent (Figure 15). This finding suggests that although the number of CD4+ cells is typically increased after treatment with Plerixafor, there is no skewing of mobilisation of the Treg population. Interestingly however, G-CSF plus Plerixafor resulted in significant increases in the Treg: CD34+ cell ratios as shown in Figure 15. This infers that patients receiving G-CSF plus Plerixafor mobilised grafts will generally receive higher absolute numbers of Tregs than those receiving G-CSF or G-CSF plus cyclophosphamide mobilised harvests as most patients receive similar CD34+ cell doses. Increased numbers of nucleated cells collected in G-CSF plus Plerixafor mobilised harvests have been described by a number of researchers (Varmavuo *et al,* 2012, DiPersio *et al,* 2009, Kean *et al,* 2011) and have been observed frequently in RMH practice. Besides mobilising different CD34+ cell subsets, Kean and colleagues showed that Plerixafor mobilised significantly more T and B lymphocytes into the peripheral blood than G-CSF alone including an increase in both effector T cells and Tregs (Kean *et al,* 2011, Kao *et al,* 2010).

The higher Treg levels observed in this study with Plerixafor plus G-CSF compared to other mobilisation regimens may be attributable to significantly higher number of T lymphocytes mobilised with this regimen. As Tregs provide a protective mechanism against GVHD these findings may have important implications for use of Plerixafor in allogeneic (HPC) transplantation if its use is extended to this population.

The criterion for use of Plerixafor at RMH is failure of at least one prior mobilisation attempt. Patients who are most likely to fail G-CSF or G-CSF plus cyclophosphamide mobilisation are those who have bone marrow involvement or progressive disease, have received a high number of chemotherapy cycles or radiotherapy, and are over 60 years of age. It is interesting to speculate if these groups of patients might have higher baseline levels of Tregs than patients who mobilise after G-CSF alone or G-CSF plus cyclophosphamide and if it is this, rather than the Plerixafor that results in the higher Treg levels seen in Plerixafor mobilised harvests . Increased Tregs levels have been demonstrated in patients with active malignancies. Suppression of anti-tumour immune responses by higher levels of Tregs would in turn necessitate higher numbers of cycles of chemotherapy to control resistant disease. Additionally Treg levels have been reported to increase with age (Lages *et al,* 2008).

It is interesting to note that the addition of cyclophosphamide to G-CSF in the mobilisation regimen did not significantly affect Treg numbers as shown in Figure 15. Following treatment of patients with cyclophosphamide, T cell numbers are notably reduced and this is followed by rapid T cell repopulation with stem cell collection taking place during the recovery phase following chemotherapy. Cyclophosphamide has been shown to decrease Treg numbers and also to reduce their suppressive function (Heylmann *et al,* 2013, Lutsiak *et al,* 2005). Lutsiak and colleagues observed that following treatment of mice with cyclophosphamide, Treg numbers and function decreased but then both levels and function returned 10 days following treatment (Lutsiak *et al,* 2005). In patients mobilised with G-CSF plus cyclophosphamide, apheresis usually takes place after a minimum of 10 days following cyclophosphamide which may explain why no decrease in Treg numbers is observed at that time point.

3.4.2 Treg levels in G-CSF mobilised and non-mobilised allogeneic HPC harvests

In allogeneic donors CD34+ cells are mobilised using G-CSF alone. If DL are required to treat cases of relapse or falling donor chimerism following allogeneic transplantation it is standard practice to re-harvest non-mobilised donor lymphocytes (DL) from the original sibling or unrelated donors. Re-calling donors for non-mobilised harvests following the initial donation may not be possible due to their lack of eligibility, for example they may have developed a medical complication that precludes further donation. They may be unwilling or unavailable to donate again. Furthermore, donor recall can have high cost implications and may involve considerable delays. In order to avoid these donor recall problems it has become accepted practice at RMH to proactively cryopreserve and store surplus G-CSF mobilised allogeneic HPC products for possible use as donor lymphocyte infusions (DLI) should this be required post-transplant. In this instance, levels of DL infused are based on the CD3 count of the original harvest. It is important to determine if Treg levels vary between G-CSF mobilised and non-mobilised products as this could affect the efficacy of the product and have clinical implications. Maury and colleagues (2010) successfully used depletion of CD4+CD25+ cells in the DL product prior to DL infusion in an attempt to improve alloreactivity and anti-tumour immune responses. After depletion of CD4+CD25+ cells from the DL products they were able to induce GVHD in two patients who had previously failed to respond to DLI therapy. Maury's team went on to treat a further four patients with fludarabine and cyclophosphamide to not only deplete donor Tregs from the recipient's circulation, but also to induce lymphopoenia to drive the homeostatic expansion and activation of the infused T cells in the DLI. In a study by Hicheri and colleagues they compared Tregs in DL products and observed that the patients who remained in a durable remission following DLI therapy had received products containing lower percentages of Tregs in total lymphocytes (Hicheri *et al,* 2008). The current study therefore aimed to establish if lower

Tregs were collected in mobilised or non-mobilised products so that clinical decisions could be made as to which product would be likely to result in optimal GVM.

When Treg levels were examined as Tregs/ml in the collected product, the Treg levels were not significantly different between mobilised and non-mobilised DLI (Figure 16(a)). However, when expressed as a percentage of CD4+ cells, Tregs in the mobilised harvests were lower than in non-mobilised harvests although this only verged on significance, indicating that other T cell subsets apart from Tregs are preferentially mobilised by G-CSF (Figure 16(c)).

Because DLI doses are calculated on the number of CD3+ cells infused, the Treg:CD3+ cell ratio was considered and compared between mobilised and non-mobilised harvests (Figure 16(b). This demonstrated a significant difference between products, with mobilised harvests exhibiting the lowest ratios. It has been reported that the relative proportions of Tregs and conventional T cells in the peripheral blood can determine immunologic activity after myeloablative transplant (Dieckmann *et al,* 2001), with a diminished ratio of Tregs to conventional T cells resulting in higher levels of GVHD post-transplant. Expressing the Treg levels in this way may relate to the potential immunosuppressive effect of the Tregs in the DL harvest. It might therefore be expected that a lower Treg: CD3+ cell ratio in DLI might result in a more potent GVM effect.

The purpose of DLI therapy in patients with falling donor chimerism or relapse is to initiate a GVM response which is typically accompanied by GVHD. Evidence of GVHD is often seen as a positive indication that the GVM process is underway with the best scenario being the achievement of anti tumour effect with tolerable GVHD. The fact that significantly lower Treg:CD3 ratio levels were observed in the pre-emptively stored surplus harvest material from donors following G-CSF mobilisation, is reassuring as it may be inferred that the lower concentrations in the graft will not diminish the desirable GVM effect mediated by effector subsets. This finding will inform future clinical decisions concerning the use of mobilised harvests to provide DL products.

3.4.3 The effect of Treg levels in patient or donor pre-harvest peripheral blood on numbers in HPC harvests

Apart from mobilisation regimen, another factor which could affect the levels of Tregs in the graft could be numbers in the peripheral blood of patients or donors prior to apheresis.

As pre-apheresis CD34 counts can used to predict the number of CD34+ cells that may be harvested (Auer *et al,* 1998, Lane *et al,* 2004) this study set out to determine if peripheral Treg counts could be used to predict the levels of Tregs that would occur in harvests. In the autologous setting, peripheral blood Treg levels measured after mobilisation and prior to apheresis showed no correlation with levels collected in harvests (Figure 17(a)). Tregs in the

healthy donors of allogeneic harvests appeared to show a weak association between peripheral pre-apheresis and harvested Tregs (Figure 17(b)). This lack of strong correlation is unexpected since T cells, including Tregs, are found in the mononuclear cell layer harvested during apheresis together with CD34+ cells. It would be expected that peripheral blood levels would therefore be linked to numbers collected in the same way as CD34+ cells.

CD34+ cells are released from reservoirs such as the bone marrow over the duration of the apheresis procedure enabling high volume apheresis with no resulting loss in the rate at which these cells are collected. At RMH extended apheresis run-times are often used to reduce the number of apheresis sessions for individual patients and donors. Although studies have demonstrated a linear correlation between CD3+ cell numbers collected and total blood volumes processed, these studies have focused on the apheresis collection of donor lymphocytes which are typically much shorter procedures (Sato *et al,* 2001). The mean apheresis run time in Sato's study of donor lymphocyte collections was 107 minutes whereas CD34 collection apheresis run-times at RMH rarely fall below 200 minutes. The same study observed that all donors showed cytoreduction in peripheral blood samples taken after the apheresis collection, with lymphocytes and platelets showing the most marked decrease of around 30% of the pre-apheresis value. This is in contrast to only a modest decline in the number of CD34+ cells in the peripheral blood following apheresis noted by Rowley and colleagues during studies on large volume apheresis collections of CD34+ cells (Rowley *et al,* 2001).

This higher lymphocyte than CD34+ cell reduction noted during and following apheresis may therefore explain the lack of relationship between pre-apheresis peripheral blood Treg values and levels collected during apheresis. There does not appear to be the same level of recruitment of CD3+ cells during apheresis as CD34+ cells and it is possible that the linear correlation between CD3+ cells collected and total blood volumes processed may not be maintained during the longer apheresis run times employed at RMH.

Since the efficiency of CD34+ cell apheresis collections can be affected by factors such as the peripheral white cell count and venous access (Verlinden *et al,* 2013) it is logical to suppose that Treg collection efficiency is also affected by other factors. Based on this data, pre-apheresis peripheral blood Treg levels cannot therefore be used to accurately predict the numbers of Tregs that would be collected. Additionally, from a practical point of view, the time required to stain and analyse Treg numbers using flow cytometry would not be a realistic proposition immediately before harvesting.

3.4.4 Treg levels in autologous and allogeneic harvests and day of collection

The time taken to achieve optimal CD34+ cell levels in the peripheral blood following administration of mobilisation regimens is based on wide-scale studies and experience within individual centres. As a regimen-induced increase in Tregs may not occur concurrently with CD34+ cells and patients and donors frequently require two and very occasionally three consecutive days of harvesting to obtain sufficient CD34+ cells, this study compared Treg levels in the harvest on different collection days.

Overall, no significant difference in Tregs was observed between harvests collected on the first and on the second day of harvesting as demonstrated in Figure 18(a). There were inadequate numbers of third day harvests for analysis but, in the two day 3 harvests tested, a small increase in Tregs was noted and this may be an area worthy of further testing. Reassuringly, the practice of extending apheresis run times to reduce the number of harvesting episodes required by patients at RMH results in very few three day collections (Wells and Smith, 2011).

Different mobilisation regimens also had no effect on the numbers of Tregs collected over different harvest days as demonstrated in Figure 18(b). In harvests mobilised by G-CSF plus cyclophosphamide it might be expected that higher Treg levels would be associated with second and third day harvests as Treg levels recover following cyclophosphamide as discussed in 3.4.1.

4.0 Results – Graft Tregs

The effect of infused graft Treg levels on clinical outcome and immune reconstitution in autologous and allogeneic patients post-transplant

4.1 Introduction

This study has noted significantly higher Treg:CD34+ cell ratios in harvests collected after mobilisation with G-CSF plus Plerixafor than in those collected following either G-CSF alone or G-CSF plus cyclophosphamide mobilisation. Patients receiving autologous transplants using harvests mobilised with G-CSF plus Plerixafor will therefore generally receive higher absolute levels of Tregs since most patients receive similar doses of CD34+ cells. It is important to assess the clinical implication of this finding in terms of disease relapse as Tregs have been reported to impair anti-tumour activity.

In allogeneic transplant recipients the Treg numbers in the graft may not only impair anti-tumour activity but may also suppress GVHD (Beres and Drobyski, 2013, Fowler, 2006, Turka and Li, 2010), therefore it is important to establish if there is an association between clinical scenarios post-transplant and Treg levels in the graft or peripheral Treg levels during immune reconstitution following transplant.

The majority of the studies examining the role of Tregs in suppressing GVM have taken place in the allogeneic setting. This study used the theories applied to the allogeneic model and tested them in the autologous setting. As GVHD and GVM appear to share some of the same immune mechanisms it is likely that higher levels of Tregs in allogeneic grafts leading to a reported decrease in the incidence of GVHD (Rezvani *et al,* 2006, Pastore *et al,* 2011, Pabst *et al,* 2007) may also lead to a decrease in anti-tumour activity. Extrapolating from this, a high number of Tregs in the grafts of autologous patients may lead to a loss of anti-tumour activity resulting in earlier disease relapse.

This study therefore incorporated an examination of Treg levels in autologous patient grafts to ascertain any relationship between levels and clinical outcome. In this patient group, outcome was measured by clinical assessment of disease status.

A number of studies have suggested that infused Tregs may provide a protective effect against T cell mediated GVHD (Wolf *et al,* 2007, Rezvani *et al,* 2006). However, because of the wide variation in donor mobilisation protocols, transplant procedures, patient cohorts and methods of Treg characterisation and enumeration in the various study groups, the results to date are difficult to interpret. This study aimed to examine Treg levels in RMH normal donor grafts for

allogeneic patients and ascertain any relationship between these levels and clinical outcome. The majority of studies to date have focussed mainly on full intensity transplants so this study examined both full intensity and RIC transplant grafts to reflect the practice at RMH. Separate analysis of the data obtained for full intensity transplant grafts was performed to determine if the results from this study concurred with those from previous studies, albeit on different cohorts of patients (Wolf *et al,* 2003, Pastore *et al,* 2011, Pabst *et al,* 2007).

Because GVHD is triggered by alloreactive donor T cells depletion of these cells from allogeneic transplant grafts can be used to reduce the incidence of GVHD, although it can result in graft failure, prolonged immunosuppression and relapse. Pastore and colleagues (2011) demonstrated that the important factor in development of GVHD is the relative proportions of Tregs and CD3 T cells in donor graft with higher proportions of Tregs appearing to suppress GVHD-inducing CD3 T cells. (Pastore *et al,* 2011). As Pastore's study included only full intensity conditioned patients this study examined CD3:Treg ratios in both full intensity and RIC transplants to determine if the relative proportions of Tregs and CD3+ cells influenced GVHD in these settings.

In addition to examining any correlation between Tregs in the graft on incidence and severity of GVHD, it is important to also consider the concurrent effect of CD34+ cell numbers infused. A number of studies have demonstrated an association between CD34 cells numbers and incidence of chronic GVHD (Mielcarek *et al,* 2002, Mohty *et al,* 2003, Zaucha *et al,* 2001) but other centres have not observed the same results (Gómez-Almaguer *et al,* 2013, Kałwak *et al,* 2010). In RMH's experience, increasing the CD34+ cell dose above a threshold of $2x10^6$/kg does not speed haematopoietic recovery, a finding which has been documented by other centres (Perez-Simon *et al,* 2003) therefore it is important to ascertain if higher CD34 doses could be detrimental in terms of increased incidence and severity of GVHD.

4.2 Data expression

4.2.1 Data expression in autologous patients

In this study, Treg numbers in autologous grafts of patients who suffered disease progression or relapse during the first year post-transplant (n=17) were compared with levels in grafts of those who achieved and sustained complete remission (n=65).

Tregs in autologous harvests measured by flow cytometry were expressed as Tregs $x10^6$/kg. Using a weight normalised dose enables a more accurate comparison of the Treg doses received by different patients.

Because of the significant differences noted with different mobilisation regimens, analysis of the data using a Treg:CD34 ratio was employed to show the relative proportions of Tregs that the patient receives in their transplant. The amount of product transplanted into the patient is based on the CD34 dose/kg with a minimum dose being $1.5x10^6$/kg. Thus the Treg:CD34 ratio indicates the number of Tregs relative to the dose of CD34+ cells.

4.2.2 Data expression in allogeneic transplant recipients

In addition to exploring the association between Tregs in the graft and GVHD, the relationship between the number of Tregs infused in the graft and clinical outcome in terms of disease relapse during the first year post-transplant was considered. In the allogeneic recipients in this study (n=53) the clinical outcome was measured not only by GVHD assessment and disease remission/relapse but additionally by donor chimerism as a low or falling donor chimerism can be indicative of graft failure or disease relapse. Specific lineage chimerism testing shows percentages of donor and recipient-derived T cells in the bone marrow and peripheral blood post-transplant at three months (n=64), six months (n=31) and one year (n=22). GVHD assessment was performed by clinicians using the Glucksberg criteria. No GVHD (n=20) and GVHD grades 1 - 4, (n=8, n=17, n=7, and n=1 respectively). Spearman rank correlation between graft Tregs and chimerism was performed.

Tregs in allogeneic harvests measured by flow cytometry were expressed as Tregs $x10^6$/kg and Treg:CD3 ratio. Data was examined for all conditioning regimens and separately for full intensity conditioning. The latter was performed in order to make a better comparison between findings at RMH and the data published from other centres which mainly focus on full intensity conditioned transplants.

4.3 Results

4.3.1 The impact of graft Treg numbers on clinical outcome in autologous patients

No significant difference was noted between Treg levels ($x10^6$/kg) in the grafts of autologous patients who showed no signs of relapse compared with those exhibiting disease progression or relapse during the first year post-transplant, 0.64 $x10^6$/kg±0.094 and 0.48 $x10^6$/kg±0.084 respectively (p=0.893) (Figure 19(a)).

When Tregs were expressed as a ratio of Tregs:CD34+ cells in the graft (Figure 19(b)) there was no significant difference noted between patients who relapsed during the first year following autologous transplant and those in remission 0.363 ± 0.103 and 0.353 ± 0.052 respectively (p=0.693).

4.3.1 The impact of graft Tregs on incidence and severity of GVHD following allogeneic transplant

Mean Treg levels measured in the donor harvests for allogeneic patients and expressed as Tregs x10^6/kg showed a consistent inverse relationship with GVHD severity during the first year following transplant. Lower Treg levels were associated with higher incidence and severity of GVHD although this difference did not reach statistical significance in numbers studied (Figure 20(a)). Mean Treg levels in the grafts of patients who did not develop GVHD during the first year were 1.86 $x10^6$/kg compared with those who developed GVHD grade 1 (1.84 $x10^6$/kg), grade 2 (1.18 $x10^6$/kg), grade 3 (0.99 $x10^6$/kg) and grade 4 (0.42 $x10^6$/kg). These data were not significantly different for the grade groupings as follows: Grades 0-1 (p=0.982), Grades 0-2 (p=0.383) and Grades 0-3 (p=0.296). Insufficient data was available for comparisons with severe GVHD grade 4.

Analysing the data from grafts of patients receiving full intensity transplants separately (Figure 20(b)) did not demonstrate a significant association between Treg levels in grafts and the incidence or severity of GVHD. The mean graft Treg levels of patients who developed no or mild GVHD was 1.16$x10^6$/kg compared with the mean of 0.75$x10^6$/kg in those who developed GVHD grades 2-3. This difference was not statistically significant (p=0.615).

When examining the relative proportions of Tregs and CD3 cells (Figure 20(c)), there was no significant difference noted between CD3:Treg ratios and the incidence or severity of GVHD (p=0.612).

4.3.2 The impact of CD34 HPC doses in the graft on the incidence and severity of GVHD

Examining Treg and CD34+ cell numbers in allogeneic grafts expressed as weight normalised units and the incidence and severity of GVHD during the first year post-transplant (Figures 21(a) and (b)) showed a significant correlation between the CD34 content of the graft and GVHD (p= 0.042). There was no significant correlation for Tregs infused in the graft (p=0.738).

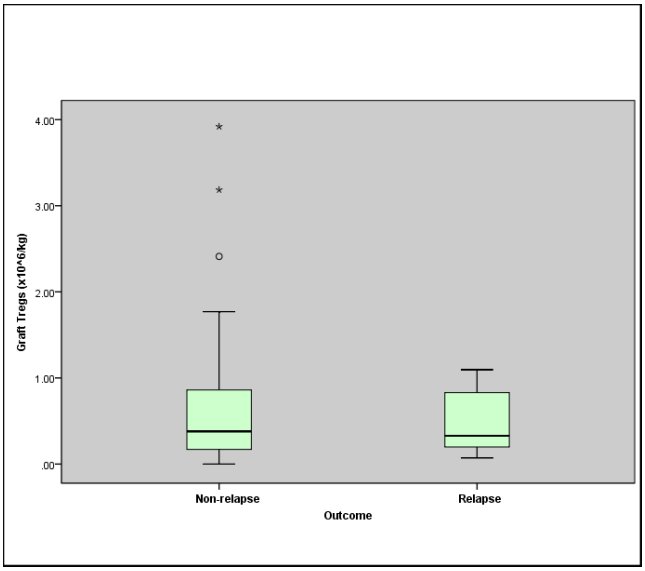
4.3.3 The impact of graft Tregs on chimerism status post allogeneic transplant

Specific lineage chimerism testing shows percentages of donor and recipient-derived T cells in the bone marrow and peripheral blood post-transplant. Treg levels in the graft were compared against percentage donor T cell chimerism in the peripheral blood of patients at three months, six months and one year post-transplant (Figures 22 (a), (b) and (c) respectively). No significant correlation was demonstrated at any of the three time points (p=0.908, p=0.397, p=0.777 respectively).

4.3.4 The effect of graft Tregs on clinical outcome in patients following allogeneic transplant

Treg/kg levels in allogeneic grafts received by patients who subsequently relapsed one year post-allogeneic transplant (mean = $0.77x10^6$/kg ±0.20) were compared with Treg/kg levels in the grafts of those patients who experienced no detectable disease relapse ($1.40x10^6$/kg ±0.31) (Figure 23). There was no significant association between Treg levels infused in the allogeneic graft and disease relapse one year following transplant. (p=0.112).

a)



b)

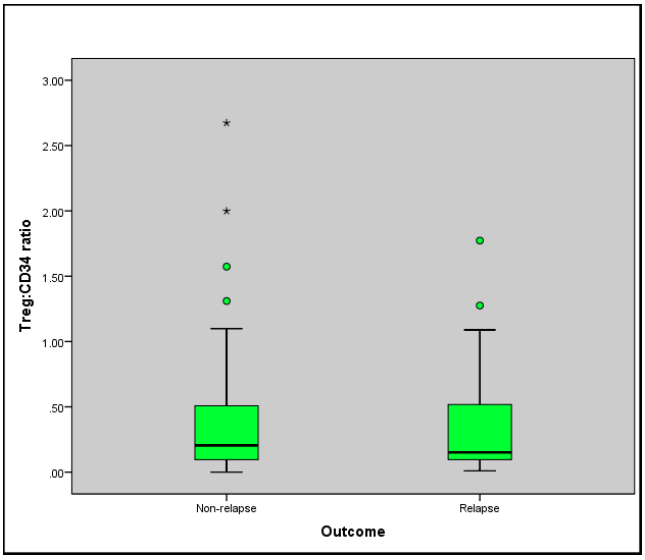
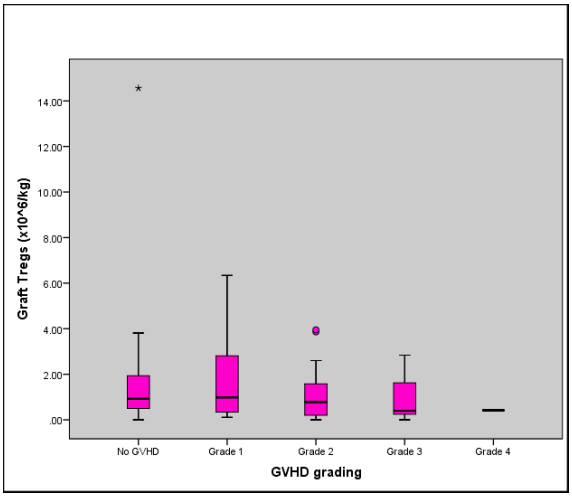


Figure 19: Levels of Tregs in autologous grafts compared with clinical outcome. (a)Tregs x10^6/kg (b) Treg:CD34+ cell ratio. Samples of autologous harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. Autologous grafts were compared in patients with disease relapse or progression (n=17) and in patients showing no disease relapse or progression (n=65) during the first year post-transplant.

a)



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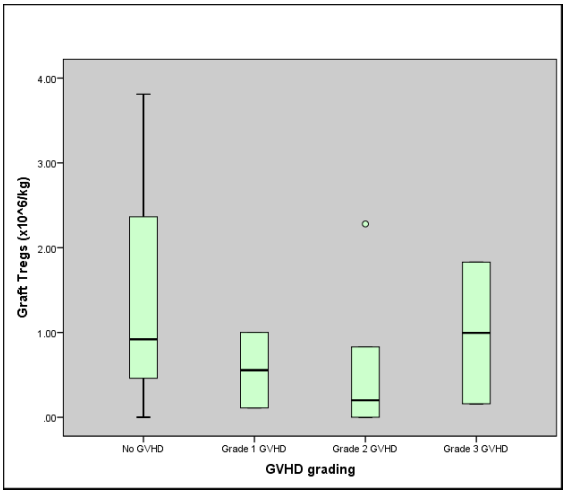

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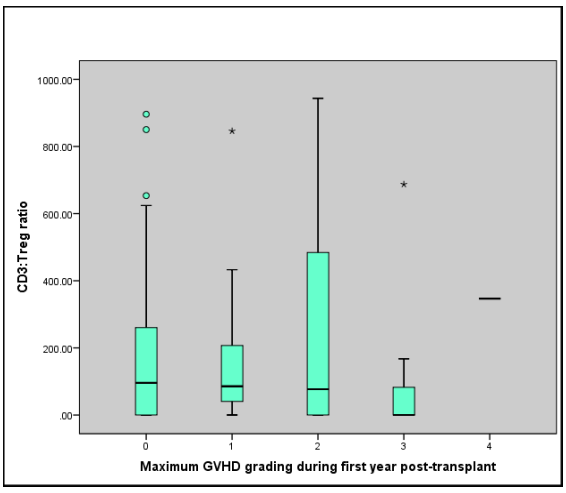
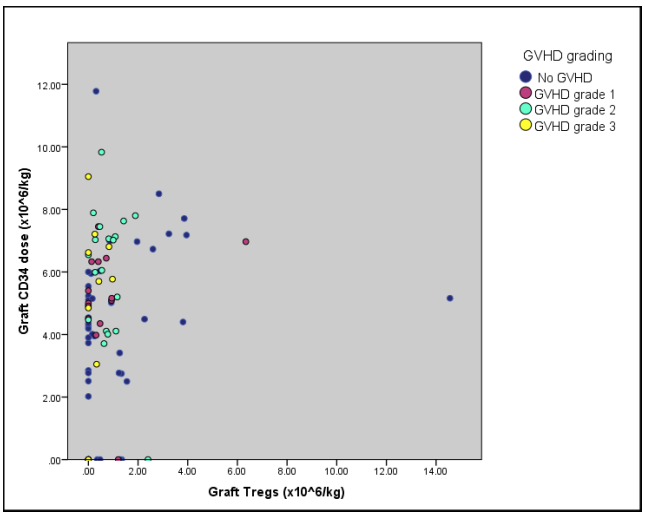


Figure 20: Treg levels in the grafts of allogeneic transplant recipients and severity of GVHD noted during the first year post-transplant. (a) Graft Treg levels x10^6/kg and GVHD grading following all conditioning regimens (b) Graft Treg levels x10^6/kg and GVHD grading following full intensity conditioning only (n=12) (c) Graft CD3: Treg ratios and GVHD following all conditioning regimens. Samples of allogeneic harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. Patients suffered no GVHD (n=20) and GVHD grades 1 - 4, (n=8, n=17, n=7, and n=1 respectively). GVHD grading is the highest grade recorded during the course of the first year post-transplant.

a)



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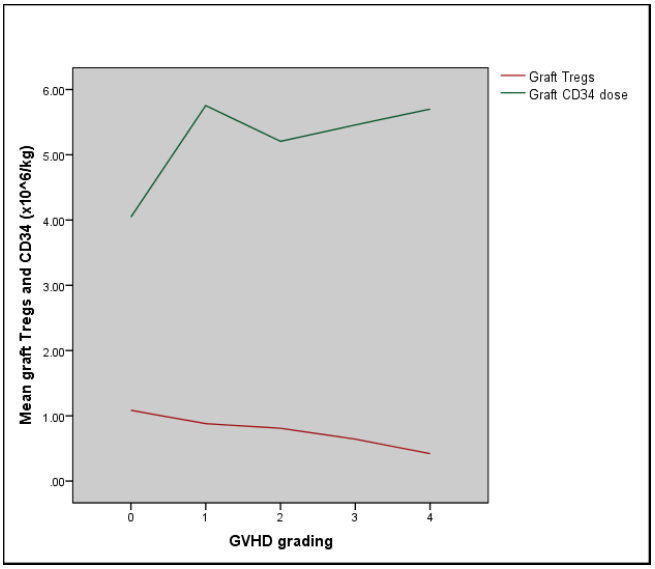
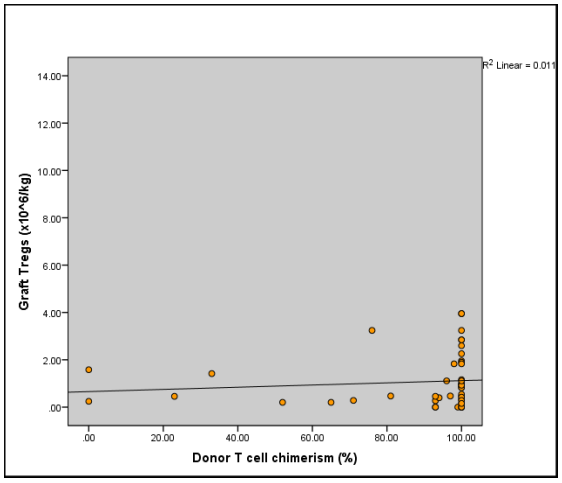


Figure 21: Graft Treg and CD34 levels compared with GVHD grading. (a) Scatter plot of Treg x10^6/kg and CD34 x10^6/kg doses of allogeneic grafts (n=53) in association with GVHD grading experienced during the first year post-transplant (b) Mean Treg x10^6/kg and CD34 x10^6/kg doses in allogeneic grafts (n=53) by maximum GVHD grading experienced during the first year post-transplant. Samples of allogeneic harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry.

a)



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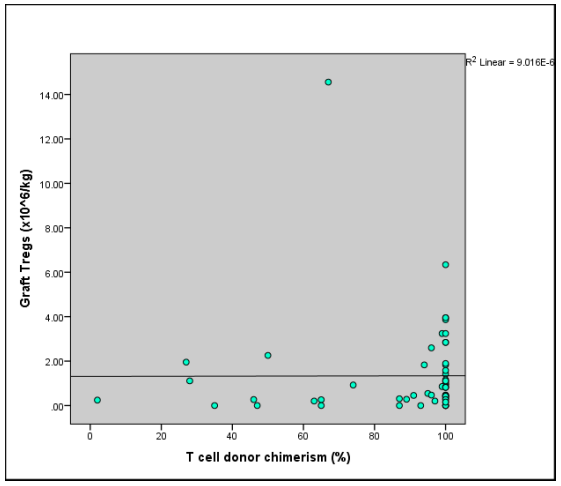

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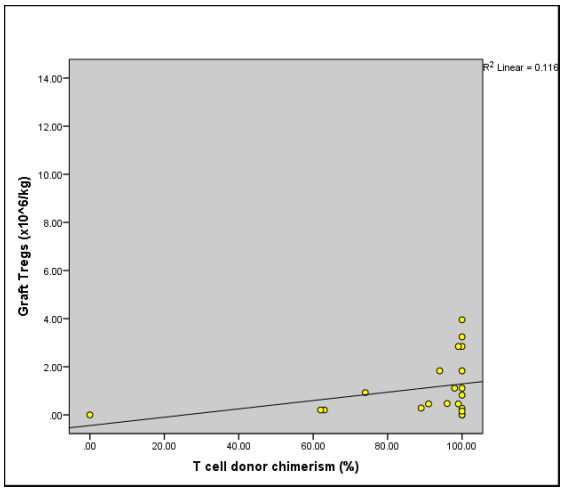


Figure 22: Comparison of Tregs in the graft and percentage donor T cell chimerism.
(a) Chimerism testing at three months (b) Six months and (c) One year post-transplant Samples of allogeneic harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Chimerism status determined by polymorphic short-tandem repeat analysis on samples taken at 3 months (n=64), six months (n=31) and one year post-transplant (n=22).

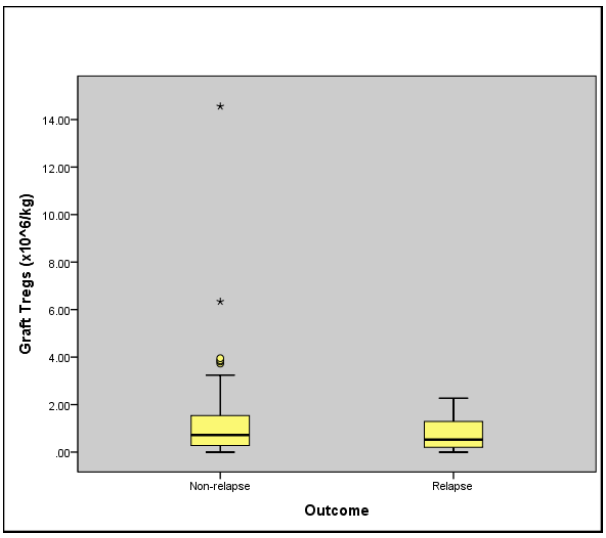


Figure 23: Levels of Tregs in allogeneic grafts and disease status. Levels of Tregs x10^6/kg in the graft are shown for patients with disease relapse or progression (n=12) and for those showing no disease relapse or progression (n=51) during the first year post-transplant. Samples of allogeneic harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

4.4 Discussion

4.4.1 The effect of graft Treg levels on clinical outcome in autologous patients

A number of studies have shown that Treg levels in grafts may not necessarily influence the extent of GVM in allogeneic transplants (Rezvani *et al,* 2006, Wolf *et al,* 2003, Pastore *et al,* 2011) but no data is available on the potential effect of Treg levels in unmanipulated autologous grafts on clinical outcome. It is important to examine this data as autologous transplantation results in different immunological dynamics to those prevalent in allogeneic transplantation. Tissue destruction and attendant immunological responses is a feature of conditioning chemotherapy in both allogeneic and autologous patients but the lack of donor cell influence in the latter group may affect clinical outcome.

As autologous transplantation is a more widely used therapeutic modality, it is important to establish whether Tregs have a negative impact on transplant success. Data from the cohort studied however, did not demonstrate any correlation between Treg levels in the graft expressed as either Tregsx10^6/kg or Treg:CD34+ cell ratios and outcome during the first year post-transplant. This is a reassuring finding as this study has demonstrated a significant increase in Treg:CD34+ levels in autologous harvests mobilised by G-CSF plus Plerixafor. It can therefore be inferred that this increase does not lead to a poorer outcome within the first year post-transplant. The study did not address relapse or disease progression occurring after this time period therefore it may be possible that graft Treg numbers could influence longer-term disease outcome. This result cannot be taken in isolation however, as the immune composition of harvests and factors affecting engraftment are complex. Any reduction in the effectiveness of treatment with Plerixafor mobilised harvests may be insignificant but as this is a relatively new agent, continued monitoring of overall survival in patients is indicated.

4.4.2 The impact of graft Tregs on incidence and severity of GVHD

Mature T cells in the donor graft can cause GVHD which remains a significant clinical complication of allogeneic transplantation. As Tregs are believed to maintain tolerance and inhibit GVHD it could be expected that patients receiving allogeneic transplants containing higher Treg numbers would experience less severe or no GVHD. In this cohort, patients with the highest levels of GVHD occurring within the first year post-transplant were noted to have received transplants with lower mean Treg values expressed as Tregs x10^6/kg, although the differences were not statistically significant with the numbers tested.

The majority of studies examining Tregs in the grafts of allogeneic transplant patients considered patients who received full intensity conditioning. The data from Pastore, Rezvani, Noel and Pabst related to patients who received full intensity allogeneic transplants (Wolf *et al,*

2007, Rezvani *et al,* 2006, Noël *et al,* 2008, Pastore *et al,* 2011). All but Noel's study demonstrated a correlation between GVHD incidence and severity and Treg levels in the graft. At RMH and many other centres, current practice involves treating the majority of patients with RIC transplants, thus this study considered the grafts of both full and RIC transplant recipients. Data was collated from RIC (n=41) and full intensity (n= 12) transplants which is representative of the relative proportions of each type of transplant performed in this and other centres. Analysis of only the RMH patients who received full intensity treatment showed no statistically significant link between Treg/kg levels in grafts and GVHD onset or severity during the first year post-transplant. However, the low numbers of patients in this group (n=12) make meaningful analysis and comparisons with data from other studies difficult.

Mielke and colleagues performed one of the few studies on patients receiving RIC transplants and observed that moderate to severe GVHD was more often seen in patients whose donors had lower levels of circulating peripheral Tregs prior to mobilisation (Mielke *et al,* 2007). However, the same study showed no relationship between Tregs in the graft or in the peripheral blood of the recipient during engraftment. It should be noted that all the grafts in Mielke's study were T-depleted *ex vivo*, unlike the grafts at RMH and the majority of centres where no manipulation takes place. Noel and colleagues also found no correlation between Treg numbers in the grafts of patients receiving RIC transplants and acute GVHD (Noël *et al,* 2008). Interestingly in the study by Wolf and colleagues, the association between grafts containing higher FoxP3 Tregs and lower cumulative incidence of GVHD was lost when the cohort was stratified based on intensity of conditioning (Wolf *et al,* 2003).

In murine and human studies it has been demonstrated that recipient Tregs are able to survive TBI conditioning and that peripheral donor Treg expansion occurs within a week of conditioning and transplant before any possible de-novo production of recipient Tregs (Matsuoka *et al,* 2010, Bayer *et al,* 2009). This expansion of recipient Tregs apparently takes place before donor-derived thymic Tregs emerge two to three weeks following transplant. Both host and donor Tregs expand following transplant, though the mechanism for this proliferation which outstrips both conventional T and B cells is not yet fully understood, but appears to be a result of CD4 lymphopoenia. Treg homeostasis differs from that of conventional T cells resulting in the skew towards higher numbers of Tregs early post-transplant. The population of host Tregs which exhibit greatest survival post conditioning are the memory Tregs (mTregs). However, following rapid expansion post-transplant, these cells undergo exhaustion (Matsuoka *et al,* 2010, Bayer *et al,* 2009). As host Treg proliferation decreases, donor-derived Treg expansion increases, initially peripherally expanded donor mTregs followed later by thymic derived de-novo generated Tregs. Thus, after about two months post-transplant the Tregs are predominantly donor, possibly as a result of competition between donor and residual host Tregs for space within the niche.

In patients receiving full intensity transplants there is rapid activation of GVHD-inducing T cell clones after conditioning, leading to early manifestation of GVHD which may in turn be controlled by relatively high Treg numbers infused. In patients receiving RIC transplants, GVHD is usually seen at later time points, so Treg levels in the graft may be of less relevance than Treg and effector T cell levels in the patient at the time when GVHD is developing. This difference in timing of onset of GVHD between full intensity and RIC transplants may explain the lack of correlation between Treg levels in grafts and GVHD incidence in the RIC transplant patients studied.

Although a number of studies have focussed on Treg levels there is increasing evidence that the absolute Treg numbers are less important than the balance between the numbers of circulating Tregs and effector T cells (Matthews *et al,* 2009, Rieger *et al,* 2006, Fujioka *et al,* 2013). Donor Tregs infused as part of the graft undergo expansion during the immediate period post-transplant and can exert a suppressive effect on donor effector T cells which mediate GVHD. Higher levels of infused Tregs may therefore confer a greater suppressive effect leading to a lower incidence of GVHD in patients receiving these grafts. In this cohort, apart from the one patient who developed grade 4 GVHD, patients with the highest graft Treg:CD3+ cell ratios showed the lowest incidence and severity of GVHD suggesting a possible association between relative numbers of Tregs to effector cells although statistically these differences did not reach significance with the numbers tested.

4.4.3 Impact of CD34+ cell numbers on incidence and severity of GVHD

Data from this study demonstrates that increasing CD34+ cell doses are significantly correlated with increased levels of GVHD. The incidence of mild cGVHD following RIC transplants is noted to improve event-free and overall survival especially in patients with AML and MDS, but the balance between GVHD and GVM is critical (Valcárcel and Martino, 2007, Martino *et al,* 2001). At RMH the approach is to limit the number of CD34+ cells transplanted unless the patient has particularly high-risk disease. With infusion of higher numbers of donor cells than this, there is a risk of disturbing the balance between GVHD and GVM in favour of GVHD due to correspondingly high numbers of effector T cells that exist concurrently with CD34+ cells in the graft. Data should be interpreted with caution however as other factors may affect outcome. An improved overall survival may enable the patient to survive long-enough to develop cGVHD, but this may not be the sole cause of the increased survival rate. Nonetheless, the study does formally support the practice of limiting CD34 doses infused in order to limit GVHD. In this centre, the maximum cut-off $CD34^{+}$ cell dose for allogeneic transplants is $8x10^{6}$/kg. This is deemed to be an acceptable level to achieve a balance between an effective anti-tumour GVM

effect and tolerable GVHD. This dose cut-off has been adopted as the result of experience rather than being based on published data as there is a lack of information in the literature. It should be noted that in many of the Treg studies in the literature, CD34+ cell doses of up to $17x10^6$/kg are given and this may result in a different pattern of GVHD incidence.

4.4.4 The effect of graft Tregs on chimerism following allogeneic transplant

As a measure of T cell chimerism, the percentages of donor T cells in recipient blood samples were analysed at 3 months, 6 months and 1 year post-transplant but no significant correlation was observed. Falling donor chimerism can be indicative of graft failure or relapse so this result is very reassuring in that it does not support a role for high levels of graft Tregs leading to subsequent disease relapse.

4.4.5 The effect of graft Tregs on disease outcome following allogeneic transplant

Relapse rate during the first year following transplant was not affected by the number of Tregs infused in this patient cohort. This observation supports the findings of Pastore and colleagues who observed that relapse rates in the patients they studied were independent of Treg numbers in the grafts they received (Pastore *et al,* 2011). Wolf and colleagues also found no correlation between graft Tregs and overall survival in RIC transplant recipients (Wolf *et al,* 2003, Pastore *et al,* 2011). They did however note a significant increased overall survival in full intensity transplant recipients who received high graft Tregs.

RIC transplantation does not induce the immediate inflammatory milieu associated with damage caused by radiotherapy and/or chemotherapy conditioning as part of full intensity transplant protocols. GVHD in these patients is also usually seen at a later stage post-transplant than following full intensity transplants. It has been demonstrated that following transplant, Tregs traffic to tumour tissue where they exert an immuno-modulatory effect on anti-tumour activity. This can be interrupted in the presence of inflammation such as that caused by full intensity conditioning or GVHD when Tregs are preferentially drawn to tissues at the site of inflammation (Dürr *et al,* 2012). Thus in RIC transplant recipients where inflammation caused by GVHD will occur at a later time point, Tregs may remain in the tumour tissue for a longer period without being trafficked to the site of GVHD induced inflammation. It could be predicted that this would result in a different pattern of response to graft Treg levels, with lower graft Treg levels exerting less of an anti-tumour effect than a graft with high Treg numbers and resulting in lower relapse rates. It is encouraging that the results of this study indicate that this is not the case.

5.0 Results - Donor lymphocytes

The effect of Treg levels on clinical outcome in allogeneic patients receiving donor lymphocyte infusions

5.1 Introduction

Following allogeneic transplant, patients are regularly monitored for both GVHD and evidence of disease relapse. Falling donor T cell chimerism can indicate the return of host derived haematopoietic cells, graft failure and subsequent relapse (Shaw *et al,* 2007). Frequently, DLI are required following RIC transplants where full donor chimerism may not be achieved in the early stages post- transplant. Falling donor chimerism is suggestive of recurrent disease and taken in conjunction with residual disease monitoring, is used to indicate the need for this treatment. It is standard practice to source directed non-mobilised DL from sibling and unrelated donors. However, in order to avoid donor recall in cases of falling chimerism or relapse, it has become accepted practice in RMH to proactively cryopreserve and store surplus G-CSF mobilised allogeneic HPC products for possible use as DLI should this be required post-transplant. This avoids the inconvenience and expense of donor recall for conventional further non-mobilised harvesting. The yield of CD3+ cells is substantially increased in G-CSF mobilised compared to non-mobilised products. It is possible that Treg numbers in DL products may affect their efficacy clinically due to suppression of donor T cell alloreactivity and therefore anti-tumour activity. In a small study performed by Hicheri and colleagues lower Treg levels in donor lymphocytes correlated significantly with favourable response (Hicheri *et al,* 2008). Because DL products are obtained in the RMH from both mobilised and non-mobilised harvests, this study investigated Treg numbers infused in donor lymphocyte products to ascertain any association with clinical outcome.

Response to DLI, either single or multiple doses, was defined as an improvement in the percentage of donor T cell chimerism or reversal of disease relapse such that no further DL infusions were required. The development of GVHD is usually observed in the presence of these clinical improvements. A minimal dose of donor lymphocytes is typically given initially and, if a response is not apparent after a period of a few months during which chimerism or disease markers are closely monitored, a slightly larger dose is then administered. Since there is a high risk of inducing GVHD with donor lymphocyte infusions, a cautious approach is usually followed with minimal increases in the CD3+ cell dose and at least a 3 month interval between doses. Failure to achieve improved donor chimerism or disease status and the absence of GVHD, usually results in a clinical decision to infuse an increased DL dose. Dosing of donor lymphocytes is stratified according to documented RMH clinical procedures, with a starting CD3+ cell dose

for falling chimerism of $5x10^6$/kg or $1x10^6$/kg for unrelated and sibling allogeneic donors respectively, and a starting dose of $1x10^7$/kg for disease relapse.

5.2 Data expression

Harvest Treg levels were measured and expressed as Tregs $x10^6$/ml and Tregs as a ratio of CD3+ cells. Doses of DLI are prepared on the basis of the harvest CD3+ cell count and the patient's weight and are expressed as CD3+ cells/kg. For this reason, Treg:CD3+ cell ratios in products were enumerated. Harvest Tregs were compared against outcome as measured by clinical response to DLI. These measures allow comparison of harvests regardless of the volume of product harvested. The same data expression was used for comparison of mobilised (n=59) and non-mobilised (n=13) harvest Tregs (Section 3). Harvests from both mobilised (n=6) and non-mobilised (n=7) donors were used for infusion in thirteen patients.

The levels in the actual doses of donor lymphocyte infusions administered (n=13) were also considered. Because doses of DLI consider the CD3+ cell count and the patient's weight, harvest Treg levels may not be a good indicator of the absolute numbers infused alongside the required CD3+ cell dose.

Doses Tregs in doses of DL administered to patients were expressed as absolute Treg numbers $x10^6$, Tregs $x10^6$/kg normalised for patient weight and as a ratio of CD3+ cells.

5.3 Results

5.3.1 The effect of Treg numbers in mobilised and non-mobilised donor lymphocyte harvests on clinical outcome

When considering the effect of G-CSF mobilisation on Tregs in DL products (section 3.3.2) no significant difference was detected between absolute Tregs/ml in mobilised harvests and in those collected without the use of a mobilising agent. However significant differences were observed between the Treg:CD3+ cell ratios in mobilised and non-mobilised harvests. It was therefore considered important to assess the effect of absolute numbers of Tregs/ml and Treg:CD3+ cell levels contained in harvests, on patient outcome following donor lymphocyte infusions as any relationship might allow the possibility of using harvested Treg levels as a predictor DLI response.

Harvests from both mobilised and non-mobilised donors were used for infusion in thirteen patients. When harvest Tregs/ml were compared in these 13 harvests against the outcome of subsequent infusions (Figure 24(a)), there was a significant correlation between lower Treg levels in the harvests and a satisfactory clinical response ($p=0.038$).

When comparing Treg:CD3 ratios in mobilised and non-mobilised harvests against response in the cohort of thirteen patients who received DLI, no significant difference was noted ($p=0.103$) (Figure 24(b)).

In section 3, Treg levels in all DL harvests were considered but in the context of this cohort of patients who actually received DLI, the levels of Tregs in G-CSF mobilised and non-mobilised harvests harvests were compared with outcome (Figure 25). In these harvests, Treg levels were significantly lower in the six harvests collected after G-CSF mobilisation (0.25 ± 0.05) than in the seven harvests collected with no mobilisation (0.73 ± 0.16) ($p=0.035$). All six G-CSF mobilised donor lymphocyte harvests elicited the desired response when doses were infused. Infusions of DL doses from non-mobilised harvests resulted in a response in five patients and no response in two patients.

5.3.2 Treg numbers infused in donor lymphocyte doses

In addition to examining Treg levels in harvests used to prepare DLI it is important to consider the actual number of Tregs in each dose administered to the patient. In this study seven patients received only one dose of donor lymphocytes, four received two doses and two three doses. All patients who received more than one dose had failed to respond to the previous dose(s). Both

the patients who failed to respond to DL treatment in this study relapsed and went on to receive chemotherapy followed by a further transplant.

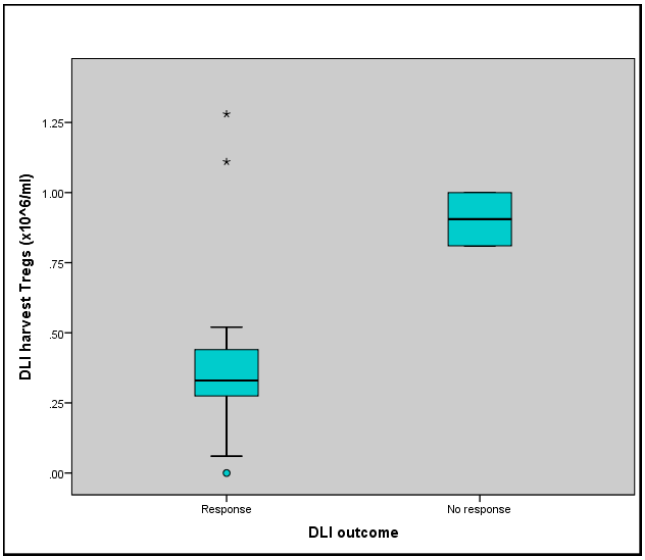
In patients receiving DLI for falling chimerism, response was defined as an adequate increase in the percentage of donor T cells requiring no further DLI or further transplant. In patients receiving DLI for persisting or relapsing disease, response was measured by morphological or disease marker remission. As it may take several months for a response to DLI to occur, the patients in this study were followed for a minimum of 6 months after the last dose of DLI was administered. Treg levels in the final dose received were used in the analysis for patients who received more than one dose of DLI.

Significantly lower absolute Treg levels ($x10^6$) were noted in the infusions given to the 11 patients who responded to DLI treatment than in the doses which elicited no response (p=0.026) (Figure 26a). When normalised for weight ($x10^6$/kg) Tregs were also significantly lower in the infusions given to patients who responded to DLI treatment than in the doses which elicited no response (p=0.30) (Figure 26b). The Treg:CD3+ cell ratio (Figure 26(c)) appeared lower in doses which produced a response in the patient (0.008 ± 0.002) than in those which did not (0.018 ± 0.004). This finding concurs with that observed with the Treg levels expressed in absolute numbers, however in this case was not statistically significant (p=0.103). In addition, CD3+ cell numbers ($x10^6$/kg) in the final doses of infused DLI were also examined against response. Higher CD3+ cell doses appeared to be associated with a failure to respond to DLI treatment however, this result did not quite achieve significance (p=0.051) (Figure 26(d)). Consideration was given to whether the number of DLI doses received by each patient affected outcome but this was not apparent (p=0.103) (Figure 27).

Since DLI infusion carries a risk of stimulating GVHD, the numbers of Tregs ($x10^6$) in DL doses were compared with the incidence and severity of GVHD experienced by patients in the six months following infusion (Figure 28(a)). No significant difference was seen between patients who suffered no GVHD (n=9), and those who experienced GVHD either grade 1 (n=2) or grade 3 (n=2) (p=0.579).

As significant differences in Treg:CD3 ratios were noted when comparing mobilised and non-mobilised harvests, the effect of this ratio in doses administered to patients was studied on incidence and severity of GVHD (Figure 28(b)). No significant difference was noted (p=0.184).

a)



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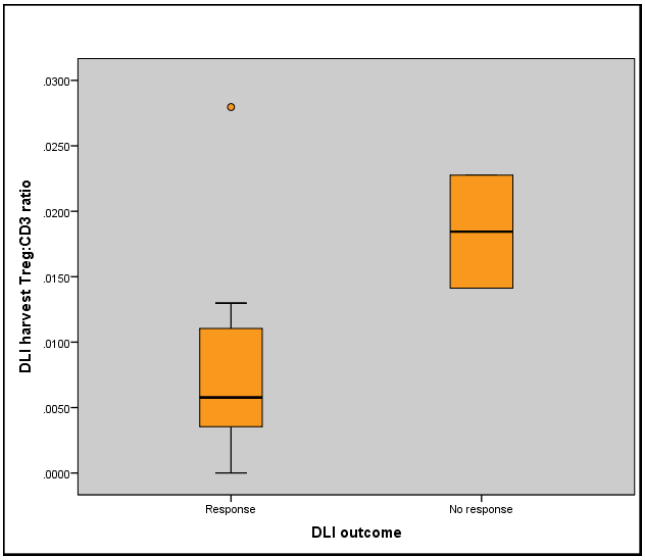


Figure 24: Treg levels in the harvests of donor lymphocyte collections compared with clinical response. (a) Harvest Tregs x10^6/ml (b) Harvest Treg:CD3 cell ratios. Treg levels in the harvests of donor lymphocyte collections were compared in patients who responded to standard dose DLI therapy (n=11)) and in those who did not respond (n=2). Samples of DLI harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. DLI response given for falling chimerism defined as an adequate increase in the percentage of donor T cells requiring no further DLI or further transplant. When DLI administered for persisting or relapsing disease, response was measured by morphological or disease marker remission.

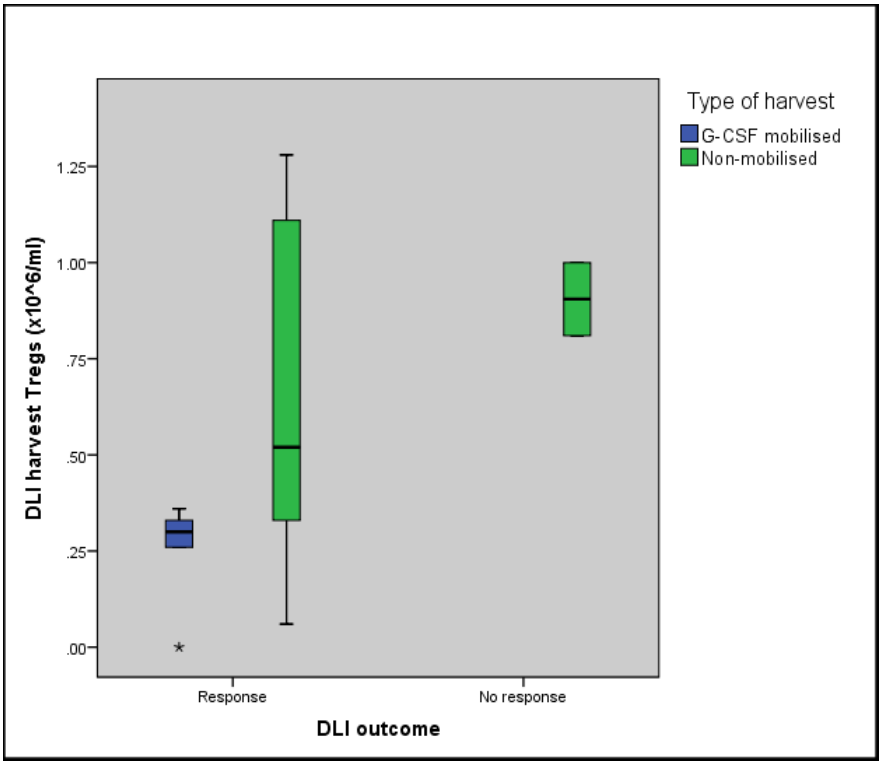


Figure 25: Treg levels in non-mobilised and G-CSF mobilised harvests of donor lymphocyte collections grouped by outcome following infusion. Treg levels in mobilised (n=6) and non-mobilised (n=7) harvests were compared against clinical response to DLI. Samples of DLI harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. DLI response given for falling chimerism defined as an adequate increase in the percentage of donor T cells requiring no further DLI or further transplant. When DLI administered for persisting or relapsing disease, response was measured by morphological or disease marker remission.

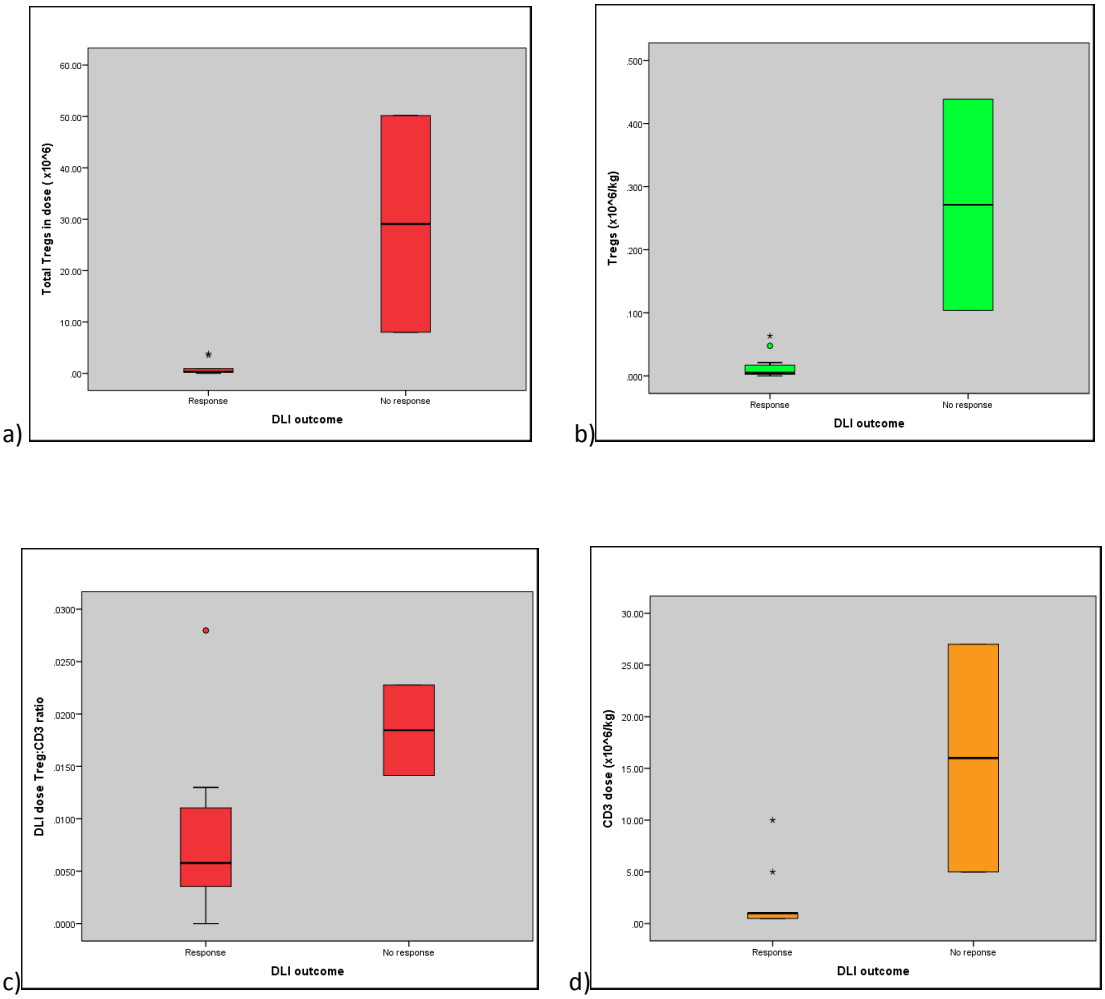


Figure 26: Tregs and CD3+ cell numbers in levels in final infused doses of donor lymphocytes compared with patient clinical outcomes: (a) DLI dose Tregs x10^6 (b) DLI dose Tregs x10^6/kg (c) DLI dose Treg:CD3 ratio (d) CD3+ cell dose x10^6/kg. Cell doses in final infusions received by patients were compared in those who responded to treatment (n=11) and those who showed no response (n=2). Samples of DLI harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. DLI response given for falling chimerism defined as an adequate increase in the percentage of donor T cells requiring no further DLI or further transplant. When DLI administered for persisting or relapsing disease, response was measured by morphological or disease marker remission.

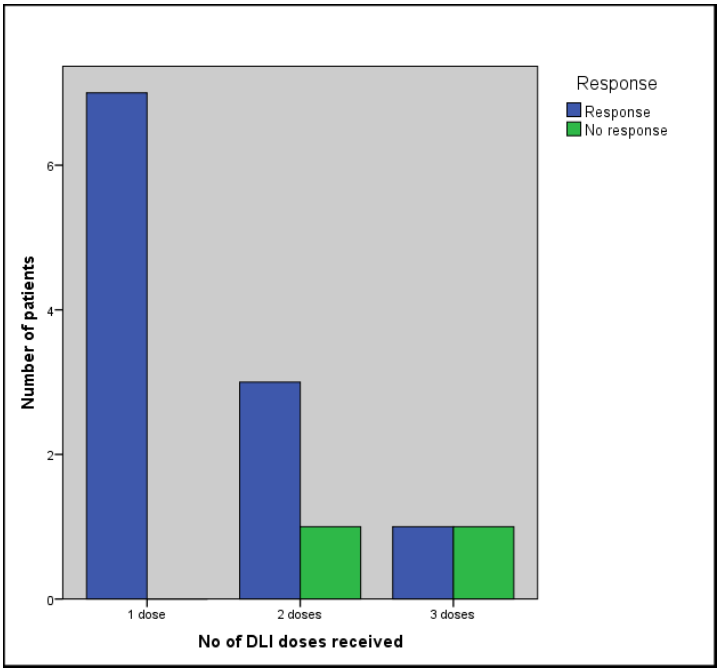
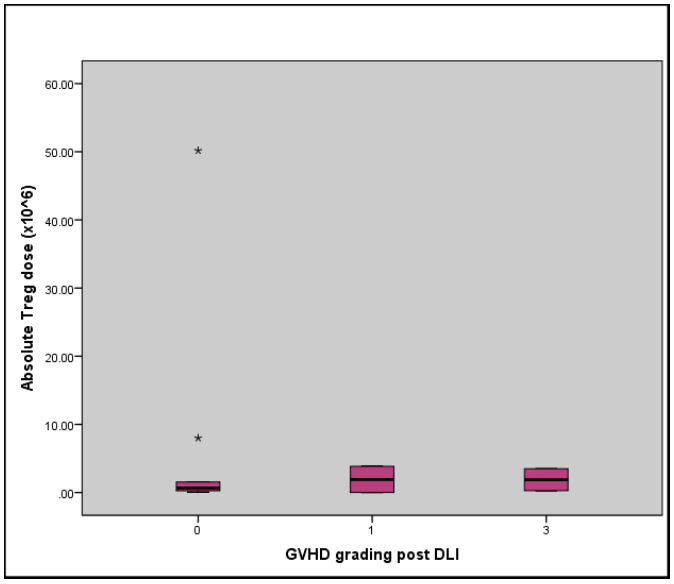


Figure 27: Number of doses of donor lymphocyte infusion received by patients and outcome. Patients received either a single dose of DLI (n=7), two DLI doses (n=4) or three DLI doses (n=2). Patients who responded to treatment (n=11) and those who showed no response (n=2). DLI response given for falling chimerism defined as an adequate increase in the percentage of donor T cells requiring no further DLI or further transplant. When DLI administered for persisting or relapsing disease, response was measured by morphological or disease marker remission.

a)



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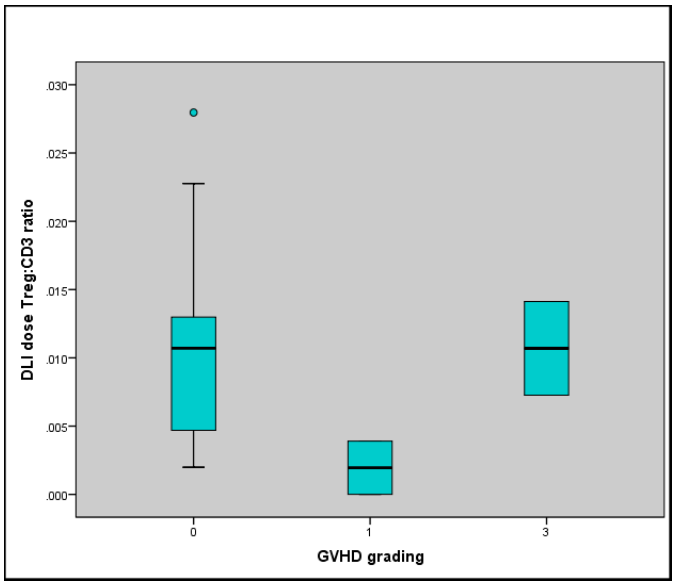


Figure 28: Treg levels in DLI doses and GVHD experienced following infusion: (a) DLI dose Tregs x10^6 against grade of GVHD experienced by patients during the six months following infusion. (b) DLI dose Treg:CD3 ratio against grade of GVHD experienced by patients during the six months following infusion. Samples of DLI harvests were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively. Patients either suffered no GVHD (n=9), grade 1 GVHD (n=2) or grade 3 GVHD (n=2). No patients experienced grade 2 GVHD.

5.4 Discussion

The majority of allogeneic transplants performed at RMH are RIC transplants whose success relies to a large extent on tumour eradication through a GVM effect rather than on myeloablation from the conditioning regimen. Undesirable effects of this treatment are a prolonged period of mixed donor and recipient chimersim, and possible graft failure or relapse. In this context, donor lymphocyte infusions are used to break tolerance and stimulate a GVM reaction driven by an alloreactive immune response. In the DLI setting the environment that the cells are infused into is not the same as at the time of the initial transplant, when the conditoning regime will have induced a neutropenic state in which there is rapid expansion of T cells including Tregs in the immediate post-transplant stage. Thus Tregs in harvests used as initial transplant grafts and harvests used for subsequent DLI should be examined separately.

Enumerating the CD3+ cells present in mobilised or non-mobilised harvests from the original donor enables multiple dose-escalating aliquots to be cryopreserved. This allows the smallest possible CD3+ cell dose to achieve either disease remission or an improvement in donor chimerism to be given to the patient, while minimising the likelihood of inducing severe GVHD. Thus the ultimate goal of DLI is to tip the balance towards GVM rather than GVHD.

Much attention has been focused on the contribution that Tregs, either in the graft or in the peripheral blood during reconstitution, may make towards prevention of GVHD following transplant. Since DLI are infused into a radically different environment from the initial transplanted cells, it is important to ascertain if Treg levels in the product affects the clinical outcome in patients receiving these infusions.

This study has demonstrated that Tregs in G-CSF mobilised products are lower than those in non-mobilised harvests. In the wider mobilisation data where 59 mobilised harvests were compared with 10 non-mobilised harvests (section 3.3.2) the difference was significant when Treg levels were expressed as Treg:CD3+ cell ratios ($p=0.022$), but not when expressed as Tregs/ml ($p=0.162$) . In the smaller cohort of harvests which were actually used for DL infusions (Figure 24), interestingly, the converse was observed with a significant difference noted between Tregs/ml in mobilised and non-mobilised products ($p=0.038$) but not between Treg:CD3 ratios ($p=0.103$). The rationale for this difference based on the data expression in the groups is unclear.

Having considered Treg levels in the mobilised and non-mobilised harvests, it was necessary to ascertain if any differences in the products, attributable to the use of G-CSF or not, prior to collection, would have an impact on clinical outcome. This was particularly important given the

RMH programme of proactive cryopreservation of surplus G-CSF mobilised allogeneic apheresis product to be used for subsequent DLI treatments. Patient responses in terms of restoration of donor chimersim or resolution of relapse were monitored in association with the absolute numbers of Tregs infused alongside the CD3+ cell doses. It was apparent that lower Treg numbers in the dose correlated significantly with a satisfactory clinical response to DLI (Figure 24), but there was no significant difference between the use of mobilised or unmobilised products and outcome (Figure 25). Since the aim of the infusion is to promote an alloreactive immune response it is possible that lower Treg numbers facilitate this response. In the immediate post-transplant setting inflammatory cytokines are released which activate alloreactive donor T cells and potentiate GVHD. In this scenario higher Treg levels have been reported to be advantageous in suppressing GVHD. There is clearly an optimal balance between GVM and GVHD but in the DLI situation, in the absence of GVHD driven by inflammatory mechanisms, Tregs may be expected to suppress the desired GVM effect. This would be a valid explanation for the correlation between lower Tregs in DL infusions and better clinical outcomes observed in this study.

The CD3+ cell content of DLs infused would be expected to correlate with outcome as the CD3+ dose determines alloreactivity. Indeed, concerns about inducing severe GVHD neccesitate the infusion of small CD3 doses initially that can be escalated if no GVHD and clinical improvement in chimersim or disease status ensues. Surprisingly, this study showed that CD3 dose was not significantly associated with successful DLI outcome defined by increased chimerism or disease remission in the cohert studied. It is apparent clinically that the majority of patients benefit at least transiently from DLI with many experiencing sustained improvement. Similarly, CD3:Treg ratios in infused doses also showed no significant association with successful DLI outcome. When comparing harvests, the proportion of Tregs to CD3+ cells is useful as it indicates the relative numbers of Tregs collected alongside alloreactive donor T cells. However when considering Treg levels in the infused doses of DLI it appears that the best indicator of the successful outcome of the infusion is the measure of Tregs/kg.

It has been reported that the relative proportions of Tregs and conventional T cells can determine immunologic activity after myeloablative transplant (Dieckmann *et al,* 2001), with a diminished ratio of Tregs to conventional T cells resulting in higher levels of GVHD post-transplant. It would therefore be expected that a lower proportion of Tregs to CD3+ cells post-DLI might result in a more potent GVM effect but this study has not found this to be the case. Differences in the immune status of the host may explain this result. At the time of initial transplant cells are infused into a neutropoenic recipient with significant inflammatory changes caused by the conditioning therapy. At the time when DLI are infused the patient is usually no

longer neutropoenic and inflammatory processes have settled. In this setting therefore, there may not be the rapid expansion of Tregs which is seen following transplant, and since a response to DLI may take several weeks or months it may not be the immediate reaction following infusion which causes the response. However it does appear that the overall Treg numbers in the DL doses are capable influencing the donor T cell mediated GVM effect in some way. Indeed, in a studies by both Miller and Guillaume and their colleagues, donor lymphocyte infusion following lymphodepletion was demonstrated to result in higher levels of GVHD attributable to T cell expansion following DLI with increased immune activation (Miller *et al,* 2007, Guillaume *et al,* 2012).

There was no demonstrable correlation between the Treg levels in infused donor lymphocyte doses and the incidence or severity of GVHD experienced in the six months following infusion in this study. This is surprising as a response to DLI is often accompanied by GVHD. It might have been expected that lower Treg levels in donor lymphocytes would lead to a higher incidence or severity of GVHD, but since this has not been confirmed it supports the idea that donor lymphocyte doses containing lower Treg levels are a superior product in terms of overall outcome.

In the total mobilised and non-mobilised cohorts considered in this study, Treg:CD3+ cell ratios were significantly lower in the former group. In those products that were actually infused, absolute Tregs were significantly lower in the mobilised harvests. Overall therefore, lower Treg levels were apparent in G-CSF mobilised rather than non-mobilised harvests which is reassuring since increasingly, products to be used as DLI are prepared from mobilised products at RMH. The prime motive for limiting doses of allogeneic products infused at intial transplant and proactively storing surplus material is to avoid donor recall and to enable 'off-the-shelf' product to be available for DLI use on demand. The lower Treg levels in these mobilised products potentially brings an additional benefit to the patient in terms of better outcome based on lack of GVM inhibition. It must however be borne in mind that the use of mobilised harvests in themselves is not an exclusive indicator of success of DLI, but their use may predispose to lower Treg levels and therefore improved outcome compared with non-mobilised products.

6.0 Results – Tregs during immune reconstitution

The influence of Treg numbers during immune reconstitution on clinical outcome in autologous and allogeneic transplant recipients.

6.1 Introduction

In addition to investigating Treg numbers in the grafts of transplant recipients, this study also measured Treg levels in the peripheral blood during immune reconstitution following transplant to identify any link with clinical outcome. Studies examining peripheral Treg numbers following transplant are hindered by transplant centre variation in conditioning regimens, patient cohorts, the method of Treg enumeration and, in allogeneic transplants, the immunosuppressive drugs used. A link between Treg levels and clinical outcome could provide a useful marker, either to predict which allograft recipients may be likely to develop GVHD or to identify those patients who may be at risk from disease relapse following autologous or allogeneic transplant. Any test used in this way would need to be reliable, reproducible and cost-effective.

6.1.1 Peripheral Tregs during immune reconstitution following autologous transplantation

This study examined peripheral Treg cell recovery following autologous transplant. Autologous transplantation involves the infusion of HPC,A collections including lymphocytes that originate from the patient. In many of these patients prior to transplant the tumour is able to evade immune surveillance, so it would be expected that in autologous, unlike allogeneic transplantation, no anti-tumour activity would result following the transplant of the patient's own cells. Immune reconstitution may be skewed or influenced by effect of mobilisation regimen on the graft or by the pattern of haematopoietic regeneration. It is therefore possible that Tregs present during this period are capable of suppressing anti-tumour activity and that an alteration in the balance of Tregs and anti-tumour effector cells may affect clinical outcome. Over and above chemotherapy-induced tumour reduction, Mirmonsef and colleagues have reported that tumour specific effector cells outcompeting Tregs during immune reconstitution can lead to a reversal of tumour tolerance following transplant in mice (Mirmonsef *et al,* 2008).

In order to build up a picture of the recovery of Tregs following autologous transplantation, overall levels were measured in the peripheral blood over the first year following transplant. Furthermore, any relationship between Tregs and outcome in terms of remission or disease relapse was assessed at one, three, six, nine and 12 months post-transplant. Absolute Treg levels and Tregs relative to numbers of CD3+ and CD4+ cells were evaluated.

6.1.2 The effect of peripheral Treg levels on clinical outcome and immune reconstitution following allogeneic transplant

Studies comparing peripheral Tregs with the incidence or severity of GVHD following allogeneic transplant have mainly focussed on patients who have received full intensity conditioning prior to transplant. Findings have been inconclusive with some researchers reporting a link between graft Tregs and GVHD, whereas others have reported a link with peripheral Tregs following transplant. An interesting question, therefore, is whether there is a demonstrable link between the number of Tregs infused in the graft and those found in the peripheral blood post-transplant.

In order to build up a picture of the recovery of Tregs following allogeneic transplantation, overall Treg levels were measured in the peripheral blood during the first year following transplant. The effect of Tregs levels on outcome in terms of GVHD, chimerism and disease relapse throughout that time period was examined.

6.2 Data expression

6.2.1 Data expression of Tregs in peripheral blood following autologous transplant

A Spearman rank correlation of graft absolute Treg numbers ($x10^6$) and peripheral blood Tregs at one month post-transplant was performed. Peripheral blood Tregs measured by flow cytometry were expressed as Tregs $x10^6$/ml and compared against outcome as measured by remission or disease relapse. Peripheral Treg:CD3+ cell ratios and Tregs as a percentage of CD4+ cells were also compared against outcome.

Longitudinal testing of samples (n=180) from autologus transplant recipients (n=85) was performed over the first year post-transplant.

6.2.2 Data expression of Tregs in peripheral blood following allogeneic transplant

A Spearman rank correlation of graft absolute Treg numbers ($x10^6$) and peripheral blood Tregs at three months post-transplant was performed. There were insufficient Treg numbers before this time point to be able to perform the correlation at an earlier time point. Peripheral blood Treg levels measured throughout the first year post-transplant were expressed as Tregs $x10^6$/ml, Tregs as a percentage of CD4+ cells and Treg:CD3+ cell ratios. Peripheral blood Tregs were compared against incidence and severity of GVHD, incidence of disease relapse and donor chimerism status during the first year post-transplant.

Longitudinal testing of samples (n=197) from allogeneic transplant recipients (n=75) was performed over the first year post-transplant.

6.3 Results

6.3.1 Tregs in peripheral blood following autologous transplants

In order to determine the potential effect of the varying Treg levels collected in harvests following different mobilisation regimens on immune reconstitution, graft Tregs levels were compared with peripheral Tregs measured at one month following autologous transplant (Figure 29(a)). No correlation was observed (ρ=0.291, p=0.189). When considering reconstitution of Tregs following autologous transplant, Treg levels were readily detectable at 28 days (Figure 29(b)) and although mean Treg levels dropped slightly at six months post-transplant before rising again, there was no significant difference in mean Treg levels throughout the first year (p=0.243).

When considering Treg levels at various time points throughout the first year post-transplant compared with outcome, the most notable difference seen as an elevation in levels, was observed at one month (Figure 30(a)). Overall, variations across the time points in those who relapsed and those who did not appeared to follow similar patterns. Patients who experienced relapse during the first year following transplant had higher Treg levels than those who remained in remission although this difference was not statistically significant (p=0.166).
Examining Treg:CD3+ cell ratios at time points throughout the first year post-transplant and outcome, the most notable difference in levels was noted at one month post-transplant (Figure 30(b)). Patients who suffered relapse during the first year following transplant appeared to show higher proportion of Tregs than those who remained in remission although this difference did not reach statistical significance (p=0.356).
Comparison of Treg percentages of CD4+ cells in all autologous patients, regardless of clinical outcome, at monthly time points throughout the first year post transplant, demonstrated significant differences (p=0.001) with highest levels at one month (Figure 31(a)).
The data was then considered in the relapse and non-relapse groups and Treg percentages were higher in the former cohort one month post-transplant although this did not reach significance (p=0.316) (Figure 31(b)).

6.3.2 Tregs in peripheral blood following allogeneic transplants

Tregs ($x10^6$) in the original allogeneic graft were compared with Treg levels ($x10^6$/ml) at 3 months post-transplant (Figure 32(a)). No correlation was observed (ρ=0.069, p=0.729). Unlike peripheral blood Tregs following autologous transplants, Treg levels ($x10^6$/ml) measured during the year following allogeneic transplant were not readily detectable in most patients until three months. Levels increased slowly until eight months after which they fell again (Figure 32(b)).

Despite this there was no significant difference in mean Treg levels throughout the first year (p=0.243).

When considered as a percentage of CD4+ cells (Figure 33(a)) Tregs increased up to six months and then dropped again. Unlike in autologous transplants (Section6.3.1), the overall CD4+ cell count following allogeneic transplant did not remain stable from one month but showed significant differences throughout the year (p=0.021) (Figure 33(b)).

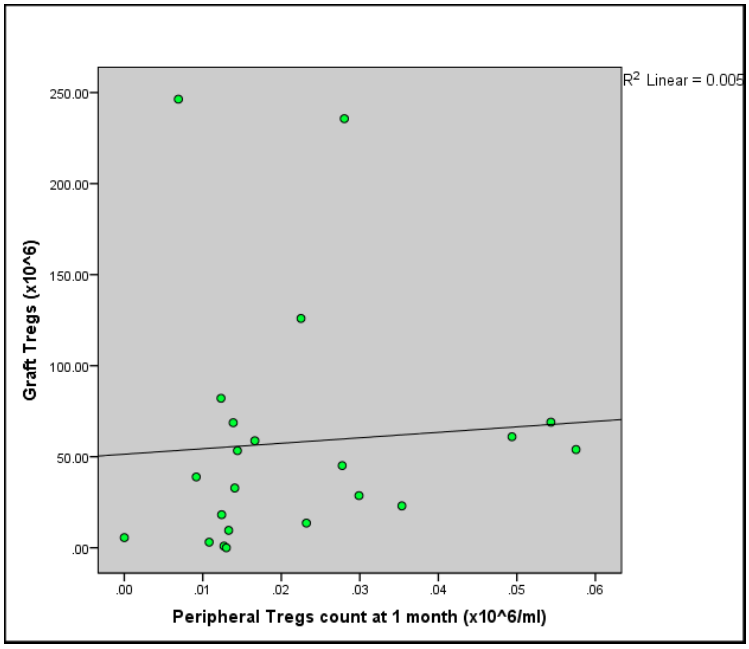
Recovery of Tregs relative to CD3+ cells showed significant differences throughout the first year (p=0.017) with the proportion of Tregs highest at six and nine months (Figure 34).

Incidence and severity of GVHD experienced by patients during the first year following allogeneic transplant was evaluated relative to Treg levels ($x10^6$/ml), as a percentage of CD4 and as a ratio of CD3+ cells (Figures 35 (a), (b) and (c). There is a lack of association between peripheral Tregs/ml and GVHD (p= 0.109) (Figure 35(a)). However, when considering mean Tregs as a percentage of CD4+ cells and GVHD grade by month it appears that a higher percentage of Tregs may offer some protection against GVHD but this lacks significance (p=0.451) (Figure 35(b)). The ratio of Tregs to CD3+ cells also appears higher up to three months post-transplant in patients with no GVHD (Figure 35(c)). However, from six months onwards this trend is lost with overall no significant difference between ratios and GVHD (p=0.445).

Peripheral Treg levels expressed as Tregs $x10^6$/ml were evaluated in patients in the first year post transplant to determine any correlation with clinical outcome in terms of the incidence of relapse (Figure 36). In both patient groups, Treg levels followed a similar oscillating pattern over the twelve month period, however in the relapse group, Treg levels appeared higher in the first six months. Conversely, after six months Treg levels in these patients were lower than in than in patients who relapsed. These differences between levels did not achieve significance (p=0.988). Mean time to relapse was six months (range two to twelve months).

When peripheral Treg levels were considered against donor T cell chimerism status in allogeneic recipients post-transplant at three, six and 12 month intervals, no significant differences were noted (p=0.720) (Figure 37 (a), (b) and (c)).

a)



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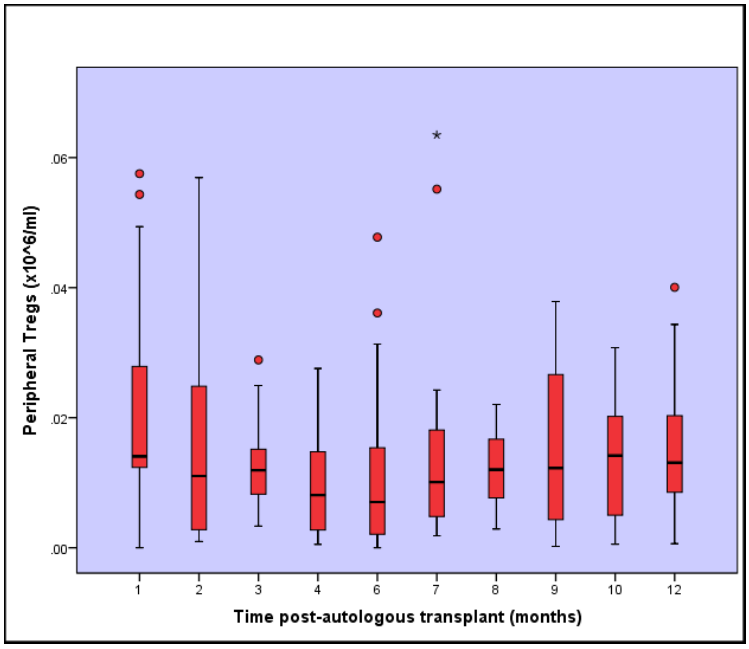
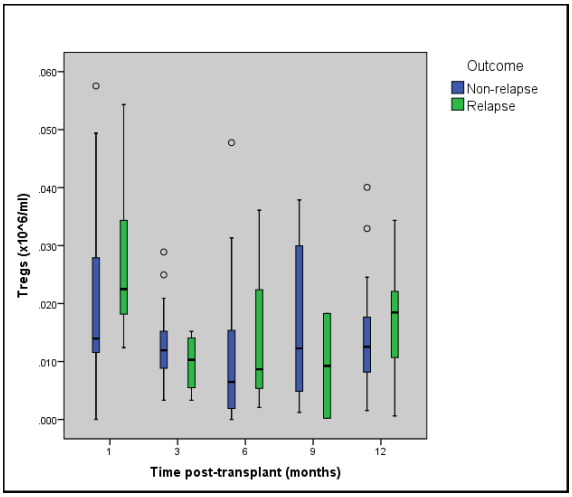


Figure 29: Treg levels in grafts and peripheral blood following autologous transplant. (a) Correlation between graft and peripheral Tregs at one month in patients following autologous transplant (n=22). Grafts collected after mobilisation with G-CSF (n=1), G-CSF and cyclophosphamide (n=18), and G-CSF and Plerixafor (n=3). (b) Treg levels in peripheral blood at time points throughout the first year post autologous transplant. Treg levels expressed as Tregs x10^6/ml. Longitudinal testing performed on 180 samples from 85 patients. Samples of autologous harvests and peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

a)



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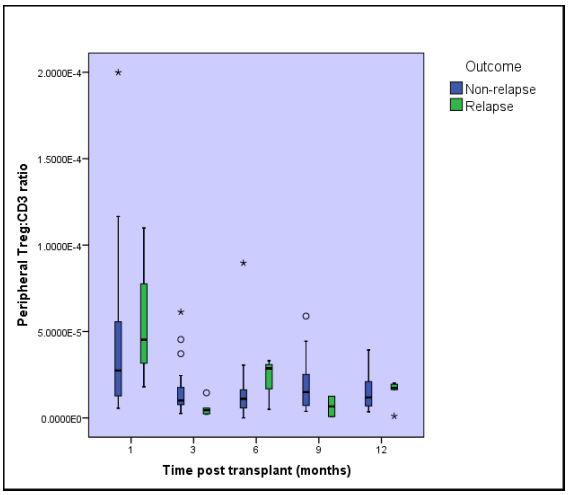

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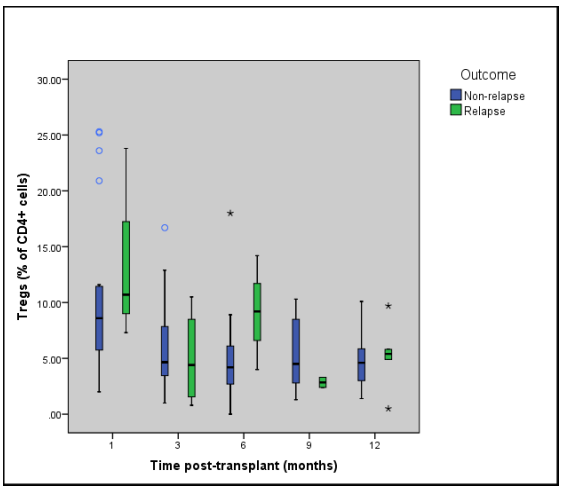
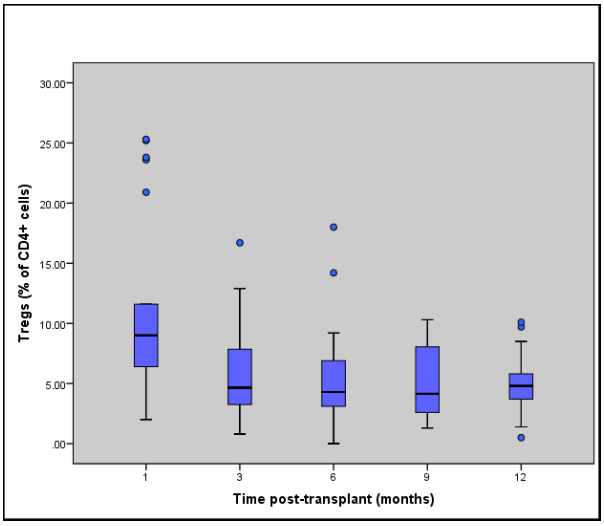


Figure 30: Treg levels in peripheral blood compared with clinical outcome following autologous transplant. (a) Tregs/ml at monthly time points (b) Treg:CD3+ cell ratios. Peripheral blood samples taken at monthly time points: 1 (n=22), 3 (n=28), 6 (n=27), 9 months (n=16) and 1 year (n=21) post-transplant in patients who relapsed (n=19) and those who did not (n=66). Samples of peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

a)



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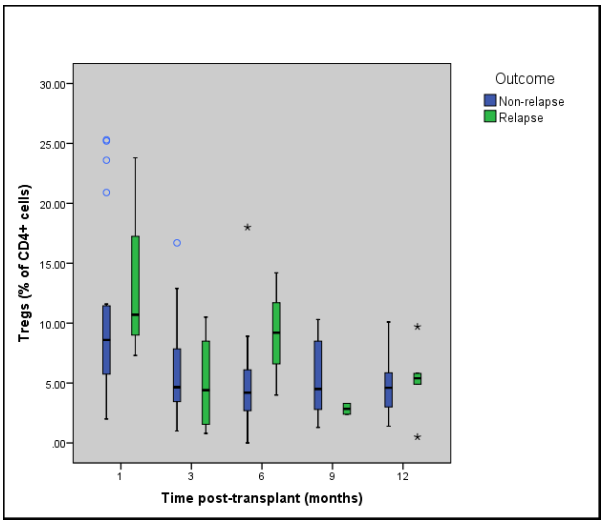
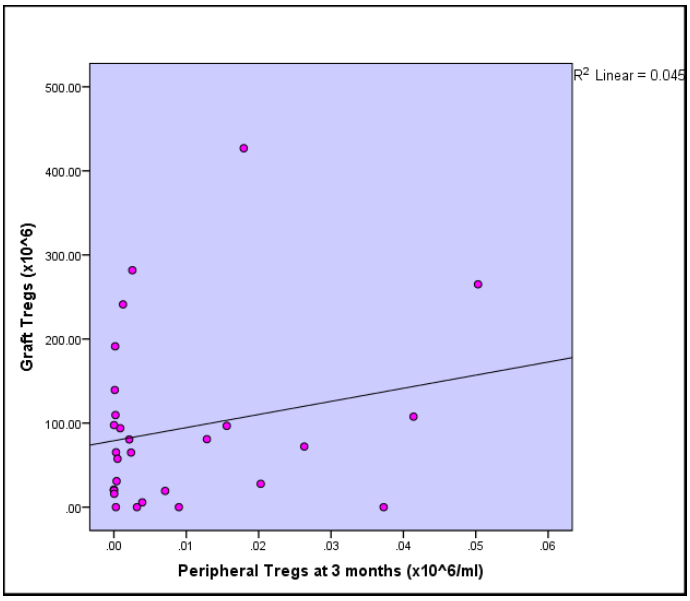


Figure 31: Peripheral Tregs as a percentage of CD4 cells following autologous transplant.
(a)Tregs as percentage of CD4+ cells – all patients (b) Peripheral Tregs as a percentage of CD4 cells by outcome (patients who relapsed (n=19) and those who did not (n=66)). Peripheral blood samples taken at monthly time points: 1 (n=22), 3 (n=28), 6 (n=27), 9 months (n=16) and 1 year (n=21) post-transplant in patients were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

a)



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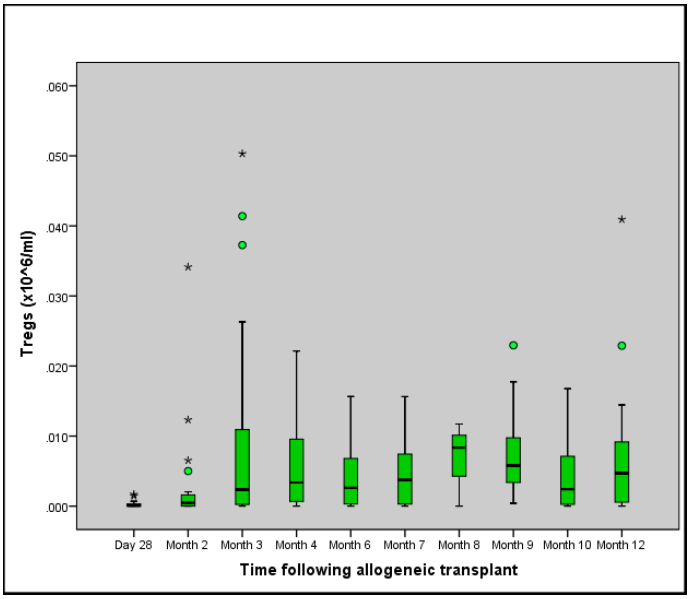
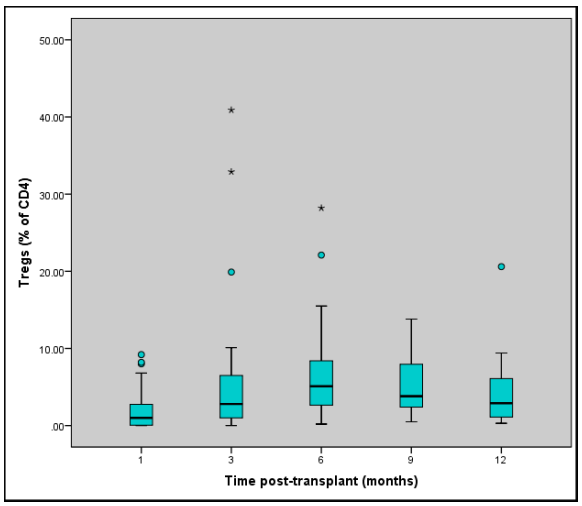


Figure 32: Treg levels in grafts and peripheral blood following allogeneic transplant.
(a) Correlation between graft and peripheral Tregs at three months in patients following allogeneic transplant (n=28). All grafts collected after mobilisation with G-CSF (b) Treg levels in peripheral blood at time points throughout the first year post allogeneic transplant. Treg levels expressed as Tregs $x10^6$/ml. Longitudinal testing performed on 197 samples from 75 patients. Samples of allogeneic harvests and peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

a)



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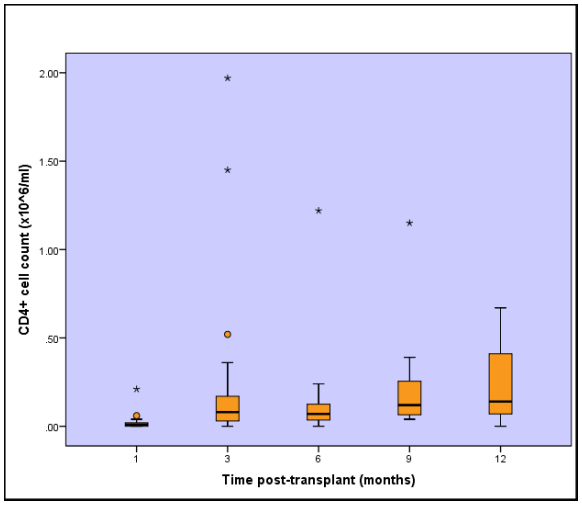


Figure 33: Treg levels and CD4+ cell counts following allogeneic transplant. a) Longitudinal testing of Tregs as a percentage of CD4+ cells in peripheral blood at time points month 1 (n=20), month 3 (n=31), month 6 (n=23), month 9 (n=11), and 1 year (n=18) post-transplant intervals. b) CD4+ counts at the same time points following allogeneic transplant. Samples of peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

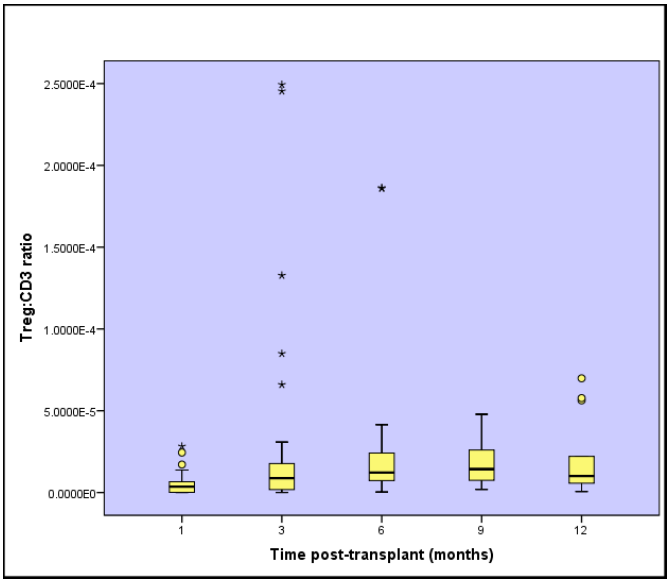
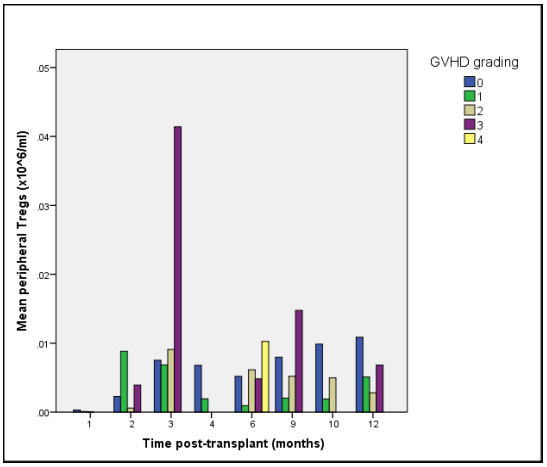


Figure 34: Treg:CD3+ cell ratios following allogeneic transplant. Longitudinal testing of peripheral blood at time points month 1 (n=20), month 3 (n=31), month 6 (n=23), month 9 (n=11), and 1 year (n=18) post-transplant. Samples of peripheral blood (n=103) were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Data represents the median (black line) and interquartile range (whiskers). Mild and extreme outliers are depicted by the symbols ° and * respectively.

a)



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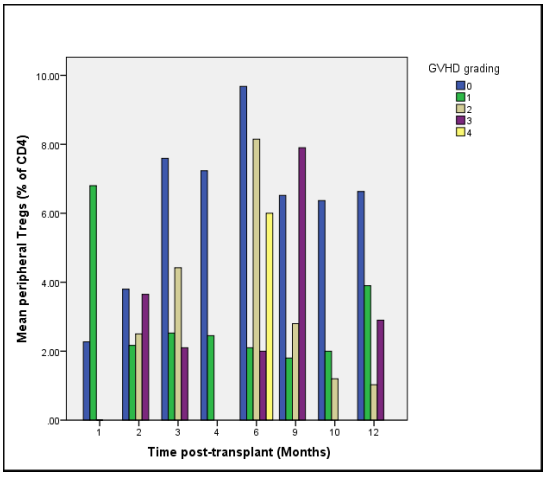

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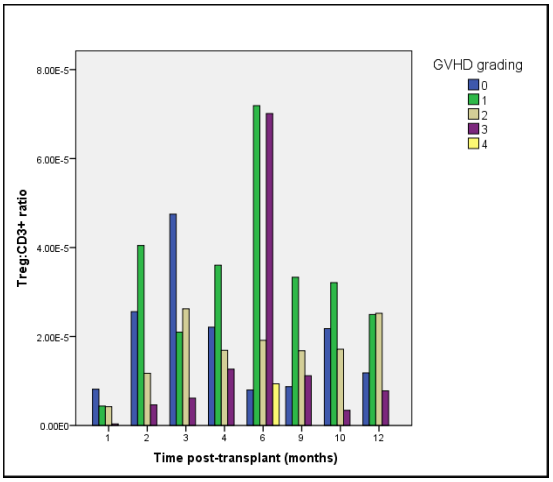


Figure 35: Mean peripheral Treg levels and GVHD grading during the first year post allogeneic transplant. a) Mean Tregs x10^6/ml b) Mean Tregs as a percentage of CD4+ cells c) Tregs:CD3+ cell ratios. Patients who did not experience GVHD over the year (n=77), and patients who suffered GVHD grade 1 (n=20), grade 2 (n=23), grade3 (n=9) and grade 4(n=1). Samples of peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry.

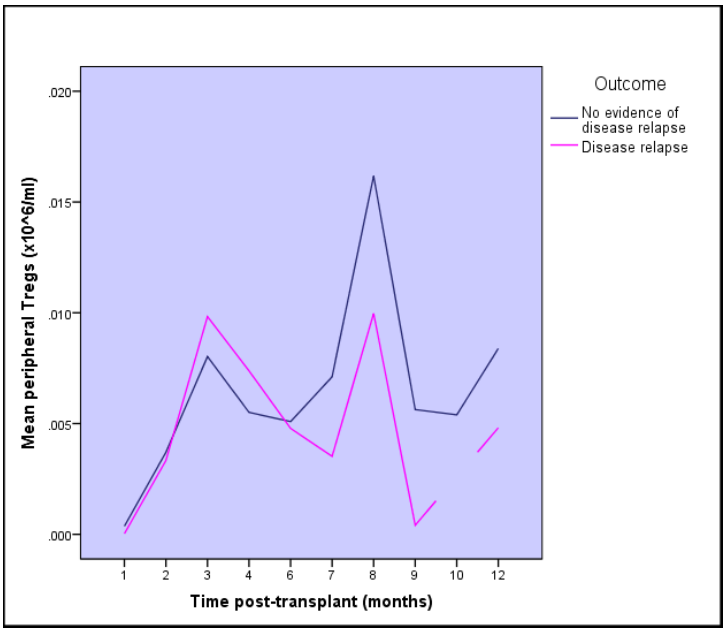
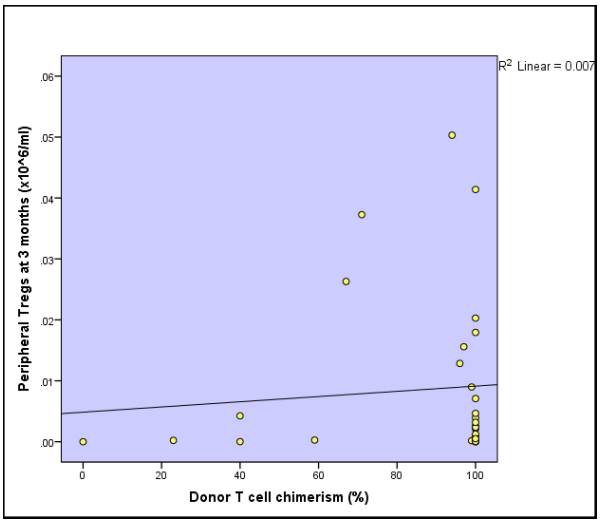


Figure 36: Peripheral Treg levels and clinical outcome during the first year post-transplant: Mean Tregs x10^6/ml in the peripheral blood of patients who suffered disease relapse (n=15) during the first year post-transplant compared levels in patients with no evidence of relapse (n=61). Samples of peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry.

a)



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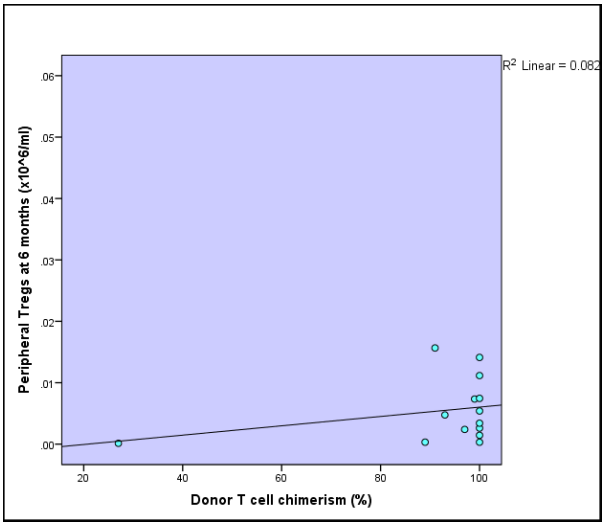

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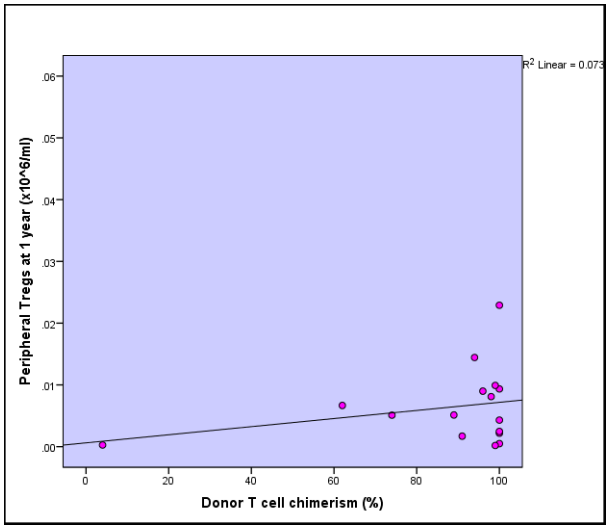


Figure 37: Peripheral blood Treg levels and percentage of donor T cell chimerism: a) 3 months, b) 6 months and c) 1 year post allogeneic transplant. Samples of peripheral blood were stained for CD3 CD4 CD25 CD127 and FoxP3 and analysed by flow cytometry. Chimerism status determined by polymorphic short-tandem repeat analysis on samples taken at 3 months (n=24), six months (n=14) and one year post-transplant (n=16).

6.4 Discussion

6.4.1 Correlation between graft and peripheral Tregs following autologous transplant

This study reported higher Treg levels harvested after mobilisation with Plerixafor when used in combination with G-CSF compared to mobilisation with either G-CSF alone or G-CSF in combination with cyclophosphamide. Because of this it was deemed important to ascertain if Treg levels in grafts harvested following these mobilisation regimens bore any relationship to peripheral Treg levels in the corresponding patients following autologous transplant. Since there were too few peripheral samples at one month for each of the mobilisation regimens, all regimens were considered together. No significant correlation was demonstrated in this study.

Immediately following autologous transplantation using PBSC, levels of CD3, CD8 and NK cells have been found to return to normal while CD4 levels fall below normal and may remain low for up to a year (Guillaume, Rubinstein and Symann, 1998). In the immediate post-transplant phase the tumour burden is minimal as it has been reduced by the high dose chemotherapy. Differing rates of reconstitution of lymphocyte subsets following transplant results in skewed proportions of the various cell types which may encourage anti-tumour activity, however, rapid expansion of Treg numbers may have the potential to suppress this. Using a syngeneic murine transplant model Mirmonsef and colleagues demonstrated restored anti-tumour activity of previously tolerant tumour specific T cells following transplant (Mirmonsef *et al,* 2008). They postulated that graft effector cells generated during the endogenous response to the tumour may expand more rapidly than Tregs during reconstitution following transplant, and are therefore able to evade Treg suppression. If this is the case then although there are likely to be many factors involved in rate of Treg expansion following transplant, the lack of correlation between graft and peripheral Treg levels is reassuring with increasing use of Plerixafor mobilised harvests.

6.4.2 Treg reconstitution following autologous transplant and impact on clinical outcome

Treg levels increased rapidly during the first 28 days following autologous transplant, a finding which supports the results from other studies (Perez-Garcia *et al,* 2009, Guillaume, Rubinstein and Symann, 1998). Following this early increase, Treg levels subsequently fell until 6 months post-transplant when they started to rise again. This does not appear to correlate with CD4 counts as these remain constant and low during the first year post autologous transplant (Guillaume, Rubinstein and Symann, 1998) (RMH data not shown), whereas CD3 counts increased rapidly until the third month, when they fell and slowly recovered over the course of the year (RMH data not shown). Although not well characterised, it is postulated that rapid Treg expansion immediately post-transplant in allogeneic recipients is driven by CD4+ lymphopoenia (Matsuoka *et al,* 2010). This may also be a factor in the rapid Treg expansion seen in this cohort

of autologous patients. Because no samples were tested during the first four weeks post-transplant due to low leucocyte cell numbers, it is possible that very low CD4+ numbers in the immediate period post-transplant may have driven this Treg expansion.

The subsequent dip and rise in numbers has not been described elsewhere but may be as a result of a number of factors. As the majority of T cells reside in lymphoid and mucosal sites and not in the peripheral blood it is possible that fluctuation in Treg numbers seen in this study reflects traffic of these cells between tissues and the periphery. Additionally, all patients in the study were suffering from malignant disease and the Treg recovery pattern may be affected by immune dysregulation observed in many cancer patients.

Alternatively the fluctuation in Tregs may be due to the recovery of at least partial thymic function following autograft resulting in a change in Treg origin from peripheral expansion to thymopoiesis. The initial immune reaction to chemotherapy-induced lymphopoenia is peripheral expansion of mature T cells originating from the graft, or of cells that survived the conditioning regimen (Hakim *et al,* 2005, Williams, Hakim and Gress, 2007). Following autologous transplant, thymic recovery may take up to 2 years and the degree of recovery is heavily age dependent (Douek *et al,* 2000). In younger patients a marked increase in thymic function is seen during the first few months following transplant so it is possible that the dip and rise in Tregs at 6 months in this cohort of mixed age patients reflects the shift in Treg origin.

Following autograft for the treatment of myeloma, many patients receive consolidation and maintenance therapy using agents such as velcade (bortezomib), lenalidomide and dexamethasone (Cavo *et al,* 2012, Bianchi and Anderson, 2014). This treatment is started at around three months post-transplant and may be responsible for fluctuations in Tregs observed in this study. Increases in Tregs have been described in patients receiving lenalidomide, either on its own (Clave, 2014) or in combination with dexamethasone (Karthick Raja Muthu Raja, 2012).

When considering peripheral blood Treg levels following transplant and clinical outcome during the first year, there was no significant correlation between Treg levels expressed as Tregs/ml, Tregs:CD3+ cell ratios or as a percentage of CD4+ cells and relapse or otherwise. Treg levels tested at one month post-transplant appeared lower in patients who suffered no disease relapse although this difference was not significant. However, it is possible that higher overall Treg numbers and Tregs relative to effector T cells at this early stage post-transplant may cause suppression of anti-tumour activity leading to subsequent disease relapse. It should be noted that in this study that patient outcomes were followed for a year post-transplant and therefore

data only reflects the effect of Treg levels on relatively early relapse. Differences between patient outcomes and Treg levels may reach significance with extension of the follow-up period. The transplant unit runs a late effects clinic to ascertain relapse or the occurrence of secondary cancers in the years following transplant. Examination of Treg levels in extended follow up would be interesting.

The number of Tregs relative to CD3+ cells and CD4+ cells were higher at one month post-transplant than at one year, with a significant difference between Tregs as a percentage of CD4+ cells at these time points ($p=0.005$). Levels of CD4+ cells recover more slowly than CD3+ cells following transplant but the highest percentage of Tregs to CD4 cells is observed in the first month. Some studies have suggested that absolute lymphocyte count at day 15 post-transplant is a predictor for clinical outcome following autologous transplant (Porrata and Markovic, 2004, Hiwase *et al,* 2008) and that recovery of lymphocyte count post-transplant is directly related to the number of T cells in the graft. Since rapid Treg expansion is thought to be regulated by CD4+ lymphocyte numbers, it is possible that patients with rapid lymphocyte recovery by day 15 have a consequently shorter period of rapid Treg expansion resulting in lower Treg levels capable of tumour suppression. This would be an interesting area for further study.

When interpreting results of studies on peripheral Treg numbers it should be noted that measurement of peripheral blood Treg levels may not be indicative of those in the tumour microenvironment. It is possible that this is where significant differences in Treg numbers may affect clinical outcome. Atanackovic and colleagues (2008) reported increased Treg numbers accumulating in the bone marrow of myeloma patients post-transplant compared with newly diagnosed myeloma patients and healthy controls. Post-transplant bone marrow biopsies could possibly yield data about the prevalence of Tregs in association with tumour in the bone marrow microenvironment. This is beyond the scope of the project but would be an interesting avenue to pursue.

6.4.3 Correlation between graft and peripheral Tregs following allogeneic transplant

The majority of research groups seeking to identify the effect of Treg numbers on the incidence of GVHD fall into two main categories: those who studied Treg levels in grafts and those who considered peripheral Treg levels post-transplant. As workers in both these categories have reported significant associations between Tregs and GVHD it might be expected that there would be a correlation between graft Tregs and those found in the peripheral blood post-transplant. Pastore and colleagues were one of the few groups to include this consideration in their study (Pastore *et al,* 2011). They reported a correlation between graft Tregs and peripheral Tregs at 1 month post-transplant in a cohort of full intensity transplants, deliberately excluding

reduced intensity transplants in order to provide a more homogeneous patient population. The current study did find a loose but insignificant association between allogeneic graft Tregs infused into patients receiving full intensity and RIC transplants taken together and peripheral Treg levels at three months post-transplant. The lack of correlation is not however surprising as it is unlikely that following infusion, graft Tregs would expand at a uniform rate in all patients. Additionally, the inclusion in the study of RIC transplants which are characterised by a longer period of mixed chimerism and delayed onset of GVHD might account for the lack of significant correlation. It is also probable that measurement of peripheral blood Tregs does not accurately reflect the entire Treg population capable of suppressing effector cells as these are distributed in tissues as well as peripheral blood.

Although there are similarities, reconstitution of Tregs in patients following allogeneic transplant was slower than in patients receiving autologous transplants. This is almost certainly due to immunosuppressive therapy which is given to all allogeneic recipients to prevent GVHD, but which also interferes with early lymphocyte reconstitution. This is supported by lower overall lymphocyte counts immediately post-transplant and the longer engraftment times seen in allogeneic patients. The consequence of this prolonged period of lymphopoenia is a more sustained but slower period of Treg expansion until they reach a peak at eight months (Figures 32(b), 33 and 34). This is also demonstrated when examining the ratio of Tregs to CD3+ cells, with increasing Tregs until nine months when the relative proportion of Tregs to overall T cells falls. Examining Tregs as a percentage of CD4+ cells illustrates Treg expansion up to six months post-transplant (Figure 42(b)), after which time CD4+ cells increase in number relative to that of Tregs. Unlike in autologous patients, Treg levels at 28 days were generally difficult to assess due to the low number of lymphocytes present and the limited blood sample size. Clearly the dynamics of Treg re-constitution differ between autologous and allogeneic patients. In the latter group relative paucity of Tregs may enable better GVM effect and consequently manifestation of GVHD is typically apparent.

A study by Bremm and colleagues was the first to demonstrate a down-regulation of CD127 expression on T cells, including Tregs, during the first few weeks post-transplant (Bremm *et al,* 2011). Although they confirmed that the use of CD127 as an additional marker for Treg determination was useful to identify Tregs where CD4+ $CD25^{high}$ gating is difficult to define, it is possible that the inclusion of this marker in the current study may have affected the results obtained in the early post-transplant samples. Nonetheless, it would be expected that lymphocyte and therefore Treg levels would be low immediately post-transplant as use of Campath 1H (Alemtuzumab) as part of allogeneic transplant conditioning induces profound depletion of all lymphocyte subsets. This in turn undoubtedly contributes to the slower recovery

of T cells including Tregs than following autologous transplants. While a caveat of allogeneic transplantation is the period of lymphopoenia with the increased risk of infection, reduced Treg numbers may work in favour of reduced tumour tolerance. The slower rate of Treg recovery is also likely to influence GVHD.

6.4.4 The effect of peripheral Treg levels on GVHD incidence and severity following allogeneic transplant

No significant association between Treg levels in the peripheral blood following transplant and the incidence or severity of GVHD was observed even when stratified for time of onset of GVHD. There may be a number of reasons for this.

The study was unable to determine Tregs at the exact time of onset of GVHD since information about GVHD was collected retrospectively and surplus peripheral blood samples were not always available at these time points. It is possible that the timing of Treg analysis may be critical to be able to correlate GVHD and Treg numbers in order to exclude the possibility that Tregs arise as a response to GVHD rather than being associated with the incidence of GVHD. It is known that GVHD impairs the reconstitution of the lymphoid compartment, therefore Treg numbers may be influenced by disturbed Treg generation in ongoing GVHD.

Samples tested for Treg levels after commencement of treatment for GVHD may not reflect the true relationship between Tregs and GVHD. Interestingly, Schneider and colleagues demonstrated that the frequency of CD4+ CD25+ Tregs increased during episodes of GVHD and that this correlated with therapeutic steroid therapy to treat the GVHD (Schneider *et al,* 2006). They were unable to determine if the increase in Tregs was directly as a result of steroid therapy or if it was part of an endogenous anti-inflammatory mechanism as a reaction to GVHD. The same study also noted that onset of GVHD was often heralded by a decrease in Tregs immediately prior to occurrence. These observations may explain some of the findings in this study.

Enumeration of Tregs would not necessarily reflect differences in Treg function following transplant or between patients. It is possible that despite the interest in numbers of Treg in the graft and following transplant that GVHD is more closely associated with their function. While Miura and colleagues reported loss of FoxP3 mRNA expression in patients with GVHD (Miura *et al,* 2004), Li's group demonstrated that incidence of GVHD correlated with a reduction in Treg frequency but no loss of suppressive function.

The current study, which focussed on Treg numbers rather than function, includes mostly patients who received reduced intensity conditioning as well as a smaller proportion who

received myeloablative therapy. These conditioning regimes will result in different inflammatory milieu and this in turn is likely to affect Treg reconstitution and patterns of effector T cell and Treg trafficking to target organs. In patients receiving RIC transplants, GVHD often develops later than in those who receive full intensity transplants, and these patients are generally more likely to suffer from chronic rather than acute GVHD, frequently with onset more than 100 days post-transplant and after the initial inflammatory insult.

As is the case with Tregs and aGVHD, the literature is divided about Treg frequencies in cGVHD. Teams led by Zorn and McIver observed decreased Treg frequency in patients suffering from cGVHD (Zorn, 2006, McIver *et al,* 2013). Li observed reduced Tregs only in patients with extensive rather than limited cGVHD (Li *et al,* 2010), whereas Clark and Sanchez observed increased numbers of CD4+CD25+ Tregs in the peripheral blood of cGVHD patients (Clark *et al,* 2003, Sanchez *et al,* 2004), a finding that was supported by Arimoto's team who measured FoxP3 expression by mRNA isolation from peripheral blood lymphocytes (Arimoto *et al,* 2007). In the current study, higher Treg levels as percentage of CD4+ cells were associated with lower incidence and severity of GVHD but this lacked significance. Similarly, the ratio of Tregs to CD3+ cells also appeared elevated up to three months post-transplant in patients with no GVHD. The reasons for lack of significant association between Treg numbers and GVHD, either developing early post-transplant or after a longer interval compared to other studies could be linked to the fact that the RMH clinical unit typically administers very much lower overall numbers of CD34+ donor cells than other centres.

An unavoidable limitation of the current study is that measurement of peripheral blood Tregs may not be indicative of Treg numbers in GVHD target organs. Within hours of infusion donor T cells migrate to the spleen and lymphoid organs where effector function is acquired after interaction with host antigen presenting cells (Wysocki *et al,* 2005). Tregs present in the lymphoid organs can prevent T cell priming and egress of effector T cells from lymphoid tissue (Engelhardt *et al,* 2012). On leaving lymphoid organs the activated alloantigen-specific effector T cells move to mucosal sites and tissue organs such as the skin, gastrointestinal tract, liver and lungs (Engelhardt *et al,* 2012). This trafficking to specific organs is controlled by chemokine-receptor and integrin-ligand interactions. Trafficking of Tregs to the same specific organs can suppress allo-reactive T cells and the relative numbers of Tregs and effector cells is a contributory factor in the prevention of GVHD (Fujioka *et al,* 2013). Some studies have benefited from being able to examine frequencies in target organs. Fondi and colleagues demonstrated lower GVHD severity when higher numbers of Tregs were present in skin biopsies of patients with skin GVHD (Fondi *et al,* 2009) and Rieger's group, who measured Tregs in intestinal mucosa of patients with GVHD, observed decreased Treg to T cell ratios in these patients (Rieger *et al,*

2006). However although these studies contribute to understanding the mechanism of Treg suppression of GVHD they do not offer a realistic predictive test for widespread use.

Allogeneic patients all receive CsA immunosuppressive therapy following transplant for a period to three to six months with gradual tapering if GVHD is not present. The immunosuppression aims to reduce the number of allo-reactive effector T cells but is simultaneously detrimental to Treg production and function (Demirkiran *et al,* 2008). Thus immunosuppression is a balance between reducing allo-reactive T cells to induce tolerance and promotion of Tregs with the same purpose. Calcineurin inhibitors such as CsA block IL2 expression essential for production of Tregs as well as blocking TCR mediated activation of calcineurin critical to their development. The increase in Treg levels in the months post-transplant are therefore not only due to increasing CD3 cell numbers but reflect reduction in CsA over this period.

6.4.5 The effect of peripheral Tregs on clinical outcome following allogeneic transplant

In patients who relapsed and also those who did not, peripheral blood Treg levels followed a similar oscillating pattern over the twelve month period following transplant. In the relapse group, although Treg levels appeared higher in the first six months, this did not achieve significance. Conversely, after 6 months Treg levels in these patients were lower than in than in patients who relapsed. Mean time to relapse was six months so this may have a loose connection to the reduction of Tregs at this time point.This study did not demonstrate any significant difference between peripheral Treg levels in patients who relapsed during the first year post-transplant and those who did not. It should be noted that in this study patient outcomes were followed for a year post-transplant and therefore data only reflects the effect of Treg levels on relatively early relapse. Extending the follow-up period may have resulted in a significant Treg level difference between patient outcomes.

In a small scale study of 16 patients Bremm and colleagues report that the highest Treg percentages were found in patients at the time of disease relapse (Bremm *et al,* 2011). Patients in Bremm's study received bone marrow (n=8), CD3 depleted PBSC (n=6) and only two received un-manipulated apheresis grafts. All patients in the study at RMH received un-manipulated apheresis grafts. This would result in a different engraftment profile and immune reconstitution from other graft sources thus comparison of data from the current and Bremm's study is difficult. In Nadal's study an increase in Tregs was reported in patients with chronic myeloid leukaemia (CML) who suffered disease relapse following transplant (Nadal *et al,* 2007).

The allogeneic transplant findings concur with the findings in autologous patients, but the same caution should be exercised when examining Tregs in peripheral blood as this may not reflect

the Treg levels in the tumour micro-environment. The results of this study established that use of peripheral Treg quantification to predict relapse in the first year post-transplant is not viable.

6.4.6 The effect of peripheral Tregs on chimerism following allogeneic transplant

As the majority of transplants in this study followed reduced intensity conditioning, the increase in full donor T cell chimerism from three to six months reflected the longer period of mixed donor and recipient chimerism seen in these patients. No correlation between donor T cell chimerism at three months, six months and one year intervals post-transplant was demonstrated. Since falling donor T cell chimerism following allogeneic transplant can herald relapse, an inverse relationship between Treg numbers and donor chimerism might have been expected. Since no such relationship was observed this supports the finding that peripheral Tregs do not appear to influence disease progression during the first year post-transplant. It should be noted that any patient requiring DLI during the first year following transplant was removed from the allogeneic follow-up part of the study at the time of the first DL treatment.

When considering the variety of conditioning regimes, the choice of immunosuppressive therapies, the manipulation or otherwise of donor grafts and the diversity of markers used for measurement of Tregs, it is not surprising that there is inconsistency between reported studies examining the effects of Tregs on GVHD and outcome. This study has not found any significant links between peripheral Treg numbers and GVHD or disease outcome following allogeneic transplant.

7.0 Results - TNFR2 as an alternative marker to FoxP3

The investigation of the efficacy of TNFR2 as an alternative marker to FoxP3 for Treg enumeration

7.1 Introduction

The identification of a suitable surface marker to identify human Tregs is important for the quantitative identification and enrichment of viable Tregs for possible therapeutic use. Chen and co-workers demonstrated that co-expression of TNFR2 (CD120b) and CD25 identified the most suppressive sub-set of Tregs (Chen *et al,* 2010) raising the possibility that this may provide a suitable surface marker to identify and enumerate Tregs without the need for time-consuming fixation and permeabilisation of cells required for FoxP3 staining. This would make testing more amenable to routine immunophenotyping laboratories and reduce testing turn-around times. Since TNFR2 expression is observed in cells that are $CD25^{high}$, $CD25^{low}$ and even cells that are $CD25^{neg}$ the use of CD25 as an additional marker alongside TNFR2 is recommended to identify Tregs. Chen and colleagues demonstrated that more than 90% of human CD4+ CD25+ TNFR2+ cells were also FoxP3+.

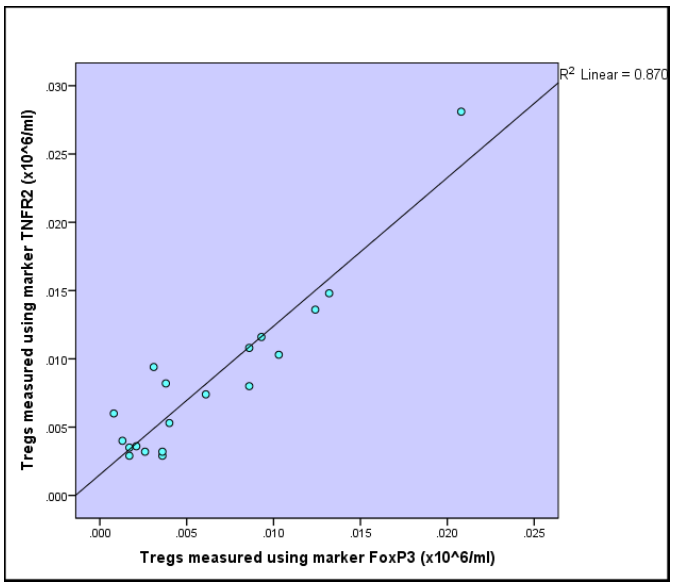
7.2 Data expression

This study used Anti -TNFR2 in combination with Anti CD4 and Anti-CD25 to test 19 peripheral blood samples in parallel with the standard FoxP3 staining method to compare the efficacy of the novel marker. Post-transplant peripheral blood samples known to show high levels of FoxP3 Tregs were chosen for testing as harvest samples tend to have lower Treg/ml levels.
Peripheral Tregs were expressed as Tregs x10^6/ml.

7.3 Results

Testing using a combination of Anti-CD3, Anti-CD4, Anti-CD25 and Anti-TNFR2 was easy and quick to perform. The results correlated closely with the results obtained using the standard FoxP3 staining method employed in this study (Figure 38(a)). Correlation between results was highly significant ($p=<0.0001$). Whereas correlation between results using both markers was strong in terms of relative levels of positive events, actual results were consistently and significantly different ($p=0.001$) with some results differing by as much as 80%. In all but two of 19 samples tested the Treg numbers obtained using the markerTNFR2 were higher than those obtained using FoxP3 (Figure 38(b)).

a)



b)

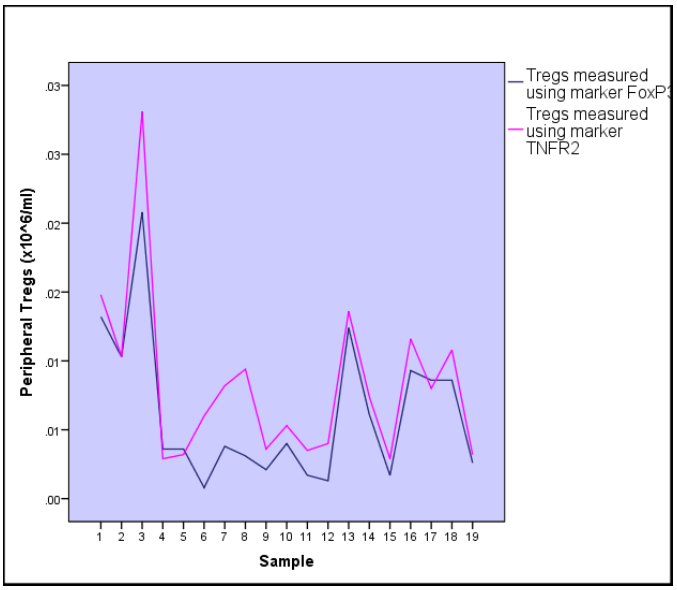


Figure 38: Treg levels using the markers FoxP3 and TNFR2: (a) Correlation between Treg levels using FoxP3 and TNFR2 (b) Comparison of Treg levels using FoxP3 and TNFR2. Post-transplant peripheral blood samples (n=19) were stained for either CD3 CD4 CD25 CD127 FoxP3 or CD3 CD4 CD25 TNFR2 and analysed by flow cytometry.

7.4 Discussion

The substitution of FoxP3 with Anti-TNFR2 used in combination with Anti-CD3, Anti-CD4, Anti-CD25 as a staining cocktail to identify and enumerate Tregs has the advantage of a significant reduction in the time required for preparation and staining. Anti-TNFR2 is a surface rather than intra-cellular marker.

The distribution of data obtained with Anti-TNFR2 correlated significantly with those obtained with the longer intracellular staining method but interestingly, the two methods do not produce comparable results. Chen and colleagues reported that the marker TNFR2 identifies the most suppressive subset of Tregs therefore it might be expected that numbers of Tregs identified by this staining method might be lower than those observed following FoxP3 staining. However, in the current study, Treg numbers identified by CD25 and TNFR2 were consistently significantly higher in 90% of the tested samples. Additionally, up-regulation of each of the markers CD25, FoxP3 and TNFR2 and down-regulation of CD127 have been reported to identify activated effector T cells as well as Tregs making TNFR2 as unreliable as FoxP3 in this respect. Indeed it is possible that TNFR2 identifies more activated T cells than FoxP3 leading to the higher results shown in this study.

Due to the typically low Treg numbers in harvested material, this methodology comparison utilised only peripheral blood samples from patients post-transplant. The use of TNFR2 suffers from the same lack of specificity with staining of activated effector cells as well as Tregs, but nonetheless the fact that this marker identifies the most suppressive Tregs may be interesting when considering clinical outcome and GVHD, and because of this might more clearly identify relationships between Treg numbers and clinical outcomes. The use of TNFR2 is clearly of interest however, further work would be required on a larger cohort of samples to validate the methodology.

8.0 Conclusions and further work

Understanding and harnessing the suppressive effects of Tregs in transplant patients presents a significant challenge, not least because of the diversity of results obtained by studies in this field. The conflicting observations demonstrate the difficulty in interpreting the wealth of data. Centre-specific factors such as disease mix, patient age, conditioning regimens, graft origin and manipulation, incidence and treatment of opportunistic infections and GVHD prophylaxis and treatment impede comparison between transplant centres and studies. This study set out to explore the influence of Tregs on clinical outcome in autologous and allogeneic patients at RMH. A major aim was to determine if routine Treg quantification - either in the graft or in the peripheral blood of patients post-transplant - could provide a useful predictor for disease relapse in autologous and allogeneic patients and falling chimerism and/or incidence of GVHD in the latter group. In order to gain an insight into the impact of Treg numbers they were quantified at various stages in the transplant process - the harvesting procedure, in transplant grafts and in the recipient's peripheral blood during immune reconstitution.

8.1 Summary of findings resulting from this study

8.1.1 The effect of mobilisation and HPC, A harvesting on Tregs

- In autologous patients, an overall increase in the absolute number of Tregs was noted in harvests mobilised by G-CSF plus Plerixafor compared to G-CSF alone and combined with cyclophosphamide although this difference did not attain significance. However a significant increase in the Treg: CD34 ratio was apparent. No link between Tregs in the grafts of autologous patients and relapse has been demonstrated within the one year follow-up period in this study. However, should an association between high Treg levels in grafts and subsequent later relapse be discovered, this increase in the Treg: CD34 ratio would be important and may indicate a case for the infusion of lower CD34 counts when using Plerixafor mobilised harvests.
- Autologous patient pre-apheresis peripheral Treg numbers do not predict the level of Tregs collected in the harvest therefore this testing would be irrelevant in the clinical setting.
- In autologous and allogeneic harvests, the number of Tregs collected in harvests is not affected by the day of harvesting which implies no selective depletion or enhancement of baseline levels by the actual harvesting process.
- In allogeneic harvests Treg numbers relative to CD3+ cells in those that have been collected following mobilisation with G-CSF, are significantly lower than in non-mobilised harvests. Cryopreservation of surplus allogeneic mobilised product for

potential use as DLI could be therefore be advantageous in terms of GVM but may have a negative impact on GVHD.

8.1.2 Tregs and clinical outcome in autologous grafts

- Treg levels in the grafts of autologous transplant recipients do not influence disease outcome during the first year post-transplant. This is reassuring when considering the finding that use of Plerixafor as part of mobilisation regimen caused increased numbers of Tregs as a ratio of CD34+ cells to be collected in the harvests. More recent guidelines for use of Plerixafor have approved its use in pre-emptive and rescue settings when it can be used in cases of failed mobilisation. However, an association between Tregs in the graft and relapse measured over a longer time period than a year post-transplant should be excluded.

8.1.3 Tregs and clinical outcome in allogeneic grafts

- There is no correlation between graft Treg levels and subsequent donor/recipient chimerism status during the first year post-transplant.
- Patients with the highest incidence and severity of GVHD received grafts with the lowest Tregs/kg and absolute Treg numbers which is what might be expected, although this was not statistically significant.
- Although low Treg doses were associated with the most severe GVHD in the cohort studied, there was no correlation between Treg levels in the graft and relapse during the first year post-transplant. Given the fact that there is a reciprocal relationship between manageable GVHD and effective GVM effect, this result is surprising. A longer follow-up of patients would be required to eliminate any correlation between Tregs in the grafts and relapse at a later time point.
- There will be many factors at play in the incidence and occurrence of falling chimerism and relapse so assessment of Treg levels as a test in isolation may be of little value in this context. It may be of value to undertake multi-variant analysis of Treg levels and clinical outcome however this would be confounded by difficulty and validity of attempting to ascertain appropriate Treg level cut-off values.

8.1.4 Tregs in donor lymphocytes and clinical efficacy

- Lower absolute Treg numbers in DLI doses were found to be significantly associated with a successful DLI outcome in terms of restoration of donor chimerism and resolution of relapse. As this study has found Treg numbers in mobilised allogeneic harvests to be lower than in non-mobilised harvests the use of G-CSF mobilised products for DLI could

be advantageous as these products may be conferring an enhanced GVM effect. The RMH practice of cryopreserving mobilised cells at the time of initial transplant for later use as DLI if required is therefore expedient in terms of both clinical outcome and cost. This finding has now been used to inform clinical practice in RMH.

- It was noted that CD3+ cell dose, and indeed CD3:Treg ratios, did not influence the outcome of DLI treatment. It is clinically accepted that higher CD3+ doses will produce a more profound therapeutic effect in terms of inducing GVM and redressing falling chimerism. Low numbers of patients receiving DLI in this study and follow up being restricted to one year may have contributed to this result. In addition, a larger patient cohort would enable data from those receiving DLI for either falling chimerism or relapse to be interrogated separately.

8.1.5 Tregs during immune reconstitution post-autologous transplant

- Peripheral Tregs at one month post-transplant were considerably lower in patients who did not relapse during the first year post-transplant although this did not reach statistical significance. Since higher Treg levels may favour tumour tolerance this observation is interesting particularly as more aggressive malignant clones in higher risk patients would be expected to recover rapidly post-autograft at the expense of normal haematopoiesis. One of the limitations of this study was that patients were followed for one year post-transplant. Extending this follow-up time would be an interesting area of further study as it might demonstrate a significant relationship between Treg levels at one month post-transplant and subsequent disease relapse.
- Peripheral Tregs at other time points post-transplant did not correlate with disease relapse and therefore peripheral Treg level is not a useful predictive tool in this context. It is likely that peripheral blood Treg numbers do not accurately reflect the levels in the tumour micro-environment but difficulty in obtaining suitable samples makes this difficult to establish or to utilise in a routine clinical setting.

8.1.6 Tregs during immune reconstitution post-allogeneic transplant

- There was no correlation observed between peripheral Treg levels and chimerism status during the first year post-transplant.
- Similarly, no correlation was noted between peripheral Tregs and incidence and severity of GVHD during the first year post-transplant. This may be due to the timing of samples tested as it was not possible to obtain patient's peripheral blood samples for testing at the point of onset of GVHD. Some samples may have been tested after start of GVHD treatment and therefore this may have affected the results. Peripheral blood Treg levels

may not accurately reflect the numbers in GVHD target organs which may be of greater significance, however the more invasive nature of obtaining suitable samples makes this difficult to establish in large patient cohorts or to utilise in a routine clinical setting.

- This study found no correlation between the incidence of relapse during the first year post-transplant and peripheral Treg numbers. Peripheral blood Treg numbers may not reflect levels in the tumour micro-environment. Additionally one of the limitations of this study was that patients were followed for one year post-transplant. Extending this follow-up time would be an interesting area of further study.

8.1.7 Methodological considerations

- The use of flow cytometry to detect the Treg marker Foxp3 is both time consuming and expensive, especially in patients where overall lymphocyte numbers are very low. Using a combination of Treg markers enables more reproducible gating but increases cost and staining time.
- The lack of suitable FoxP3+ controls reduces the reliability of the test. Ideally, the use of a control would enable validation of methodology as individual patient samples are not of sufficient size and cellularity to be used for more than one or two tests.
- Routine Treg enumeration early post-transplant and during periods of lymphopoenia would necessitate larger volume samples than were available for this study.
- The use of the surface marker TNFR2 in combination with the other surface markers CD3, CD4 and CD25 suggest a promising and less time-consuming alternative to intracellular staining of FoxP3, but although results correlated significantly, they were not comparable. These markers are identifying different cell populations with TNFR2 being more prevalent on a more suppressive sub-population of Tregs. Only post-transplant peripheral blood samples were tested using anti-TNFR2 therefore further testing of a variety of harvested material would be required to validate the method which due to its simplicity would be more appropriate for routine testing.

8.2 Future work

- Consider testing a wider cohort of patients receiving DLI
- Confocal studies to visualise differences between staining methods
- Consider longer follow-up of patients post-transplant

9.0 Recommendations

The practice of limiting the infusion of CD34+ doses to $8.0x10^6$/kg in patients receiving allogeneic transplants is valid and should be continued.

The use of surplus mobilised product for DLI should continue and if possible be extended to include more patients. Not only does this method provide the benefits of quick access to DLI if required and considerable cost savings but it demonstrates better clinical outcomes than with the use of non-mobilised products.

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Appendix 1

GVHD grading scheme

Clinical grading scheme for acute GVHD, according to Glucksberg

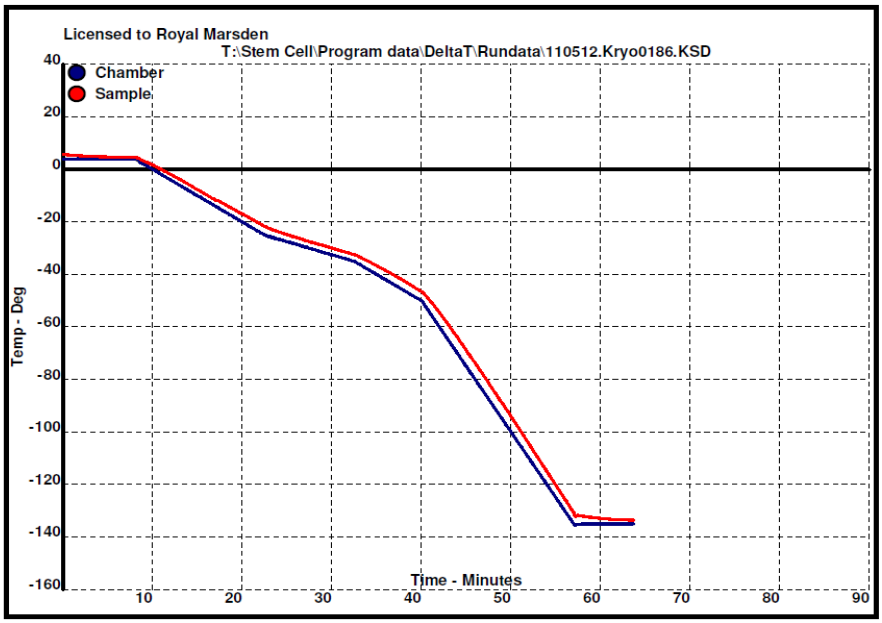
Criteria for individual organ systems			
Organ	Skin	Gut	Liver
Grade	% of body surface area covered by rash	Diarrhoea volume (ml/day)	Bilirubin level (mg/dl)
0	0	≤500	<2
1	<25	> 500	2-3
2	25-50	>1000	3-6
3	Generalised erythroderma	>1500	6-15
4	Generalised erythroderma with bullous formation and desquamation	Severe abdominal pain with or without ileus	>15

Clinical grading scheme for acute GVHD, according to Glucksberg

Overall grade	Skin grade	Gut grade	Liver grade	Decreased clinical performance
0	0	0	0	None
1	1-2	0	0	None
2*	1-3	1	1	Mild
3*	2-3	2-3	2-4	Marked
4	2-4	2-4	2-4	Extreme
*Grades 2 and 3 must involve either gut or liver (or both) at the indicated organ grades, in addition to skin involvement.				

Appendix 2

Freeze profile used in controlled rate freezer for cryopreservation of samples



No. of Samples : 1
Janet's Cells

JJ
KryoFile Start Time : 11-May-2012 10:32
User Aborted the Run
STOPPED BY JJ
KryoFile End Time : 11-May-2012 11:41

Appendix 3

FoxP3 staining method

Mononuclear cells were separated from peripheral blood using Ficoll density gradient centrifugation.

Apheresis harvests are mononuclear cell-rich and were therefore used without preparation.

Buffers were allowed to reach room temperature. Working solutions of BD Pharmigen Human FoxP3 Buffer were prepared prior to each staining episode.

Cells were washed, spun and diluted with BD Pharmigen Stain Buffer to give a final concentration of 10^6cells/ml.

10µl Anti-CD3, and 20µl of Human Regulatory T cell cocktail (Anti-CD4, Anti-CD25, Anti-CD127) were added to each 100µl of cells used, the contents vortexed, and the tube incubated at room temperature in the dark for 20 minutes.

Following incubation, 2mls of stain buffer were added to each tube and these were centrifuged for 250g for 10 minutes. The wash buffer was removed and the cells gently resuspended in the residual buffer.

To fix the cells, 2mls of diluted Human FoxP3 buffer A were added, mixed and the tubes incubated for 10 minutes at room temperature in the dark.

Tubes were centrifuged at 500g for 5 minutes and the fixative removed. When using cryopreserved and thawed cells care was taken not to remove the cell pellet as it was buoyant at this stage.

Cells were washed and resuspended in 2mls of stain buffer and further centrifuged at 500g for 5 minutes. The buffer was removed.

To permeabilise the cells the pellet was resuspneded in the residual stain buffer and 0.5mls of freshly prepared working solution Human FoxP3 Buffer C was added to each tube. Cells were mixed and incubated at room temperature in the dark for 30 minutes.

Cells were washed in 2mls of stain buffer, centrifuged at 500g for 5 minutes and the buffer removed. The wash step was repeated.

Either 20µl Anti-FoxP3 antibody or 20µl of the PE isotype control was added to each tube as appropriate and mixed.

Cells were incubated at room temperature for 30 minutes in the dark.

Cells were immediately washed twice in stain buffer, resuspended in a suitable volume of stain buffer and analysed.

Cells were analysed as quickly as possible following staining.

TNFR2 staining method

Mononuclear cells were separated from peripheral blood using Ficoll density gradient centrifugation.

Apheresis harvests are mononuclear cell-rich and were therefore used without preparation.

Buffers were allowed to reach room temperature. Working solutions of BD Pharmigen Human FoxP3 Buffer were prepared prior to each staining episode.

Cells were washed, spun and diluted with BD Pharmigen Stain Buffer to give a final concentration of 10^6cells/ml.

10µl Anti-CD3, and 20µl of Human Regulatory T cell cocktail (Anti-CD4, Anti-CD25, Anti-CD127) were added to each 100µl of cells used. Either 10µl of TNFR2 or 10µl of the PE isotype control were added to each tube, the contents vortexed, and the tube incubated at room temperature in the dark for 20 minutes. Although the CD127 marker was not used for this staining method it was part of the staining cocktail.

Following incubation the cells were washed by adding 2mls of stain buffer to each tube and centrifuging at 250g for 10 minutes. The wash buffer was removed and the cells gently resuspended in the residual buffer before repeating the wash stage.

Cells were resuspended in a suitable volume of stain buffer and analysed.

Appendix 4

Patient selection

Autologous donors by diagnosis

Diagnosis	Number of patients	Number of harvests
MM	66	92
NHL	3	4
HD	7	8
CLL	1	2
Plasmacytoma	3	3

Mobilised and non-mobilised harvests by type of allogeneic donor

	Mobilised harvests	Non-mobilised harvests
Siblings	20	10
VUDs	39	3
Total	59	13

Peripheral blood and harvest pairs by diagnosis and type of donor

	Total number of patients	Total number of harvests
Autologous		
MM	26	35
NHL	5	7
HD	1	1
Plasmacytoma	2	2
Allogeneic		
Sibling donors	9	11

Day of harvesting

Diagnosis	Day 1 harvest (n=49)	Day 2 harvest (n=28)	Day 3 harvest (n=2)
MM	41	24	2
NHL	2	1	
HD	3	1	
CLL		1	
Plasmacytoma	3	1	

Patients receiving autologous transplants

Diagnosis	Patients (n=85)
MM	71
Lymphoma	7
HD	3
Plasmacytoma	3
CLL	1

Patients receiving allogeneic transplants

Daignosis	Patients (n=75)
ALL	15
Lymphoma	9
CLL	11
AML	30
MM	2
MDS	3
Myelofibrosis	2
hd	2
CML	1