A Study of Regulatory T Cells in Allogeneic Haematopoietic Stem Cell Transplantation

A thesis submitted to the University of Oxford in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

Biomedical and Clinical Sciences

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Trinity Term 2012

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ABSTRACT

A Study of Regulatory T Cells in Allogeneic Haematopoietic Stem Cell Transplantation

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Thesis submitted for the degree of Doctor of Philosophy

Trinity Term, 2012

Allogeneic haematopoietic stem cell transplantation (alloHSCT) is an established therapy for many haematological disorders. Unfortunately, the new donor-derived immune system may damage host cells (Graft-versus-Host Disease (GvHD)), causing significant morbidity and mortality. Since regulatory T cells (Tregs) can modulate immune responses, it was hypothesised that Treg numbers in the haematopoietic stem cell grafts and/or peripheral blood may influence the development of GvHD and other transplant-related complications.

In this project, a prospective observational clinical study of putative Tregs in human alloHSCT was performed in Oxford. Flow cytometry and methylation-specific qPCR assays were developed to quantify putative Tregs and lymphocyte populations within the grafts and post-transplant blood samples. Although low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell numbers were not associated with increased incidence of GvHD, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells in the graft (as a percentage of total $CD4^+$ T cells) were independently associated with poor engraftment, increased non-relapse mortality and inferior overall survival. Similarly, falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts over the first three months post-transplant were associated with higher non-relapse mortality and inferior overall survival. In view of these novel findings, strategies that increase $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in alloHSCT may improve clinical outcomes.

One possible route for increasing Tregs is through cellular therapy. This project therefore tested the hypothesis that $CD4^+CD25^+FOXP3^+$ Tregs can be produced *in vitro* from conventional $CD4^+$T cells. In the presence of TGFβ and Azacitidine, FOXP3 was expressed in the majority of activated $CD4^+$ T cells. These cells also had a demethylated *FOXP3* TSDR enhancer which is specific to natural Tregs (Baron *et al.,* 2007). However, most of these cells produced pro-inflammatory cytokines, for example, TNFα. Therefore, under these conditions, FOXP3 expression was not sufficient to produce a Treg phenotype. It is proposed that current focus for generating Tregs for human clinical trials should be directed towards improving isolation and expansion of *ex vivo* isolated Tregs.

DECLARATIONS

I declare the thesis I am submitting is entirely my own work except where otherwise indicated.

Patient consent and post-transplant blood samples/data were collected with the assistance of the bone marrow transplant (BMT) doctors (Dr Andy Peniket, Dr Patrick Medd and Dr Tim Littlewood; Department of Haematology, Churchill Hospital, Oxford, UK) and the clinical nurse specialists (Denise Wareham, Mandy Ellis and Lara Rowley; Department of Haematology, Churchill Hospital, Oxford, UK).

Sindu Nair (Blood Research Laboratory, NHSBT, Oxford, UK) helped with processing and flow cytometry analysis of the clinical blood samples. I designed the methylation-specific real-time qPCR assay and samples were tested by Hannah Tudor (Oxford BRC Molecular Diagnostic Service, Oxford, UK). DNA sequencing was performed by the DNA Sequencing Service (Weatherall Institute of Molecular Medicine (WIMM), Oxford, UK). Dr Wei Zhang (Blood Research Laboratory, NHSBT, Oxford, UK) supplied the data for Figure 6.15.

This work has not been submitted, either partially or in full, for another qualification of this University or for a qualification at any other institution.

ACKNOWLEDGEMENTS

I wish to thank my supervisors: Dr Andrew Peniket, Professor David Roberts and Professor Herman Waldmann, for all the advice and support throughout this project. I would like to thank Wendy Slack, Sindu Nair and Dr Wei Zhang and all the members of the Blood Research Laboratory and NHSBT (Oxford) for all their help over the last few years. I must thank Hannah Tudor, Dr Anna Schuh and members of the Oxford BRC Molecular Diagnostic Service (Oxford) for helping to develop and run the Treg PCR assay. I would also like to thank all the doctors, nurses and staff in the Department of Haematology (Oxford). In particular, I must thank the BMT specialist nurses: Denise Wareham, Mandy Ellis and Lara Rowley, and Dr Patrick Medd for helping to collect the never ending supply of blood samples and clinical data. I would also like to show my gratitude to Professor Dave Collett (NHSBT, Bristol) and Dr Patrick Medd for helping me with the statistics and answering my many questions. I must also give special thanks to all the patients that took part in this study as they formed the basis for this whole project.

I would like to thank the Wellcome Trust and the Medical Sciences Doctoral Training Centre (University of Oxford), for their generous funding and support, without which this project would not have been possible. I would also like to thank the Department of Haematology (Oxford), the Nuffield Department of Clinical Laboratory Sciences (University of Oxford), NHS Blood and Transplant, the Biomedical Research Council (Oxford) and the Oxford University Hospitals NHS Trust for their funding and use of facilities and staff during this project.

Finally, I would like to give my biggest thanks to my family and my wife. Their never-ending support and encouragement has been hugely important to me. Thank you.

ABBREVIATIONS

List of commonly used abbreviations:

AA	Aplastic anaemia
AD	Active disease
aGvHD	Acute graft-versus-host disease
ALL	Acute lymphoid leukaemia
alloHSCT	Allogeneic haematopoietic stem cell transplants
AML	Acute myeloid leukaemia
ATG	Anti-thymocyte globulin
BM	Bone marrow
BMT	Bone marrow transplant
BS	Bisulphite specific
cGvHD	Chronic graft-versus-host disease
CLA	Common leucocyte antigen
CLL	Chronic lymphoid leukaemia
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CR	Complete response
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBC	Full blood count
FMO	Fluorescence minus one
FOXP3	Forkhead box P3 (Human)
Foxp3	Forkhead box P3 (Murine)
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCSF	Granulocyte colony stimulating factor
GMP	Good Manufacturing Practice
GvHD	Graft-versus-host disease
GvT	Graft-versus-tumour
HL	Hodgkin's Lymphoma

HLA	Human leucocyte antigen
HR	Hazards Ratio
HSC	Haematopoietic stem cells
HvG	Host-versus-graft
IFNγ	Interferon gamma
IL17	Interleukin 17
IL2	Interleukin 2
iTregs	Inducible regulatory T cells
LH	Lithium heparin
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MFI	Median fluorescence intensity
MS	Methylation specific
MSRT-qPCR	Methylation-specific real-time quantitative polymerase chain reaction
Ne	Neutrophils
NFAT	Nuclear Factor of Activated T cells
NHL	Non-Hodgkin's Lymphoma
NHS	National Health Service
NHSBT	National Health Service Blood and Transplant
NIH	National Institutes of Health
NK	Natural killer
NRM	Non-relapse mortality
nTregs	Natural regulatory T cells
OS	Overall survival
OUH	Oxford University Hospitals
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem
PBSCH	Peripheral blood stem cell harvest
PCR	Polymerase chain reaction
Plts	Platelets
PR	Partial response
Rel	Relapse
RFS	Relapse free survival
RIC	Reduced intensity conditioning

RT	Room temperature
SSC	Side scatter
TBI	Total body irradiation
TCR	T-cell receptor
TGFβ	Transforming growth factor beta
TNFα	Tissue necrosis factor alpha
Tregs	Regulatory T cells
TRM	Transplant related mortality
TSDR	Treg-specific demethylated region
UCB	Umbilical cord blood

Chapter 1

Introduction

1. INTRODUCTION

Allogeneic haematopoietic stem cell transplantation is the transfer of haematopoietic stem cells (HSC) from a healthy donor into an immunosuppressed host to generate a new haematopoietic system and re-establish functional immunity. Over the last 50 years, it has become conventional therapy for many haematological disorders, particularly for treating malignant conditions such as leukaemia and lymphoma. Over 25,000 allogeneic haematopoietic stem cell transplants (alloHSCT) are now performed each year and in 2009 there were 12,400 transplants in Europe alone (Gratwohl *et al.,* 2010; Baldomero *et al.,* 2011). 11,442 of these were first transplants with 70% treating leukaemia, 17% lymphoproliferative disorders and 5% bone marrow failure (Baldomero *et al.,* 2011). As techniques continue to advance and transplant related mortality (TRM) improves, alloHSCT are being increasingly used to treat solid tumours and non-malignant disorders such as haemoglobinopathies and primary immune deficiency.

1.1 HISTORY OF ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

The general principle of allogeneic HSC transplantation started after the Second World War while researching the effects of ionising radiation on the human body. Following the development of atomic warfare, bone marrow (BM) and haematopoietic cells were known to be sensitive to radiation and in 1949 Jacobson and colleagues showed that lethally irradiated mice could be protected by shielding their spleen or femurs (Little and Storb, 2002). Crucially, in 1951, Lorenz and colleagues showed that mice could be rescued from lethal irradiation by intravenous infusion of BM, although it was not until several years later that it was recognised that this was due to the transfer of cells (Lorenz *et al.,* 1951; Barnes and Loutit, 1954; Thomas and Blume, 1999). During the same period, the use of radiation to treat leukaemia was being recognised, although many patients died from relapse, infection or BM failure (Osgood and Seaman, 1952; Hulbert, 1954). This generated interest in the possibility of using radiation followed by transfer of BM to treat life-threatening malignancies.

In the early 1950s, attempts at human allogeneic HSC transplantation were unsuccessful due to failed engraftment (Bortin, 1970). However, in 1957, Dr Donnall Thomas reported transient engraftment in a patient receiving chemotherapy and radiotherapy (Thomas *et al.,* 1957). Two years later, significant progress was made when he reported the first successful human bone marrow transplants (BMT) in which two patients with leukaemia were treated with lethal dose radiation and given BM from their identical twins (Thomas *et al.,* 1959; Thomas and Blume, 1999). In the same year, Georges Mathé, a French oncologist and immunologist, treated five Yugoslavian physicists, who had been exposed to radiation following a nuclear reactor accident, with allogeneic BM (Mathé *et al.,* 1959; Jansen, 2005). Although engraftment was reported, these transplants were later rejected. However, in 1965, Georges Mathé then reported the successful treatment of a patient with leukaemia using an allogeneic BMT (Mathé *et al.,* 1965; Jansen, 2005). This patient survived for 20 months, suffering from a secondary disease, now known as Graft-versus-Host Disease (GvHD).

Following the discovery of human leucocyte antigens (HLA) by Jean Dausset in 1958, there was increased recognition for HLA matching, both for engraftment and to prevent GvHD (Dausset, 1959; Thomas and Blume, 1999). At the same time, high dose therapy enabled treatment of the disease and immunosuppression of the host to prevent graft rejection. Following the work in animal models, it was also shown that GvHD could be reduced by the administration of immunosuppressant drugs, in particular Methotrexate (Thomas *et al.,* 1962; Storb *et al.,* 1986). Consequently, in 1968, Dr Robert Good reported the first successful alloHSCT for a non-malignant condition in which a young boy was successfully treated for severe combined immunodeficiency syndrome (SCID) using BM from his HLA-matched sister (Gatti *et al.,* 1968). In 1973, the first successful unrelated BMTs for non-malignant conditions were reported (Speck *et al*., 1973; Foroozanfar *et al.,* 1977; O'Reilly *et al*., 1977). Six years later, an unrelated BMT for leukaemia was performed at the Fred Hutchinson Cancer Centre, once again, under the supervision of Dr Donnall Thomas (Thomas *et al.,* 1979).

In the 1970-80s, the formation of national and international BM registries increased the availability of HLA-matched donors and in the late 1980s it was recognised that HSC could also be obtained from peripheral blood (Juttner *et al.,* 1988; Thomas and Blume, 1999). The availability of HSC grafts was further increased when in 1989 the first successful transplant using umbilical cord blood (UCB) was performed (Gluckman *et al.,* 1989). Over the last 10 years, significant advances have been made to reduce GvHD and TRM, in particular, through the use of reduced-intensity conditioning (RIC) regimes, T-cell depletion of the graft and improved supportive care (discussed below) (Slavin *et al.,* 1985; Giralt *et al.,* 1997; Schmidt *et al.,* 1991; Little and Storb, 2002).

1.2 CURRENT ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

1.2.1 PRE-TRANSPLANT CONDITIONING

Prior to allogeneic HSC transplantation, recipients are given preparative conditioning containing chemotherapy, radiotherapy and/or immunotherapy. This conditioning has two main purposes. First, it has a myeloablative effect that depletes the recipient's haemopoietic cells, reduces any residual tumour and empties the BM niche for the incoming graft. Second, it has a profound immunosuppressive effect to reduce the risk of rejection, either of the graft by the host (Host-versus-Graft (HvG)) or of the host by the graft (Graft-versus-Host (GvH)). The extent to which the conditioning regimen contains these two components can be manipulated, depending upon the recipient, the disease being treated and the risk of rejection. Conventional regimens use intensive conditioning with strong myeloablation and today, the most common intensive regimens are Cyclophosphamide/Total Body Irradiation (TBI) and Cyclophosphamide/Busulphan (Bu/Cy) (Bacigalupo *et al.,* 2009). Cyclophosphamide and Busulphan are alkylating agents which cause cross linking of deoxyribonucleic acid (DNA) and have myeloablative and immunosuppressive properties. Although these regimens have strong anti-tumour effect, they cause considerable tissue damage and are associated with high toxicity and TRM (Shi-Xia *et al.,* 2011; Topcuoglu *et al.,* 2012). For this reason, myeloablative conditioning (MAC) regimens are generally restricted to younger patients (<55 years) without significant co-morbidities and with high-risk disease.

To reduce the toxicity of conditioning and to increase the availability of alloHSCT to older patients, there has been increasing use of reduced intensity (RIC) or non-myeloablative (NMA) conditioning over the last 15 years (Baldomero *et al.,* 2011). These regimens are less myeloablative but provide sufficient immunosuppression to allow donor engraftment. Disease eradication is dependent upon the donor-derived T cells recognising residual tumour as 'non-self', producing an immune mediated Graft-versus-Tumour (GvT) response (Sandmaier *et al.,* 2007). As they are less intensive, they have significantly lower toxicity and TRM, allowing transplants to be performed in older patients or

in those with co-morbidities (Topcuoglu *et al.,* 2012). Today, many regimens exist, differing in the balance between myelosuppression and immunosuppression. In general, most use Fludarabine (purine analogue that inhibits DNA synthesis) plus an alkylating agent (Melphalan, Cyclophosphamide or Busulphan) or low dose TBI (Bacigalupo *et al.,* 2009). In several retrospective reviews, RIC regimens are generally associated with lower TRM but with higher rates of disease relapse compared to MAC (Shi-Xia *et al.,* 2011; Topcuoglu *et al.,* 2012). Overall survival (OS) appears similar although longer follow-up will be required.

1.2.2 DONOR SELECTION

A major factor in the advancement of alloHSCT was a greater understanding of how the immune system differentiates self from non-self and the identification of the human leucocyte antigens (HLA). HLA proteins are expressed on the surface of all cells and are crucial for the interaction between themselves and the immune system (Little *et al.,* 2011). The HLA proteins are encoded by a related group of genes on the short arm of chromosome six that show extensive polymorphism (Little and Parham, 1999). HLA Class I molecules (A, B and C) are present on almost all nucleated cells and platelets and consist of a transmembrane protein with three structural domains (α1, α2, α3) in association with β_2 microglobulin (chromosome 15). The antigen presenting site, located between the α1 and α2 domains, can present short peptides (8-10 amino acids) derived from proteasome degradation of intracellular proteins to the T-cell receptor (TCR) of $CD8^+$ T cells (Little *et al.,* 2011). HLA Class II molecules (DP, DQ and DR) are found on antigen presenting cells (APC), including dendritic cells, macrophages, B cells and activated T cells. The class II molecules are formed from two transmembrane proteins, each with two structural domains (α1, α2 and β1, β2). The antigen presenting site, located between the α1 and β1 domains, presents fragments of extracellular protein taken up by the APC and degraded by proteolysis. The antigen is presented to the TCR of $CD4^+$ T cells (Little *et al.,* 2011).

In relation to alloHSCT, the recipient and donor should ideally be matched for all HLA antigens as mismatches increase the risk of rejection, either by HvG or GvH, and reduce overall survival (Loiseau *et al.,* 2007, Passweg *et al.,* 2011). The availability and choice of donor is primarily determined by the size of the family, genotype of family members, the HLA type and the ethnicity of the recipient. Full siblings have a one in four chance of being HLA-matched unless they are a genetically identical twin. If a matched sibling is not available, an HLA-matched unrelated donor (MUD) may be found through the national or international BM registries. Certain ethnic groups or HLA types are under-represented in these registries and some populations have more diversity of HLA types. Therefore, finding a suitable donor is not always possible (Majhail *et al.,* 2012). In such situations, several options are available. The first is the use of UCB which contains a higher concentration of HSC than adult blood (Bender *et al.,* 1994). Many UCB banks now exist worldwide and due to the increased plasticity of the UCB grafts, only HLA matching for HLA-A, B and DR is required (Stanevsky *et al.,* 2009). The disadvantage of UCB transplantation, however, is the small size of most UCB samples and the associated delayed engraftment compared to conventional transplants (Rocha and Gluckman, 2009; Stanevsky *et al.,* 2009). Recent analysis of the outcomes from UCB transplants continues to show encouraging results and supports their use as an alternative source of HSC (Brunstein, 2011a). The second option is to use an HLA-mismatched donor, either at one or two HLA loci or by using an HLA-haploidentical donor. Both are associated with increased risk of graft rejection and GvHD but may be appropriate for high-risk disease or when other options are not available (Reisner *et al.,* 2011).

1.2.3 STEM CELL SOURCE

Traditionally, a BM harvest involves multiple aspirations from the posterior iliac crests performed under general anaesthesia. While providing sufficient HSC for transplantation, this procedure requires hospital admission and has a small risk of morbidity and mortality due to the general anaesthesia and temporary discomfort from the harvest site (Siddiq *et al.,* 2009). An alternative

approach is to use a peripheral blood stem harvest (PBSCH) in which HSC are collected from the donor's peripheral blood using cytapharesis. This is generally more comfortable for the donor, only requiring access into a peripheral vein, and can be performed as a day case procedure. To acquire sufficient HSC for transplantation (typical minimum target dose $4x10^6$/kg $CD34^+$ cells), the stem cells are mobilised with sub-cutaneous granulocyte colony stimulating factor (GCSF) for three to five days prior to harvesting. This can be associated with minor side effects including headache, bone and splenic discomfort, and there is a small risk of failing to mobilise (Siddiq *et al.,* 2009). The choice of which HSC source to use is dependent on both the donor's preference and the transplant procedure. Today, most alloHSCT use PBSCH because of the greater convenience and PBSCH are associated with faster neutrophil and platelet engraftment (Baldomero *et al.,* 2011; Nagler *et al.,* 2012). However, it has also been reported that PBSCH have a higher risk of GvHD possibly due to higher donor T-cell doses (Eapen *et al.,* 2007). Therefore, for those transplants where the GvT response is not so important, e.g. aplastic anaemia, BM harvests are still preferred to minimise the morbidity and mortality from GvHD (Führer, 2008).

1.2.4 GRAFT MANIPULATION

To reduce the risk of GvHD, alloHSCT can be performed with T-cell depletion. T cells can be depleted from the *ex vivo* HSC graft by antibody labelled magnetic beads, using either negative selection of T cells or positive selection and infusion of HSC. Alternatively, T-cell depletion can be performed *in vivo* by the administration of T-cell depleting agents to the recipient during conditioning. The most common agents used are anti-thymocyte globulin (ATG) and Alemtuzumab (Norlin and Remberger, 2011). ATG is a polyclonal antibody, prepared in rabbits or horses, raised against thymocyte antigens (Simpson, 2003). Alemtuzumab is an humanised monoclonal antibody directed against CD52, a glycosylphosphatidylinositol anchored protein on the surface of all lymphocytes (Riechmann *et al*., 1988). Although infused into the recipient pre-transplant, the half-life of both these antibodies is such that they reduce T cells in both the recipient and the graft

(Chakraverty *et al.,* 2010). The decision to use T-cell depletion is dependent on the risk of HvG/GvHD and the preference of the transplant centre. The optimal protocol still remains contentious. While some centres, including Oxford, use Alemtuzumab in unrelated donor transplants only, other UK centres use Alemtuzumab in both sibling and unrelated donor transplants. Although Alemtuzumab reduces the occurrence of GvHD, it has been associated with increased incidence of disease relapse and infection (Pérez-Simón *et al.*, 2002; Chae *et al.,* 2008; Malladi *et al.,* 2009). However, overall survival appears similar between these two strategies (Malladi *et al.,* 2009).

1.3 ENGRAFTMENT AND IMMUNE RECONSTITUTION

Reconstitution of the haematopoietic system is achieved through engraftment of the donor HSC into the BM niche. For successful engraftment there must be sufficient HLA matching between donor and host, suppression of the host's native immune system and a sufficient number of donor HSC. In clinical HSCT, HSC are often assessed using CD34 expression; a glycoprotein present on the surface of HSC and early progenitors (Link and Arseniev, 1997). The $CD34^+$ dose in the graft is a significant predictor of engraftment in both autologous and allogeneic HSCT (Kamel *et al.,* 2005; Specchia *et al.,* 2006; Pastore *et al.,* 2008). The source of the HSC can also affect the rate of engraftment with PBSCH producing faster engraftment, possibly due to higher numbers of haemopoietic progenitors and mature T cells (Bittencourt *et al.,* 2009). Engraftment is also aided by the presence of donor $CD8^+$ T cells and natural killer (NK) cells in the graft to overcome residual host immunity (Gandy *et al.,* 1999, Kim *et al.,* 2006). Several studies have shown that successful engraftment is strongly associated with the $CD8^+$ T-cell and NK cell dose in the graft (Cao *et al.,* 2005; Kim *et al.,* 2006). In contrast, depletion of $CD4^+$ T cells from the graft does not appear to increase graft rejection (Martin *et al.,* 1999).

Following alloHSCT, patients remain immunosuppressed for many months. Pre-transplant conditioning and immunosuppressive therapy produce an initial period of profound cellular and humoral deficiency, especially when using T-cell depletion. Following an initial aplastic period, neutrophils recover approximately 14-21 days post-transplant. NK cells are the first lymphocyte population to recover, primarily due to expansion of $CD16^-CD56^{bright}$ cells (Dulphy *et al.,* 2008; Seggewiss and Einsele, 2010). T-cell recovery takes longer (6-12 months) as thymic function is reduced due to age, chemo/radiotherapy, T-cell depletion and GvHD, limiting the production of naïve T cells. Thymic function can be assessed by measuring T-cell receptor rearrangement excision DNA circles (TRECs) and these remain low for the first 3-6 months (Fallen *et al.,* 2003). Much of the early T-cell reconstitution is due to homeostatic expansion of donor memory T cells, often with a limited T-cell repertoire, and CD4:CD8 T-cell ratios are reversed due to delayed $CD4^+$

T-cell recovery (Fallen *et al.,* 2003; Seggewiss and Einsele, 2010). B cells and circulating immunoglobulins are also markedly reduced and may remain low for many months due to reduction of B cell precursors in the bone marrow (Seggewiss and Einsele, 2010).

1.4 GRAFT-VERSUS-HOST DISEASE

Graft-versus-Host Disease (GvHD) is a multi-systemic disorder caused by donor T cells reacting to alloantigens on the host (recipient) cells (Ferrara *et al.,* 2009). Despite improvements in alloHSCT regimens and immunosuppressive therapy, GvHD continues to cause significant morbidity and mortality following transplantation (Pasquini *et al.,* 2010; Ruutu *et al.,* 2012).

1.4.1 ACUTE GRAFT-VERSUS-HOST DISEASE

Conventionally, acute GvHD (aGvHD) was defined as occurring within 100 days of alloHSCT or donor lymphocyte infusion (DLI) and has an incidence of 30-50% in HLA-matched alloHSCT despite prophylaxis (Przepiorka *et al.,* 1995; Weisdorf, 2007). However, in 2005, the National Institutes of Health (NIH) proposed a re-classification based upon clinical features rather than time. Acute GvHD is now defined by the presence of diagnostic features (maculopapular rash, diarrhoea and/or cholestatic jaundice) and can be classic (within 100 days), persistent (extends beyond 100 days), late onset (starts after 100 days), recurrent (both before and after day 100) or overlap (when both acute and chronic GvHD features are present at the same time) (Filipovich *et al.,* 2005).

A) PATHOGENESIS

The pathogenesis of aGvHD can be summarised into (Ferrara *et al.,* 2009; Morris and Hill, 2007):

A) **Conditioning**: During pre-transplant conditioning there is damage to host tissues causing activation of the host's APCs, release of pro-inflammatory cytokines, up-regulation of HLA antigens and presentation of host peptides (Cooke *et al.,* 1998; Zhang *et al.,* 2002). Damage to the mucosa of the gastrointestinal (GI) tract allows passage of lipopolysaccharides across the intestinal wall, also activating the immune system (Hill *et al.,* 1997).

B) **T-cell activation**: Donor T cells within the graft and with specificity to host antigens interact with host APCs causing activation, proliferation and differentiation of the donor T cells. Donor naïve $CD4^+$ T cells differentiate into Th1, Th2 and Th17 effector cells releasing interleukin (IL) 2/interferon-γ (IFNγ), IL4/IL10 and IL17 respectively (Antin and Ferrara, 1992; Nikolic *et al.,* 2000; Carlson *et al.,* 2009). Donor naïve $CD8^+$ T cells differentiate into cytotoxic T-lymphocytes releasing perforin, granzymes and Fas ligand (Graubert *et al.,* 1997; Shresta *et al.,* 1998). The role of donor memory $CD4^+$ and $CD8^+$ T cells is less clear since studies in mice have suggested that memory T cells do not cause aGvHD (Zhang *et al.,* 2004; Beilhack *et al.,* 2005).

C) **Host cell damage**: The pro-inflammatory response causes migration and activation of effector cells, including NK cells, neutrophils, macrophages and cytotoxic T cells causing tissue damage, cell death and apoptosis, classically involving epithelial tissues (Welniak *et al.,* 2007; Blazar *et al.,* 2012).

In HLA-mismatched transplants, the main host antigens driving GvHD are the HLA antigens and the risk of GvHD is linked to the degree of mismatch (Anasetti and Hansen, 1994; Welniak *et al.,* 2007). However, even in HLA-matched transplants, GvHD still occurs and is due to differences between the donor and host in minor histocompatibility antigens i.e. polymorphic peptides encoded outside the major histocompatibility complex (MHC) and presented by the HLA antigens (Goulmy *et al.,* 1996). For example, HY is a male specific antigen expressed on all cells and may mediate GvHD in female-to-male transplants (Miklos *et al.,* 2005; Ferrara *et al.,* 2009). The main risk factors for aGvHD are HLA-mismatch, unrelated donors, female-to-male transplants, intensive conditioning and increasing recipient age (Ferrara *et al.,* 2009; Jagasia *et al.,* 2012).

B) CLINICAL FEATURES

Acute GvHD classically causes a maculopapular skin rash, jaundice and/or diarrhoea (Filipovich *et al.,* 2005; Ferrara *et al.,* 2009). The skin is the most commonly affected site (60-80%), followed by GI tract (50-56%) and the liver (20-50%) (Ferrara *et al.,* 2009). However, patterns of aGvHD may differ between different transplant regimens and/or conditioning protocols and a recent retrospective analysis of Oxford's RIC sibling allografts found the GI tract to be the most frequently affected site (65%) (Medd *et al.,* 2011). The characteristic features of aGvHD are:

A) **Skin Rash**: Erythematous, maculopapular rash that can involve the whole body, classically but not exclusively seen on the palms and soles. This can progress to bullae and ulceration (Ferrara *et al.,* 2009). Histology shows apoptosis in the epidermal basal layer, lichenoid inflammation, vacuolar change and lymphocytic infiltration (Shulman *et al.,* 2006).

B) **Liver**: Cholestatic jaundice with histological features of bile duct destruction, cholestasis, lobular and portal lymphocytic infiltration (Shulman *et al.,* 2006; Ferrara *et al.,* 2009).

C) **Gastrointestinal Tract**: Presents with nausea, anorexia and watery diarrhoea that can progress to large volume bloody diarrhoea and severe abdominal pain/ileus (Ferrara *et al.,* 2009). Histological features include apoptotic enterocytes, lymphocytic infiltration, crypt destruction and abscesses (Shulman *et al.,* 2006)

C) GRADING

The grading criteria of aGvHD was originally established by Glucksberg and colleagues (1974) and later modified by the Consensus Conference (Przepiorka *et al.,* 1995) (Table 1.1)

Stage	Skin rash	Liver (Bilirubin)	Gut (Diarrhoea)
1	<25%	34-50 mmol/l	>500ml or nausea
2	25-50%	51-102 mmol/l	1000-1500ml
3	>50%	103-255 mmol/l	>1500ml
4	Erythroderma	>255 mmol/l	Severe pain/ileus

Functional Grade	Skin	Liver	Gut
I	Stage 1-2	0	0
II	Stage 3 or	Stage 1 or	Stage 1
III-IV	Stage 4 or	Stage 2-4 or	Stage 2-4

Table 1.1: Consensus Conference Grading of Acute GvHD

Grading of aGvHD using the Consensus Conference Classification (Przepiorka *et al.,* 1995). Individual stage for the skin, liver and gut (1-4) and the overall functional grade (0-IV).

D) PREVENTION AND TREATMENT

Acute GvHD can be reduced by careful selection of the donor, modification of the graft and reducing the intensity of conditioning to limit tissue damage. However, primary systemic immunosuppression using pharmacological agents is also required, particularly early post-transplant. The most common immunosuppressant drugs are the calcineurin inhibitors, Ciclosporin (CsA) or Tacrolimus (TAC), and the anti-folate drug, Methotrexate (Storb *et al.,* 1986; Mrsic´ *et al.,* 1990). Ciclosporin and Tacrolimus block calcineurin, a calcium-dependent phosphatase enzyme that activates the transcription factor Nuclear Factor of Activated T cells (NFAT), and inhibits the

production of IL2 (Matsuda and Koyasu, 2000; Dumont, 2000). Since IL2 is required for T-cell activation, proliferation and cytokine secretion, blocking its production causes significant immunosuppression. In the absence of significant GvHD, calcineurin inhibitors can usually be withdrawn from three months post-transplant. Methotrexate is usually given at low doses early post-transplant and its omission from T-replete conditioning regimens has been associated with an increased incidence of GvHD (Kumar *et al.,* 2002; Medd *et al.,* 2011).

First line treatment of aGvHD is with corticosteroids, which have both anti-inflammatory and immunosuppressive actions (Deeg *et al.,* 2007). Mild aGvHD (grade 1) of the skin and mouth may be treated with topical corticosteroids only. However, grade II-IV aGvHD usually requires systemic treatment, typically methylprednisolone 1-2mg/kg/day, tapered according to response (Dignan *et al.,* 2012b). Approximately 50% of patients fail to respond to steroids. 'Steroid-refractory GvHD' is usually defined as progression after three days, no change after five to seven days or incomplete response after 14 days (Martin *et al.,* 1990; Macmillan *et al.,* 2002). Failure to respond to steroids is associated with poor prognosis with mortality as high as 95% (Weisdorf *et al.,* 1990; Arai *et al.,* 2002). In these patients, second line therapy is required, although the optimal treatment remains unclear because of the lack of randomised controlled trials and small numbers with limited responses. Many agents have been tried and current guidelines recommend extra-corporeal photopheresis, anti-tumour necrosis factor α antibodies, mammalian target of rapamycin inhibitors, mycophenolate mofetil or interleukin-2 receptor antibodies (Dignan *et al.,* 2012b).

1.4.2 CHRONIC GRAFT-VERSUS-HOST DISEASE

A) CLINCAL FEATURES AND CLASSIFICATION

Chronic GvHD (cGvHD) is a multi-systemic disorder with many features similar to autoimmune disease. It was conventionally defined as any GvHD occurring after 100 days post-transplant and was classified into limited or extensive based on a long-term study of 20 Seattle patients (Shulman *et al.,* 1980) (Table 1.2). In 2005, the National Institutes of Health (NIH) consensus proposed a new classification and grading system to define the extent and severity of cGvHD and proposed standardised indications for treatment (Filipovich *et al.,* 2005). In particular, cGvHD was defined by the presence of at least one diagnostic symptom or at least one distinctive feature confirmed by biopsy or other tests, without any features of aGvHD, irrespective of the time (Table 1.3). A new global assessment of cGvHD severity was also defined based upon the number of organs involved and the involvement of each affected organ (Mild: one or two organs scoring 1 (except lung); Moderate: three or more organs scoring 1, any organ scoring 2, lung scoring 1; Severe: any organ scoring 3, lung scoring 2) (Table 1.4).

Several groups have retrospectively reviewed and reclassified their GvHD data using the NIH Consensus Criteria. While some centres have shown a significant difference in non-relapse mortality (NRM) and overall survival (OS) between those patients with late acute and classic chronic GvHD, other groups have not (Arora *et al.,* 2009; Vigorito *et al.,* 2009). Likewise, the clinical significance of the new grading of mild, moderate and severe cGvHD still remains unclear. However, as retrospective studies are often limited by the availability of clinical and histopathology data, the Chronic GvHD Consortium has now established a multi-centre prospective cohort study to further evaluate this new classification (Arai *et al.,* 2011; Chronic GvHD Consortium, 2011). Initial results from the first 298 patients suggest that the global severity scores for cGvHD are significantly associated with NRM and overall survival with reported two year OS rates of 62%, 86% and 97% for severe, moderate and mild cGvHD respectively (Chronic GvHD Consortium, 2011). Further prospective evaluation of the NIH Consensus Criteria will be required.

Limited
Either or both
Localised skin involvement
Hepatic dysfunction due to chronic GvHD
Extensive
Either
Generalised skin involvement
Or
Localised skin involvement and/or hepatic dysfunction due to chronic GvHD
Plus (at least one)
Liver histology showing chronic aggressive hepatitis, bridging necrosis or cirrhosis
Involvement of eye (Schirmer's test with <5mm wetting)
Involvement of minor salivary glands or oral mucosa demonstrated on labial biopsy
Involvement of any other target organ

Table 1.2: Classification of Chronic GvHD

Symptoms and signs defining limited and extensive chronic GvHD (Shulman *et al.,* 1980).

Organ	Diagnostic	Distinctive
Skin	Poikiloderma; lichen planus; sclerosis; morphea	Depigmentation
Nails		Dystrophy; longitudinal ridging /splitting; onycholysis, pterygium; nail loss
Hair/scalp		New onset alopecia; scaling papulosquamous lesions
Mouth	Lichen, hyperkeratotic plaques, restriction	Xerostomia; mucocele; mucosal atrophy; pseudomembranes, ulcers
Eyes		New onset dry, gritty or painful; conjunctivitis; keratoconjunctivitis sicca; confluent areas of punctate keratopathy
Genitalia	Lichen planus; scarring or stenosis.	Erosions; fissures; ulcers
GI Tract	Oesophageal web; strictures or stenosis in the upper mid third	
Lung	Bronchiolitis with lung biopsy	Bronchiolitis with PFTs and radiology
Muscles	Fasciitis; joint stiffness; contractures secondary to sclerosis	Myositis or polymyositis

Table 1.3: NIH Consensus Conference Features of Chronic GvHD

Diagnostic and distinctive symptoms and signs of cGvHD as proposed by the NIH Consensus Conference Classification of Chronic GvHD (Filipovich *et al.,* 2005).

	Score 0	Score 1	Score 2	Score 3
Performance	Asymptomatic Fully active KPS 100%	Restricted in strenuous exercise KPS 80-90%	Self-care >50% of time KPS 60-70%	Self-care <50% of time KPS <60%
Skin	No symptoms	<18% BSA No sclerotic features	19-50% BSA Superficial sclerotic features	>50% BSA Deep sclerotic features
Mouth	No symptoms	Mild but not limiting intake	Moderate with limitation of intake	Severe with major limitation of intake
Eyes	No symptoms	Mild dry eye not affecting ADL	Moderate dry eye without visual impairment	Severe dry eye affecting ADL or loss of vision
GI Tract	No symptoms	Symptoms without weight loss (<5%)	Symptoms, weight loss (5-15%)	Symptoms, weight loss (>15%)
Liver	Normal	High Bil/AST < 2 x normal	Bil > 3 or Bil/AST 2.5 x normal	Bil or AST > 5 x normal
Lungs	No symptoms FEV1>80%	Mild FEV1 60-79%	Moderate FEV1 40-59%	Severe FEV1<39%
Joints/Fascia	No symptoms	Mild tightness Normal or mild decreased ROM Not affecting ADL	Tightness with moderate decreased ROM and mild limitation of ADL	Contractures with significant decrease ROM and limitation of ADL
Genital Tract	No symptoms	Mild and no effect on coitus and minimal discomfort with examination	Moderate and with mild dyspareunia or discomfort with examination	Advanced and severe pain with coitus or inability to insert speculum

Table 1.4: NIH Consensus Conference Grading of Chronic GvHD

Summary of symptoms/signs used to grade cGvHD (Filipovich *et al.,* 2005). KPS (Karnofsky Performance Score), BSA (body surface area), ADL (activities daily living), FEV1 (forced expiratory volume), Bil (Bilirubin), AST (Aspartate transaminase), ROM (range of movement).

The incidence of cGvHD varies widely (5-75%) reflecting the diversity in the study populations, transplant and conditioning protocols, GvHD prophylaxis and the definitions of cGvHD (Remberger *et al.,* 2005; Sabry *et al.,* 2009; Castagna *et al.,* 2011; Marsh *et al.,* 2011). The most consistently reported risk factors for cGvHD are the presence of aGvHD, use of PBSCH, HLA-mismatch, female-to-male transplants, unrelated donors and increasing recipient and donor age (Wojnar *et al.,* 2006; Eapen *et al.,* 2007; Moon *et al.,* 2010; Alsultan *et al.,* 2011; Flowers *et al.,* 2011). Furthermore, several risk factors associated with increased mortality from cGvHD have also been identified, including progressive onset, extensive skin involvement, thrombocytopenia at diagnosis, high bilirubin and low Karnofsky score (Lee *et al.,* 2002; Akpek *et al.,* 2003). Recently, data from 5353 patients with cGvHD were used to develop a GvHD risk score by incorporating 10 patient, transplant and cGvHD variables (Arora *et al.,* 2011). Patients with GvHD were stratified into six risks groups with five year OS ranging from 91% (85-97%) in the lowest risk group to 4% (0-9%) in the highest risk group. Although further testing is required, these scores may provide useful methods to predict survival and direct targeted treatment.

B) TREATMENT

Treatment for cGvHD is often prolonged and is a balance between reducing the morbidity and mortality from the GvHD against increasing the risk of infection or side effects from the therapy. Retrospective analysis of 80 RIC T-replete sibling allografts performed in Oxford demonstrated that the median duration of immunosuppression therapy for cGvHD was 41 months (Medd *et al.,* 2011). Similarly, Flowers and colleagues (2002) reported a median duration of cGvHD treatment of 2 years for BM transplants and 3.5 years for PBSCH transplants. Although mild symptoms may be treated with topical treatment only, systemic therapy is recommended in those patients with moderate to severe chronic GvHD, based on the NIH Consensus grading (Filipovich *et al.,* 2005). First line therapy is corticosteroids (1mg/kg/day) and/or prolonged use of calcineurin inhibitors (Dignan *et al.,* 2012a). However, although calcineurin inhibitors may reduce the total steroid dose required, there are still no randomised clinical trials demonstrating clear survival benefit of a using

this combined approach (Koc *et al*., 2002; Martin *et al*., 2011). Steroids are then reduced, to minimise steroid related side effects, while maintaining control of GvHD. For those patients that fail to respond or progress on treatment, second line therapy will be required. However, as with aGvHD, the optimal second line therapy remains unclear and may vary depending on the site and organs involved. Current UK guidelines recommend the use of extra-corporeal photopheresis (skin, mouth, liver), mammalian target of rapamycin inhibitors, Rituximab (chimeric monoclonal antibody against CD20) (skin, musculoskeletal), Pentostatin (purine analogue) or Imatinib (tyrosine kinase inhibitor) (skin, lung) (Dignan *et al.,* 2012a).

1.5 OTHER COMPLICATIONS OF HAEMATOPOIETIC STEM CELL TRANSPLANTATION

1.5.1 FAILED ENGRAFTMENT

Failure of the donor HSC to engraft in the BM niche is a serious but relatively infrequent complication of alloHSCT (Shaw *et al.,* 2005). Primary graft failure, defined as not achieving a neutrophil count of greater than $0.5x10^9$/l by 28 days post-transplant, occurs due to the host's native immune system rejecting the new graft (HvG) (Craddock and Chakraverty, 2011). It has a higher incidence in HLA-mismatched transplants (5%) where the presence of donor HLA-specific antibodies is significantly associated with graft failure (Mattsson *et al.,* 2008; Yoshihara *et al.,* 2012). Secondary graft failure occurs when the new graft fails following an initial period of engraftment and is usually due to late HvG rejection, infection or drug toxicity (Craddock and Chakraverty, 2011). Mortality from graft failure is high due to the prolonged neutropenia but patients may be rescued by a second HSCT.

1.5.2 RELAPSE

Disease relapse remains a major complication of alloHSCT. The disease type, the status at transplant and presence of high risk factors e.g. poor risk cytogenetics in acute myeloid leukaemia, are all associated with the incidence of relapse (Craddock *et al.,* 2010). Full intensity conditioning decreases the risk of relapse by reducing residual tumour burden. However, overall survival is often not improved due to the higher TRM (Barrett and Battiwalla, 2010). Conversely, factors that reduce the intensity of the GvT response, such as T-cell depletion or increased exposure to post-transplant immunosuppression, increase relapse (Craddock *et al.,* 2010; Soiffer *et al.,* 2011).

As well as the traditional morphological blood film and bone marrow examination, disease relapse may now be determined using molecular or cytogenetic testing and detecting loss of donor chimerism. These sensitive tests enable early detection of relapse, allowing intensification of the

GvT response before morphological relapse has occurred. The GvT effect can be maximised by rapid withdrawal of immunosuppression or administration of more donor lymphocytes: both strategies, however, are associated with a significant risk of GvHD and their success is dependent on the type of disease and the extent of the tumour burden (Bloor *et al.,* 2008; Roddie and Peggs, 2011).

1.5.3 INFECTION

Over the last 20 years, significant improvements in TRM have been due to improved supportive care, including the use of antibiotic, antiviral and antifungal therapies. However, gram-negative and gram-positive bacterial infections remain common during the early neutropenic period and require prompt treatment with antibiotics. Upon neutrophil engraftment, patients are treated with pneumocystis prophylaxis (Co-trimoxazole or Pentamidine) followed by life-long Penicillin against encapsulated bacteria. In relation to viral infections, CMV used to be the most common cause of infectious mortality following alloHSCT (Craddock and Chakraverty, 2011). However, CMV prophylaxis (Aciclovir) and CMV DNA polymerase chain reaction (PCR) monitoring with pre-emptive treatment (Ganciclovir/Valganciclovir) have significantly reduced morbidity and mortality (Hebart and Einsele, 2004). The main risk factors for CMV infection post alloHSCT remain CMV seropositive recipients, T-cell depletion, GvHD and prolonged immunosuppression (Nachbaur *et al.,* 2003; Ljungman *et al.,* 2006). Fungal infections also cause significant morbidity post-transplant and are associated with profound neutropenia, immunosuppression, GvHD and corticosteroid therapy (Fukuda *et al.,* 2003; Kobayashi *et al.,* 2007). Prophylaxis with Fluconazole has reduced Candidiasis infection while early treatment with liposomal Amphotericin or triazole antifungals for antibiotic resistant fevers and/or clinical suspicion of fungal infection has improved mortality (Robenshtok *et al.,* 2007; Pagano *et al.,* 2010).

1.6 REGULATORY T CELLS

Over 40 years ago, the existence of a population of thymic-derived suppressor T cells with the ability to induce peripheral immune tolerance was first proposed (Germain *et al.,* 2008). However, this hypothesis failed to gain widespread acceptance, partly due to the absence of specific phenotypic markers. However, in 1995, Sakaguchi and colleagues (1995) described a population of $CD4^+$ cells that expressed CD25, the IL-2 receptor alpha-chain. When $CD4^+CD25^-$ cells, isolated from BALB/c nu/+ mice, were transferred into BALB/c nu/nu mice, they induced a widespread autoimmune disease that could be prevented by co-transfer of donor $CD4^+CD25^+$ cells. The $CD4^+CD25^+$ T cells subsequently became known as regulatory T cells (Tregs) and in 2001, human $CD4^+CD25^+$ Tregs were demonstrated (Shevach, 2001; Stephens *et al.,* 2001). These seminal findings initiated widespread interest in human Tregs over the next decade, particularly in the areas of autoimmunity, solid organ transplantation and alloHSCT.

1.6.1 HUMAN TREG MARKERS

Although Tregs were initially characterised by their high expression of CD25, it was known that CD25 was also expressed in activated human T cells, B cells, macrophages and some neoplastic cells (Erber and Mason, 1988; Zola *et al.,* 1989; Choy *et al.,* 1990). In addition, there was no universal definition of what constituted $CD25^{high}$ expression, making direct comparison between studies difficult. In 2003, the transcription factor forkhead box P3 (Foxp3) was found to be specifically expressed in natural $CD4^+CD25^+$ Tregs (nTregs) in mice and is thought to be the master regulator of Treg differentiation and function (Fontenot *et al.,* 2003; Hori *et al.,* 2003). Scurfy mice have functional mutations and/or deficiency of Foxp3, causing a deficiency in $CD4^+CD25^+$ T cells and severe multi-systemic autoimmune disorder (Brunkow *et al.,* 2001; Fontenot *et al.,* 2003; Lahl *et al.,* 2007). Conversely, ectopic expression of Foxp3 in mouse naïve $CD4^+$ T cells produces suppressive functional activity as well as expression of other nTreg phenotypic markers e.g.

cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid induced TNF receptor related protein (GITR) (Fontenot *et al.,* 2003; Hori *et al.,* 2003). Human FOXP3 was demonstrated in human $CD4^{+}CD25^{+}$ Tregs at both the transcript and protein level and mutations in the FOXP3 gene cause the IPEX syndrome i.e. Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (Bennett *et al.,* 2001; Yagi *et al.,* 2004, Roncador *et al.,* 2005). FOXP3 is a member of the forkhead/winged-helix transcription factors and its gene is located on the short arm of the X chromosome at position Xp11.23 (49,106,897-49,121,288; -1). This gene contains eleven coding exons with three 5' non-encoding exons and the *FOXP3* promoter has been located to -6221bp upstream of the translational start site (Mantel *et al.,* 2006; Lal and Bromberg, 2009). As well as TATA, GC and CAAT boxes, the promoter also contains important binding sites for the transcription factors NFAT and activator protein 1 (AP1). Downstream from the *FOXP3* promoter, are several highly conserved non-coding loci that enhance and/or stabilise FOXP3 expression. A transforming growth factor-β (TGFβ) sensitive enhancer contains binding sites for the transcription factors NFAT and SMAD3, the latter being induced by binding of TGFβ to its surface receptor (Tone *et al.,* 2008). A 'Treg-specific-demethylated region' (TSDR) is also thought to be important for stable FOXP3 expression and contains binding sites for the transcription factors cAMP response element-binding (CREB) and STAT5. Crucially, DNA cytosine-guanine (CpG) dinucleotides within this region are demethylated in $CD4^{+}CD25^{high}FOXP3^{+}$ nTregs, allowing transcription factors to bind to the DNA and enhance FOXP3 expression (Baron *et al.,* 2007; Huehn *et al.,* 2009).

In contrast to mice, several variants of the FOXP3 protein have been found in human $CD4^{+}$ T cells, including the 1296bp full length version, an 1191bp isoform lacking the 105bp exon 2 (FOXP3Δ2), an 1215bp isoform lacking the 81bp exon 7 (FOXP3Δ7) and an isoform lacking both exons 2 and 7 (FOXP3Δ2Δ7) (Smith *et al.,* 2006). The role and contribution of each isoform in Treg differentiation and function remains unclear, particularly as the FOXP3Δ2Δ7 isoform may lack suppressive function (Mailer *et al.,* 2009).

Although the discovery of FOXP3 allowed significant advances in Treg biology, FOXP3 cannot be used to isolate functional $CD4^+CD25^{high}FOXP3^+$ Tregs because it is an intracellular protein. As with CD25, many other surface markers expressed on $CD4^+CD25^{high}FOXP3^+$ Tregs, such as CTLA-4 and GITR, are also expressed on non-Treg cells and therefore, lack Treg specificity. However, using the expression of more than one surface antigen has improved the purity of human $CD4^+CD25^{high}FOXP3^+$ Treg isolation over using CD25 alone. Expression of the alpha-chain of the IL7-receptor (CD127) inversely correlates with FOXP3 expression and $CD4^+$ T cells expressing low levels of the CD127 contain a high proportion of $FOXP3^+$ suppressive cells (Liu *et al.,* 2006; Seddiki *et al.,* 2006b). Similarly, the α-chain of the integrin VLA-4 (α4β1) (CD49d), is expressed on the majority of pro-inflammatory mononuclear cells but not on $CD4^+FOXP3^+$ Tregs. Therefore, combining these two molecules to isolate $CD25^+CD127^-CD49d^-$ cells may improve the purity of $CD4^+CD25^{high}FOXP3^+$ Treg isolation (Kleinewietfeld *et al.,* 2009). Another surface antigen, Glycoprotein A repetitions predominant (GARP/LRRC32), is a transmembrane protein that has been reported to be expressed on activated human $CD4^+CD25^{high}FOXP3^+$ Tregs but not on B cells, CD8 T cells, monocytes, NK cells, NKT cells, dendritic cells or activated $CD25^+$ T cells (Wang *et al.,* 2009). GARP is believed to be a carrier for TGFβ latency associated peptide and its expression correlates with suppressive function making it a possible marker for functional Tregs (Tran *et al.,* 2009). However, its widespread use in *ex vivo* $CD4^+CD25^{high}FOXP3^+$ Treg isolation has not been reported, probably because Tregs must be activated for it to be expressed.

An additional reason for seeking other specific markers for human Tregs is that in humans, unlike mice, FOXP3 expression has been reported in activated conventional T cells. *In vitro*, activated $CD4^+CD25^-$ T cells showed transient expression of FOXP3, at both the messenger ribonucleic acid (mRNA) and protein level (Pillai *et al.,* 2007; Wang *et al.,* 2007). Although early studies demonstrated that these $FOXP3^+$ cells could suppress *in vitro* proliferation of autologous $CD4^+CD25^-$ cells, other groups have since shown that activation-induced FOXP3 expression does not confer suppressive function (Pillai *et al.,* 2007; Wang *et al.,* 2007). Therefore, FOXP3 may not

necessarily be a specific marker of human Tregs, particularly under conditions that induce T-cell activation.

As our understanding of Tregs has improved, it has become clear that Tregs can be divided into subpopulations. Like conventional T cells, $CD4^+CD25^+$ Tregs exist in both naïve and effector forms in cord blood, the thymus, peripheral blood, lymph nodes and spleen (Valmori *et al.,* 2005; Seddiki *et al.,* 2006a). The presence of naïve $CD4^+CD25^{bright}CD45RA^+$ Tregs declines with age and in adult blood the ratio of $CD45RA^+$:$CD45RO^+$ Tregs is approximately 2:1 (Seddiki *et al.,* 2006a). Miyara and colleagues (2009) further characterised $CD4^+FOXP3^+$ T cells based upon the expression of CD45RA and the level of FOXP3 expression. *In vivo*, naïve $CD45RA^+FOXP3^{low}$ Tregs are in a resting state with low numbers expressing the proliferation marker Ki-67. Upon TCR stimulation, naïve $CD45RA^+FOXP3^{low}$ Tregs up-regulate FOXP3 and CD25, differentiate into effector $CD45RA^-FOXP3^{high}$ Tregs and are able to proliferate. Effector $CD45RA^-FOXP3^{high}$ Tregs are activated cells, express higher levels of Ki-67 and CTLA-4 and were highly proliferative *in vivo*. Both populations expressed low levels of IL2 and IFNγ and were able to suppress the proliferation of $CD4^+CD25^-$ responder cells. *In vitro*, these cell populations exhibited different characteristics: the naïve $CD45RA^+FOXP3^{low}$ Tregs were highly proliferative, became $FOXP3^{high}$ and were suppressive; the effector $CD45RA^-FOXP3^{high}$ Tregs were suppressive, proliferated poorly and underwent apoptosis. A third population of $CD45RA^-FOXP3^{low}$ T cells were also identified but expressed high levels of pro-inflammatory cytokines and were not suppressive, in keeping with $FOXP3^+$ activated T cells.

1.6.2 TREG DEVELOPMENT

In both humans and mice, naturally occurring Tregs (nTregs) are produced in the thymus, as demonstrated in the early studies that showed neonatal thymectomy in mice around three days caused profound depletion of the peripheral $CD4^+CD25^+$ cells and a severe autoimmune disease which could be prevented by early administration of $CD4^+CD25^+$ cells (Asano *et al.,* 1996). In

addition, naïve $CD4^+CD25^+CD45RA^+CD45RO^-FOXP3^+$ Tregs express platelet endothelial cell adhesion molecule (PECAM-1) (CD31), a cell marker suggestive of recent thymic emigration (Haas *et al.,* 2007). The conditions required for Treg development in the thymus still remain unclear. TCR signalling appears crucial as $CD4^+CD25^+Foxp3^+$ Tregs arise from a population of thymocytes with high affinity TCR to self-antigen and avoid negative selection within a specific region of the thymic medulla called the Hassall's corpuscles (Jordan *et al.,* 2001; Watanabe *et al.,* 2005; Yuan and Malek, 2012). Similarly, CD28 co-stimulation, CD40L and Il2 are also critical for Treg generation in the thymus. CD28, B7 and/or Il2 deficient mice contain significantly lower numbers of Treg precursors and functional $CD4^+CD25^+Foxp3^+$ Tregs in the thymus and periphery (Tai *et al.,* 2005; Bayer *et al.,* 2007). It has been proposed that TCR and CD28 co-stimulation make Treg thymocyte precursors sensitive to IL2, which then induces CD25 and Foxp3 up-regulation (Burchill *et al.,* 2008; Yuan and Malek, 2012). Although Foxp3 expression is required for functional Tregs, the relationship between Foxp3 expression and Treg commitment remains uncertain, raising questions as to at what stage Treg fate is established. Foxp3 expression has been detected in $CD4^+CD8^+$ thymocytes before commitment to the single positive $CD4^+CD8^-$ stage (Nunes-Cabaço *et al.,* 2010). The absence of Foxp3 does not prevent the development of the Treg 'genetic signature', although these cells do lack suppressive function (Lin *et al.,* 2007). Therefore, it has been hypothesised that Treg commitment in the thymus may not initially require Foxp3 expression, but that is it needed later to confer Tregs with suppressive function (Gavin *et al.,* 2007).

$CD4^+CD25^{high}Foxp3^+$ Tregs can also be produced in the periphery by induction of FOXP3 in naïve $CD4^+$ $FOXP3^-$ T cells (inducible Tregs, iTregs). In mice, iTregs can be induced *in vivo*, as demonstrated by the detection of $CD45.1^+CD4^+CD25^+$ suppressive cells after infusion of naïve $CD45.1^+CD4^+CD25^-$ into $CD45.2^+$ mice (Liang *et al.,* 2005). This effect was still seen in thymectomized mice but is dependent on CD28 co-stimulation (Liang *et al.,* 2005; Guo *et al.,* 2008). Both *in vitro* and *in vivo* studies in mice have shown that TGFβ further enhances TCR-induced Foxp3 expression in naïve $CD4^+CD25^-$ T cells. The induced $Foxp3^+$ cells have a Treg phenotype, are hypoproliferative, have suppressive function and do not produce Th1/Th2 pro-

inflammatory cytokines (Chen *et al.,* 2003a, Fu *et al.,* 2004). In humans, the ability to produce functional iTregs remains less clear and the extent to which inducible Tregs contribute to peripheral immune tolerance in humans is unknown. Although *in vitro* TCR stimulation of naïve $CD4^+CD25^-$ in the presence of TGFβ or retinoic acid can induce FOXP3 expression, this may not induce suppressive function (Allan *et al.,* 2007; Tran *et al.,* 2007; Nagar *et al.,* 2008). However, Lu and colleagues (2010) have reported that the combination of suboptimal TCR stimulation of human naïve $CD4^+$ T cells with TGFβ and all trans-retinoic acid (ATRA) can produce $CD4^+CD25^+FOXP3^+$ T cells *in vitro*, with both phenotype and function similar to $CD4^+CD25^{high}$ nTreg. Similarly, it has been reported that the addition of Rapamycin to *in vitro* TCR/TGFβ stimulation of human naïve $CD4^+CD25^-$ T cells can produce $FOXP3^+$ Treg cells with potent *in vitro* and *in vivo* suppressive function (Valmori *et al.,* 2006; Hippen *et al.,* 2011). The stability of the induced $FOXP3^+$ Tregs remains to be fully elucidated but importantly these cells produced similar inhibition of GvHD in a xenogenic model compared to $CD4^+CD25^{high}$ nTregs (Hippen *et al.,* 2011). Although it remains to be determined if these strategies can increase human iTregs *in vivo*, they may provide a useful strategy for developing iTregs for cellular therapy.

1.6.3 TREG FUNCTION

Tregs are able to produce peripheral immune tolerance by inhibiting the proliferation and cytokine secretion of T, B, NK, NK-T and antigen presenting cells. Many mechanisms for the suppressive function of Tregs have been proposed, including cell contact dependent and independent methods. However, the contribution of each mechanism *in vivo* remains uncertain or whether Tregs use different methods depending on the local environment and the cells being inhibited (Sakaguchi *et al.,* 2009). Of note, many of these hypotheses have been based upon *in vitro* studies and it remains unclear whether they occur *in vivo*. Likewise, the optimal *in vitro* assay to assess Treg function remains controversial as the assays may not reflect *in vivo* activity (Sakaguchi *et al.,* 2010).

Cell contact independent mechanisms include the sequestration of IL2 and the production of inhibitory cytokines such as IL10 and IL35. IL2 has a critical role in Treg biology since it is involved in $CD4^+CD25^+Foxp3^+$ Treg development, is required for maintenance of nTregs and enhances the expression of functional molecules such as CTLA-4 (Bayer *et al.,* 2007; Barron *et al.,* 2010). As Tregs express high levels of CD25, Tregs may preferentially absorb IL2, causing Bim-mediated apoptosis in effector cells due to relative cytokine deficiency (Pandiyan *et al.,* 2007). In keeping with this hypothesis, high dose IL2 abrogates $CD4^+CD25^+$ Treg function (Takahashi *et al.,* 1998). However, in contrast, Tran and colleagues (2009b) demonstrated that blocking IL2 binding does not affect suppressive function. IL10 (human cytokine synthesis inhibitory factor (CSIF)) is an anti-inflammatory cytokine that is important for maximal $CD4^+CD25^+Foxp3^+$ Treg function (Collison *et al.,* 2009). $CD4^+CD25^+Foxp3^+$ Treg-produced IL10 can suppress Th17 cell responses and is important for inhibiting IFNγ production by $CD4^+$ T cells in inflamed tissues (Chaudhry *et al.,* 2011; Sojka and Fowell, 2011).

Cell contact dependent mechanisms include CTLA-4, cell surface expressed TGFβ and granzyme mediated cell apoptosis (Shevach, 2009). CTLA-4 on the surface of $CD4^+CD25^+$ Tregs can down-regulate CD80 and CD86 expression on APC, preventing activation of effector T cells though TCR-MHC-APC interactions (Misra *et al.,* 2004). In mice, CTLA-4 deficiency did not prevent development or maintenance of $Foxp3^+$ Tregs but the mice developed a lethal autoimmune disorder. Specific CTLA-deficient $Foxp3^+$ Tregs were unable to prevent increased CD80 and CD86 expression in dendritic cells upon stimulation and caused lethal autoimmunity (Wing *et al.,* 2008). The contribution of TGFβ to Treg function is less clear as Treg function is not inhibited by anti-TGFβ (Shevach, 2009). Activated $FOXP3^+$ Tregs do, however, express GARP and latent TGFβ on their cell surface (Tran *et al.,* 2009). As TGFβ can induce FOXP3 expression in TCR stimulated non-Tregs, GARP-latent TGFβ complex may induce $FOXP3^+$ Tregs in areas of on-going inflammation i.e. infectious tolerance. However, small interfering RNA (siRNA) knockdown of TGFβ only produced a partial reduction of suppression, in keeping with the hypothesis that Treg suppression probably acts through multiple pathways (Shevach, 2009).

1.7 REGULATORY T CELLS IN ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

In alloHSCT, a delicate balance must be attained between adequate immune suppression and sufficient immune activity. The fundamental aim of the transplant immunotherapy is to prevent graft rejection and GvHD whilst allowing a GvT response and protection from bacterial, fungal and viral infections. Although many conventional immunotherapies reduce GvHD, they increase the risk of disease relapse and infection and are associated with side effects and toxicity (Furlong *et al.,* 2000; Kobayashi *et al.,* 2007; Craddock *et al.,* 2010). Therefore, new approaches to alloHSCT and/or novel immunotherapies are still required.

Over the last 10 years, there has been interest in the role and potential use of Tregs in alloHSCT. In mouse GvHD models, depletion of $CD4^+CD25^+$ Tregs from BM grafts given to lethally irradiated MHC-mismatched mice significantly increased the severity and mortality from GvHD (Johnson *et al.,* 2002; Taylor *et al.,* 2002). Conversely, co-transfer of a one to one ratio of $CD4^+CD25^+$ Tregs with $CD4^+CD25^-$ effector T cells from C57BL/6 mice into MHC mismatched BALB/c mice prevented the lethal GvHD seen with the transfer of $CD4^+CD25^-$ effector T cells alone (Hoffmann *et al.,* 2002). Even more significant was the discovery that isolated $CD4^+CD25^+$ Tregs, co-transferred with $CD4^+CD25^-$ conventional T cells to an MHC mismatched mouse with leukaemia, were able to prevent GvHD but did not prevent an effective GvT response (Edinger *et al.,* 2003). Similar responses have since been demonstrated using engineered Tregs, either by lentiviral expression FOXP3 or induction of FOXP3 using hypomethylating agents (Cao *et al.,* 2010; Choi *et al.,* 2010). How Tregs prevent GvHD but maintain GvT remains to be determined but it may be that different cell types/immune pathways have differing sensitivity to Treg suppression (Edinger *et al.,* 2003; Li *et al.,* 2009; Cao *et al.,* 2010).

1.7.1 REGULATORY T CELLS AND GRAFT-VERSUS-HOST DISEASE

Although the adoptive transfer of Tregs in mouse transplant models has shown great promise, the relationship between Tregs and GvHD in human alloHSCT is less clear. Based upon mouse data, it was proposed that GvHD in human alloHSCT could be due to absolute and/or relative deficiency of Tregs post-transplant (Clark *et al.,* 2004; Stanzani *et al.,* 2004). This hypothesis has been supported by several studies that demonstrated that there are reduced numbers of $CD4^{+}CD25^{high}$ cells, $CD4^{+}FOXP3^{+}$ cells, $CD4^{+}CD25^{high}FOXP3^{+}$ cells or FOXP3 mRNA in the peripheral blood of patients with GvHD compared to non-GvHD alloHSCT recipients (Miura *et al.,* 2004; Zorn *et al.,* 2005; Rezvani *et al.,* 2006; Rieger *et al.,* 2006; Schneider *et al.,* 2006; Magenau *et al.,* 2010). Magenau and colleagues (2010) demonstrated that $CD4^{+}CD25^{hi}FOXP3^{+}$ cell frequency at the start of the GvHD reduced linearly with increasing GvHD severity, correlated with the maximum grade of GvHD and predicted response to treatment. Other groups, however, have found increased numbers of $CD4^{+}CD25^{+}$ cells in patients with cGvHD while other studies were unable to demonstrate any correlation between $CD4^{+}CD25^{+}FOXP3^{+}$ cells or FOXP3 mRNA levels and the presence of GvHD (Clark *et al.,* 2004; Sanchez *et al.,* 2004; Meignin *et al.,* 2005; Arimoto *et al.,* 2007). The discrepancy between these findings may be due to the different criteria used to define Tregs, particularly since it is now recognised that CD25 and FOXP3 can be up-regulated in non-Tregs. While some studies expressed Treg numbers as the percentage of the total lymphocytes, others expressed the results relative to $CD3^{+}$ cells, $CD4^{+}$ T cells or as absolute counts. Variation in the study population and the use of GvHD treatment may also affect the findings. Also, Tregs in the blood may not reflect what is happening at the tissue level. Tissue staining of intestinal biopsies from alloHSCT patients showed increased numbers of $CD3^{+}$, $CD4^{+}$, $CD8^{+}$ and $FOXP3^{+}$ lymphocytes compared to healthy control samples. However, patients with GvHD had significantly fewer $FOXP3^{+}$ cells per $CD8^{+}$ lymphocyte compared to patients without GVHD or with non-GvHD inflammation (Rieger *et al.,* 2006). In a similar study analysing skin biopsies, T cells and $FOXP3^{+}$ cells were increased in rashes due to GvHD and drug reactions compared to healthy control samples. The $FOXP3^{+}$/T-cell ratios were reduced, although not statistically significant, compared

to healthy controls and drug reactions. In addition, $FOXP3^+$ cells were higher in those patients that were responding to GvHD therapy or had lower grades of GvHD (Fondi *et al.,* 2009).

For clinical alloHSCT, it is also important to consider whether Treg deficiency is associated with the prospective development of GvHD. If so, strategies to increase Tregs may be able to reduce the risk of GvHD. Stanzani and colleagues (2004) demonstrated that patients transplanted with higher numbers of donor $CD4^+CD25^+$ cells had a greater incidence of aGvHD. However, since CD25 is expressed in activated T cells, the association reported in this study was not specific to Tregs alone. In a subsequent study of 58 alloHSCT patients, the number of $CD4^+FOXP3^+$ cells in the PBSCH was an independent predictor of aGvHD (Wolf *et al.,* 2007). Subgroup analysis showed this affect was confined to the MAC transplants only, although the number of RIC transplants was relatively small. In a similar study of unrelated myeloablative transplants, low numbers of $CD4^+FOXP3^+$ cells in the PBSCH were also significantly associated with an increased risk of aGvHD, although this was not significant on multivariate analysis (Pabst *et al.,* 2007). Recently, the $CD3^+/CD4^+CD25^{high}FOXP3^+$ ratio has been shown to be associated with cumulative risk of aGvHD and was an independent predictor of grade II-IV aGvHD (Pastore *et al.,* 2012). However, none of the studies have shown any association between graft Tregs and cGvHD, NRM or OS by multivariate analysis. This association may also be limited to transplants using PBCSH only since a retrospective analysis of $CD4^+FOXP3^+$ cells in BM grafts did not show any association with the incidence of aGvHD (Rosenzwajg *et al.,* 2011). Few studies have investigated whether peripheral blood Tregs post-transplant can predict the onset of GvHD. Matthews and colleagues (2009) demonstrated that day 30 $CD4^+CD25^{high}FOXP3^+$ T cells were similar in patients that did and did not develop aGvHD. However, patients who subsequently developed aGvHD had a significantly higher effector $CD4^+$ T-cell/$CD4^+CD25^{high}FOXP3^+$ Treg ratio compared to those without GvHD.

1.7.2 INCREASING REGULATORY T CELLS POST-TRANSPLANT

Using Tregs in alloHSCT may be a promising strategy to prevent and/or treat GvHD while maintaining the GvT response. In recent years, much of the focus has been on the isolation and transfer of Tregs at or around the time of transplantation. Unfortunately, the isolation of human Tregs for clinical use has been hampered by the lack of Treg-specific surface markers, low Treg numbers in the peripheral blood and limitations of Good Manufacturing Practice (GMP). Most strategies use GMP-grade magnetic bead selection of $CD25^{high}$ cells with or without depletion of $CD8^+$ T cells, $CD19^+$ B cells and/or $CD127^{high}$ cells. These 'Treg-rich' products contain 40-60% $CD4^+CD25^+FOXP3^+$ T cells with *in vitro* suppressive function (Hoffmann *et al.,* 2006a; Wichlan *et al.,* 2006; Peters *et al.,* 2008). Using this approach, two clinical trials of adoptive transfer of Treg products have been performed in humans. The first was a phase I study in which patients with a high risk of relapse were pre-emptively given up to $5x10^6$/kg cells (>50% $FOXP3^+$), prior to DLI without immunosuppressive therapy (Edinger and Hoffmann, 2011). In nine patients, there were no adverse events related to the cell infusions. In the second study, 28 HLA-haploidentical transplants were given 2-$4x10^6$/kg isolated donor $CD4^+CD25^+$ Tregs (69±14% $FOXP3^+$) four days prior to receiving $CD34^+$ cells and 0.5-$2.0x10^6$/kg conventional T cells (Di Ianni *et al.,* 2011). The administration of donor $CD4^+CD25^+$ Tregs into a lymphopenic environment was with the aim of allowing pre-activation and homeostatic expansion of Tregs *in vivo* (Nguyen *et al.,* 2007). Despite the absence of other immunosuppression, only two patients developed grade II-IV aGvHD. Both studies provide encouraging results for Treg cellular therapies and support the need for further trials.

Due to the low number of human Tregs obtained by isolation, there has been interest in developing *in vitro* Treg expansion protocols. Higher numbers of Tregs may be required for prophylactic therapy in T-replete transplants where the number of conventional T cells given is in the order of 1-$5x10^8$/kg. Similarly, higher numbers of Tregs may be required to treat GvHD when immune reconstitution has occurred. Initial strategies used *in vitro* CD3/CD28 co-stimulation of the $CD4^+CD25^+$ 'Treg-rich' isolations in the presence of high dose IL2 (Earle *et al.,* 2005; Hoffmann *et*

al., 2006b). However, small numbers of contaminating non-Tregs rapidly expanded in culture, reducing the purity of the final product, and there are concerns that some $FOXP3^+$ cells may be activated T cells. In addition, repetitive *in vitro* stimulation was associated with down-regulation of FOXP3 in $CD4^+CD25^{high}$ nTregs (Hoffmann *et al.,* 2009). Therefore, refinement of these protocols has been proposed. First, is the combined use of CD25 and CD45RA to isolate naïve $CD4^+CD25^+CD45RA^+$ Tregs only and improve Treg purity by depleting contaminating effector T cells. Naïve $CD4^+CD25^+CD45RA^+$ Tregs are highly proliferative *in vitro* and have a more stable phenotype and function following culture (Hoffmann *et al.,* 2006b). The second modification is the addition of Rapamycin as mTOR inhibition prevents expansion of conventional T cells allowing preferential expansion of $CD4^+CD25^+FOXP3^+$ Tregs (Battaglia *et al.,* 2005; Strauss *et al.,* 2007; Strauss *et al.,* 2009). Such protocols have reported 50-100 fold expansion (Strauss *et al.,* 2007; Strauss *et al.,* 2009). Rapamycin and other agents such as ATRA may also convert conventional T cells into $FOXP3^+$ Tregs. Although the functionality and stability of these cells remains contentious, recent reports suggest that *ex vivo* expansion of $CD4^+CD25^+CD127^{dim}$ Tregs in the presence of Rapamycin and ATRA may be superior to expansion with Rapamycin alone, with the expanded cells having higher suppressive function (Golovina *et al.,* 2011). To date, only Brunstein and colleagues (2011b) have reported results using expanded human Tregs to prevent GvHD. 23 double UCB transplant patients were given expanded $CD4^+CD25^+$ Tregs (86% purity) at a dose of $1\text{-}30x10^5$/kg on day one, with 13 of these patients receiving an additional dose of $30x10^5$/kg on day 15. Tregs were obtained by CD25 bead isolation from third party UCB and expanded with CD3/CD28 beads and high dose IL2 (300IU/ml) for 18 days. There were no reported adverse events from the Treg infusions and the incidence of grade II-IV aGvHD was reduced compared to 108 historical controls. However, unlike the other studies, patients continued to receive immunosuppression (Mycophenolate and Ciclosporin/Rapamycin). To date, there are also a few case reports of expanded human Tregs being used in steroid refractory GvHD. Edinger and Hoffmann (2011) reported compassionate use of expanded Tregs (>95% $FOPX3^+$) for treatment resistant aGvHD. Likewise, Trzonkowski and colleagues (2009) reported the administration of

$1x10^5$/kg expanded $CD4^+CD25^+CD127^-$ Tregs (90% $FOXP3^+$) to a patient with refractory cGvHD with subsequent improvement of symptoms. Clearly, these responses may have occurred independently of Treg therapy and are not proof of the effectiveness of Tregs to treat GvHD. However, they are tentative first steps to Treg cellular therapy and highlight the feasibility of these methods.

While adoptive transfer of human Tregs shows encouraging results, there are significant hurdles to overcome with regards cost, feasibility and practical considerations, particularly when isolating and expanding Tregs. Ideally, the ultimate goal of Treg therapy should be to preferentially expand Tregs *in vivo*. Modification of current immunotherapy strategies may promote Treg expansion and/or function, particularly in the early stages of immune reconstitution. For example, ciclosporin reduces the level of FOXP3 expression in $CD4^+CD25^+$ cells, significantly reduces *in vitro* and *in vivo* function of allo-stimulated $CD4^+CD25^+$ Tregs and reduces $CD4^+CD25^+$ Treg expansion (Coenen *et al.,* 2006; Zeiser *et al.,* 2006; Coenen *et al.,* 2007). On the other hand, Rapamycin suppresses conventional T cells but does not reduce FOXP3 expression or *in vivo* $CD4^+CD25^+$ Treg function and is being used in the *in vitro* expansion of isolated $CD4^+CD25^+$ Tregs (Battaglia *et al.,* 2005; Strauss *et al.,* 2007; Zeiser *et al.,* 2008; Strauss *et al.,* 2009). *In vivo* studies in mice have also shown that the combination of IL2 and Rapamycin can selectively expand $CD4^+CD25^{high}$ Tregs (>95% $FOXP3^+$) and is associated with a reduction of lethal aGvHD (Shin *et al.,* 2011). In several phase II clinical studies, the combination of prophylactic Rapamycin and Tacrolimus has shown effective prevention of GvHD and lower NRM compared to historical controls (Ho *et al.,* 2009; Rodriguez *et al.,* 2010). However, there have been concerns with the increased rates of thrombotic microangiopathy.

Novel therapies to increase Tregs *in vivo* are also being investigated. One promising strategy is the use of pharmacological drugs to modify the epigenetic regulation of the *FOXP3* gene, increasing stable FOXP3 expression and Treg function. Epigenetics are heritable mechanisms that regulate gene expression without altering the genomic DNA nucleotide sequence. These include DNA

methylation and histone acetylation and methylation which have both been reported in the *FOXP3* gene (Lal and Bromberg, 2009). In mice, *in vitro* inhibition of DNA methylation using the hypomethylating agents Azacitidine (Aza) and Decitabine (Dec) or by knocking out DNA methyltransferase 1 (DNMT) induced Foxp3 expression in proliferating naïve $CD4^+CD25^-$ T cells (Polansky *et al.,* 2008; Lal *et al.,* 2009; Moon *et al.,* 2009; Choi *et al.,* 2010). In contrast to TGFβ, Azacitidine-induced $Foxp3^+$ cells showed complete demethylation of the TSDR Foxp3 enhancer, although sorted $Foxp3^-$ cells from the same cultures remained methylated at this site. However, there was no difference in the methylation status at a control CpG locus between the $Foxp3^+$ and $Foxp3^-$ Azacitidine treated cells and only $Foxp3^+$ cells had Foxp3 mRNA expression (Polansky *et al.,* 2008). When combined with TGFβ, Azacitidine increased the proportion of cells expressing Foxp3 compared to TGFβ alone and were reported to express similar surface markers and levels of cytokine mRNA to $CD4^+CD25^+Foxp3^+$ nTregs (Polansky *et al.,* 2008; Lal *et al.,* 2009; Moon *et al.,* 2009). The Aza/TGFβ-induced $Foxp3^+$ cells had improved Foxp3 stability upon repeated *in vitro* TCR stimulation compared to TGFβ alone and also had suppressive function *in vitro* and in *in vivo* mouse models of colitis and allogeneic pancreatic islet cell transplant (Polansky *et al.,* 2008; Lal *et al.,* 2009). In relation to alloHSCT, Choi and colleagues (2010) demonstrated that Decitabine treated naive $CD4^+CD25^-$ T cells from B6 mice (approximately 60% $Foxp3^+$) were able to protect lethally irradiated BALB/c mice from GvHD when given T-cell depleted (TCD) BM and conventional T cells from the same B6 mice. Mice given Decitabine treated naive $CD4^+CD25^-$ T cells had less weight loss and improved survival compared to those given PBS treated naive $CD4^+CD25^-$ T cells, in keeping with less GvHD. Using the same basic GvHD model, lethally irradiated BALB/c mice were also given a TCD BM from B6 mice followed by Tcon on day 11 and treated with *in vivo* Azacitidine/PBS on days 15, 17, 19 and 21. The delayed infusion of Tcon and the DNMT1 inhibitors was to allow recovery of blood counts in order to minimise problems with the myelosuppressive effects of the Azacitidine. Mice treated with Azacitidine had no GvHD, had a significant increase in the percentage of $FOXP3^+$ Tregs in the blood and all survived with successful engraftment. Using a luciferase-expressing murine leukaemia, Choi and colleagues (2010) were

also able to demonstrate that although Azacitidine could suppress GvHD, it did not suppress the GvT effect. In proliferating human $CD4^{+}CD25^{-}$ T cells, *in vitro* Azacitidine can also increase FOXP3 expression at both the mRNA and protein level (Nagar *et al.,* 2008; Lal *et al.,* 2009). Adding Azacitidine to TGFβ also increased the proportion of cell expressing FOXP3 compared to TGFβ alone. However, although Azacitidine treated $FOXP3^{+}$ cells had *in vitro* suppressive function, Nagar and colleagues (2008) demonstrated that they produced pro-inflammatory cytokines, including IFNγ and TNFα. Therefore, it remains unclear whether these agents can be used to produce stable, functional human Tregs for clinical use.

1.8 AIMS AND HYPOTHESES

Despite prophylactic immunosuppression, GvHD continues to cause significant morbidity and mortality following alloHSCT. In addition, the immunosuppressive therapies used to control GvHD are associated with increased risk of disease relapse, infection and side effects. Therefore, alternative strategies to prevent GvHD are being sought. Following from the work in mouse allogeneic transplant models, it was proposed that GvHD may be associated with low numbers of Tregs and may affect immune reconstitution post-transplant (Schneider *et al.,* 2006, Rezvani *et al.,* 2006, Magenau *et al.,* 2010). Increasing Tregs post-transplant may control GvHD while allowing the beneficial GvT response (Edinger *et al.,* 2003). However, the association between low Tregs and GvHD in human alloHSCT has remained controversial, as not all studies have demonstrated low Tregs in GvHD. Furthermore, accurate measurement of Tregs has remained difficult due to their low numbers post-transplant, lack of specific surface markers and expression of CD25 and FOXP3 in activated conventional T cells. These factors have also made the isolation and expansion of human Tregs for clinical use challenging.

The primary aim of this study was to establish whether Tregs in human alloHSCT are associated with GvHD. In particular, it was to test the hypotheses that low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells (putative Tregs) in the HSC grafts (Chapter 4) and/or in the peripheral blood post-transplant (Chapter 5) are independently associated with an increased incidence of acute and chronic GvHD, non-relapse mortality and inferior overall survival. In addition, this study would test the hypothesis that patients with active GvHD have lower $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells (Chapter 5). Since T-cell immunity is also important for engraftment, relapse and preventing infections, the secondary aims of this study were to test the hypotheses that low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the HSC grafts (Chapter 4) and/or the peripheral blood (Chapter 5) are associated with delayed engraftment, increased relapse and increased CMV reactivation. To test these hypotheses,

this project would perform a prospective observational clinical study of human alloHSCT in Oxford, measuring $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and lymphocyte subsets in the HSC grafts and at regular intervals in the first year post-transplant. This would be correlated against prospectively collected clinical data regarding the patient, donor, type of transplant, GvHD symptoms and transplant outcomes. This study aimed to develop and test reliable, accurate and reproducible methods to measure putative Tregs (Chapter 3) in the peripheral blood using traditional flow cytometry and a recently described methylation specific real time quantitative polymerase chain reaction (MSRT-qPCR) to detect the demethylated TSDR in the *FOXP3* gene (Sehouli *et al.,* 2011). By comparing the two methods, this study would test the hypothesis that detecting FOXP3 protein expression by flow cytometry may over-estimate nTreg numbers post-transplant due to expression of FOXP3 in activated conventional T cells.

An additional aim of the clinical study was to determine the pattern of cellular immune reconstitution post-alloHSCT in our patient cohort and to investigate $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell reconstitution (Chapter 5). This project would test the hypothesis that low and/or falling $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the first three months post-transplant are significantly associated with increased GvHD. By examining $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell reconstitution, this project would analyse which pre-, peri- and post-transplant factors are significantly associated with low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells post-transplant. This analysis may provide useful information for predicting which patients have low Tregs post-HSCT, determining which factors are important for Treg recovery and whether transplant regimens can be modified to increase Tregs.

Finally, in view of the difficulties with current Treg isolation and expansion, this study would also test the hypothesis that FOXP3 can be induced in $CD4^+CD25^-$ T cells through *in vitro* TCR stimulation (Chapter 6). In particular, it would test the hypothesis that TCR stimulation in the presence of TGFβ and Azacitidine can induce FOXP3 and demethylate the *FOXP3* TSDR enhancer, required for stable FOXP3 expression. The study will aim to determine whether these induced $FOXP3^+$ $CD4^+$ T cells have a Treg-like phenotype or are activated conventional T cells. These *in vitro* methods may provide useful strategies to produce functional $FOXP3^+$ cells for human cellular therapy trials. In addition, they may provide useful insights into how FOXP3 can be induced *in vivo* in $CD4^+$ T cells post-transplant and how Tregs and immune reconstitution may be modified to prevent GvHD.

Chapter 2

Methods

2. METHODS

This chapter describes the materials and methods used in this project.

2.1 CLINICAL STUDY

2.1.1 STUDY DESIGN

This project performed a prospective, observational study of putative Tregs and immune reconstitution in alloHSCT. All adult alloHSCT patients (>16 years) treated at the Oxford University Hospitals (OUH) NHS Trust were eligible for this study. Patients were excluded if they were unable to give informed written consent. Clinical details about the recipient, donor and transplant were acquired from the patient's records and clinical data were collected for up to three years post-transplant. An aliquot of the HSC graft (1-4ml) was collected from the Stem Cell Services Laboratory (National Health Service Blood and Transplant (NHSBT), Oxford, UK) on the day of transplant. Peripheral blood (PB) samples (5-10ml) were collected at +30, +60, +90, +120, +150, +180, +270 and +365 days post-transplant.

Sample size was calculated using aGvHD as the primary outcome. The incidence of grade II-IV aGvHD in Oxford is approximately 37% (Medd *et al.,* 2011). Previous studies analysing graft $CD4^+CD25^+FOXP3^+$ Tregs and aGvHD have shown a difference in the incidence of grade II-IV aGvHD of 21-64% (Pabst *et al.,* 2007; Wolf *et al.,* 2007; Pastore *et al.,* 2012). Based upon these values, this study was designed to detect a difference of 25% with an estimated incidence of grade II-IV aGvHD of 50% and 25% in the low and high Treg groups respectively. Using a one sided test with an α value of 0.05 and a β value of 0.2, the estimated sample size was 92.

2.1.2 STUDY OUTCOMES

The primary study outcomes were:

- **Acute GvHD (aGvHD):** The time from transplantation to onset of aGvHD or last follow-up. Acute GvHD was defined according to the Consensus Conference Classification (Przepiorka *et al.,* 1995) and the NIH Consensus Criteria (Filipovich *et al.,* 2005) (Table 1.1).

- **Chronic GvHD (cGvHD):** The time from transplantation to onset of cGvHD or last follow-up. Chronic GvHD was defined according to the Shulman classification (Shulman *et al.,* 1980) and the NIH Consensus Criteria (Filipovich *et al.,* 2005) (Tables 1.2-1.4).

- **Relapse:** The time from transplantation to relapse (morphological and molecular) or last follow-up.

- **Non-Relapse Mortality (NRM):** The time from transplantation to death (without prior relapse) or last follow-up.

- **Overall Survival (OS):** The time from transplantation to death (all causes) or last follow-up.

Other outcomes studied were:

- **Neutrophil engraftment:** The time from transplantation to the first of three consecutive days with a neutrophil count $\geq 0.5x10^9$/l.

- **Platelet engraftment:** The time from transplantation to the first of three consecutive days with a platelet count $\geq 50x10^9$/l.

- **CMV activation:** The time from transplantation to detectable CMV DNA by PCR amplification (> 1000 copies/ml).

2.1.3 ETHICS

Oxford Research Ethics Committee (REC) and OUH NHS Trust approval was obtained for this study. All data were handled in accordance with the Data Protection Act, the OUH NHS Trust guidelines and the Declaration of Helsinki. Patient confidentiality was maintained at all times and samples and data were stored using an anonymous identification number. Blood samples and GvHD data collection forms were stored in the Blood Research Laboratory (John Radcliffe Hospital, Oxford, UK) accessible to authorised staff only.

2.1.4 RECRUITMENT AND CONSENT

All patients who were due to have an alloHSCT in Oxford were identified by the principal investigator. Patients were approached for entry into this study during their pre-transplant appointments or admission to the ward. All participants were required to provide informed, written consent prior taking part in this study.

2.1.5 CLINICAL DATA

Data about the patient, recipient and transplant were obtained from the patient's records, NHSBT (Oxford) and the OUH NHS computer systems. HLA-typing results were obtained from Maggie Sutton (Transplant Immunology, Churchill Hospital, Oxford, UK). Chimerism results and molecular relapse monitoring were obtained from Dr Anna Schuh (Oxford BRC Molecular Diagnostic Service, John Radcliffe Hospital, Oxford, UK). CMV PCR results were obtained from the Department of Microbiology (John Radcliffe Hospital, Oxford, UK). Prospective clinical data were collected during out-patient appointments or from the inpatient ward by the attending physician/BMT specialist nurse (Figures 2.1-2.2). At the end of the study, all participants' notes were reviewed by the principal investigator to confirm the clinical data.

2.1.6 DISEASE AND STATUS AT TRANSPLANT

Due to the different indications for alloHSCT, it was necessary to group related diagnoses. In this study, the diagnoses were grouped into: (**A**) Leukaemia (Acute Myeloid Leukaemia (AML), Acute Lymphoid Leukaemia (ALL), Myelodysplastic Syndrome (MDS)); (**B**) Lymphoproliferative (Non-Hodgkin's Lymphoma (NHL), Hodgkin's Lymphoma (HL), Chronic Lymphoid Leukaemia (CLL) and Myeloma); (**C**) Other (Myeloproliferative and Chronic Myeloid Leukaemia (CML), Aplastic Anaemia (AA)). Disease status at transplant was defined as complete response (CR), partial response (PR) or active disease (AD) (Cheson *et al.*, 1990; Bladé *et al*., 1998; Cheson *et al*., 2007).

2.1.7 TRANSPLANT PROTOCOL AND IMMUNOSUPPRESSION

All patients were transplanted by the OUH NHS Trust Haematology Department. Patients transplanted with a RIC regimen received Fludarabine (Flu) 125mg/m^2 (25mg/m^2 IV days -7 to -3) and Melphalan (Mel) 140mg/m^2 (day -1); or Fludarabine 120mg/m^2 (30mg/m^2 IV days -6 to -3) and Cyclophosphamide (Cy) 1200mg/m^2 (300mg/m^2 IV days -6 to -3). The MAC regimens used were Cyclophosphamide 120mg/kg (60mg/kg days -6 and -5) and 14.4Gy TBI fractionated over four days (days -4 to -1); or Cyclophosphamide 120mg/kg (60mg/kg days -3 to -2) and Busulphan (Bu) 16mg/kg (3.2mg/kg IV days -8 to -4); or Cyclophosphamide 200mg/kg (50mg/kg IV days -5 to -2) and ATG 11.25mg/kg (3.75mg/kg days -4 to -2). To reduce GvHD, patients conditioned with Cy/TBI, Cy/Bu or Cy/ATG received Methotrexate 15mg/m^2 on day +1 and 10mg/m^2 on day +3, +6 and +11 whilst Flu/Mel sibling allografts received Methotrexate 5mg/m^2 on day +1, +3, +6 and +11. All transplants from an unrelated donor and/or HLA-mismatched also received Alemtuzumab 60mg (30mg IV days -8 to -7). GvHD prophylaxis was provided by Ciclosporin 1.5mg/kg IV from day -1, converting to oral Ciclosporin when tolerated, aiming for trough levels of 200-300ng/ml. If Ciclosporin could not be tolerated, GvHD prophylaxis was switched to Tacrolimus. In the absence of GvHD, Ciclosporin/Tacrolimus were tailed to stop from day 60 onwards. GvHD was treated with systemic corticosteroid therapy (Prednisolone or Methylprednisolone, 1-2mg/kg/day).

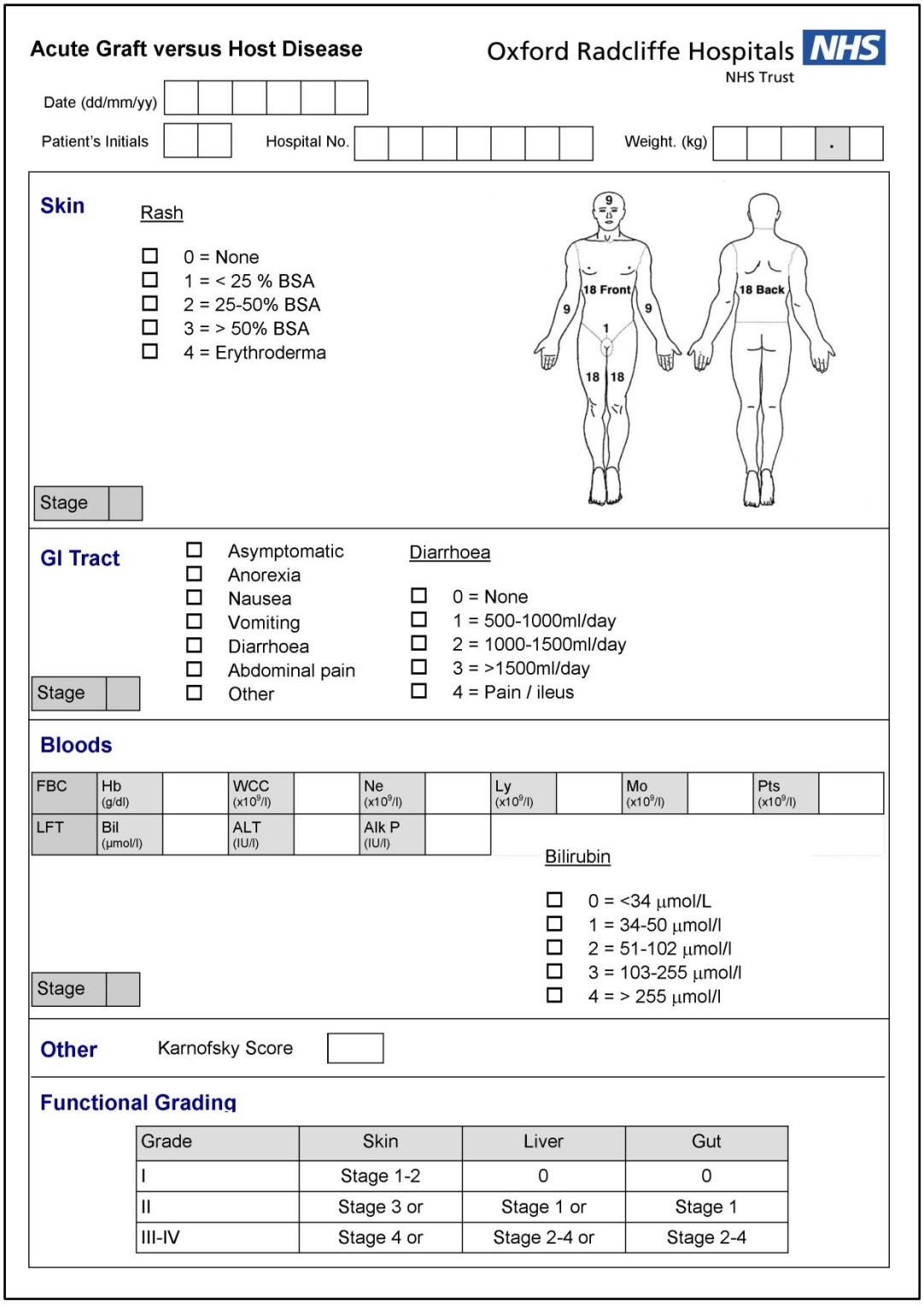
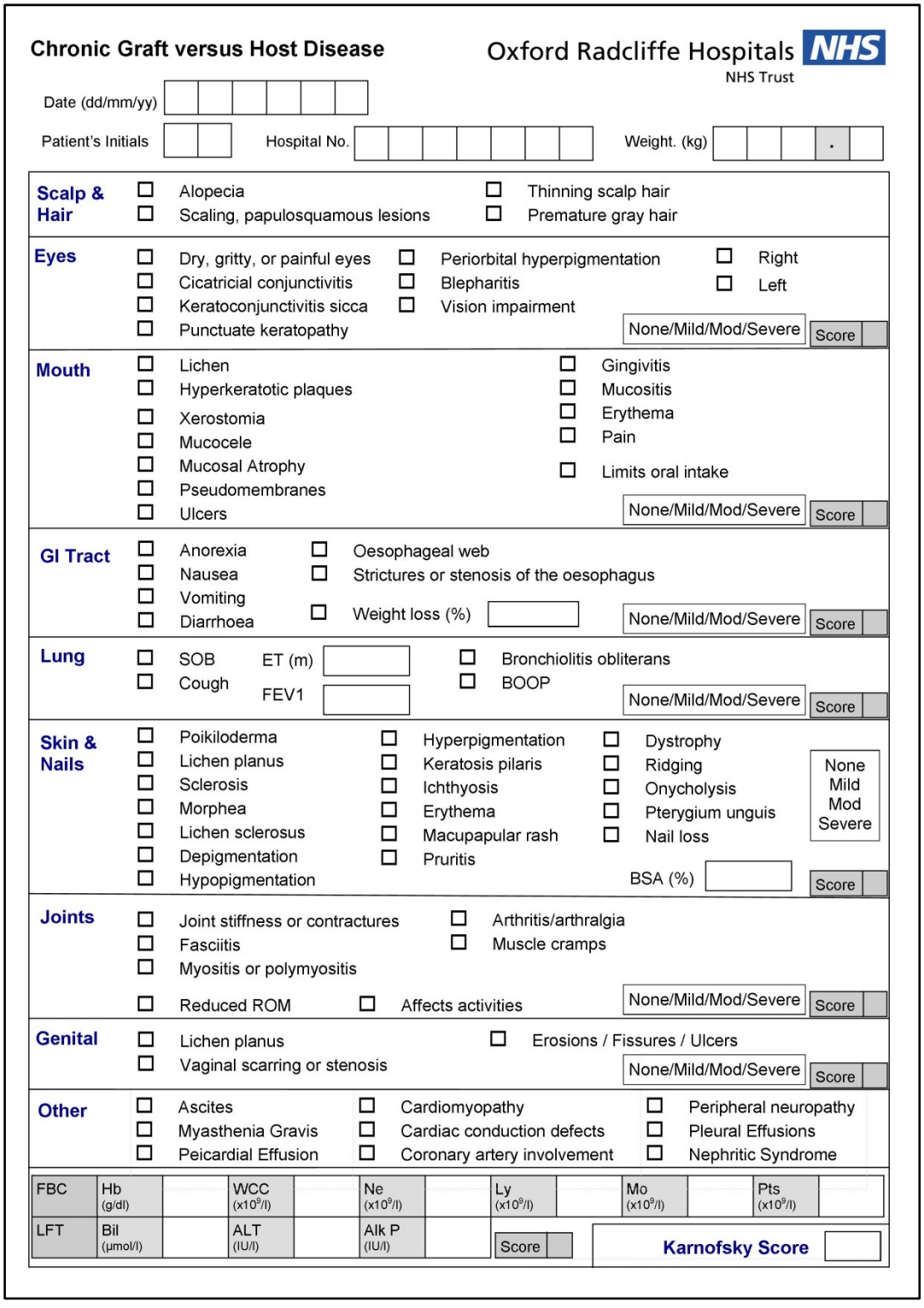


Figure 2.1: Acute GvHD Data Collection Form

Acute GvHD data collection form was designed using the Consensus Conference Grading of acute GvHD (Przepiorka *et al.,* 1995).



Chronic Graft versus Host Disease

Oxford Radcliffe Hospitals NHS
NHS Trust

Date (dd/mm/yy)

Patient's Initials | Hospital No. | Weight. (kg)

Scalp & Hair
- ☐ Alopecia
- ☐ Scaling, papulosquamous lesions
- ☐ Thinning scalp hair
- ☐ Premature gray hair

Eyes
- ☐ Dry, gritty, or painful eyes
- ☐ Cicatricial conjunctivitis
- ☐ Keratoconjunctivitis sicca
- ☐ Punctuate keratopathy
- ☐ Periorbital hyperpigmentation
- ☐ Blepharitis
- ☐ Vision impairment
- ☐ Right
- ☐ Left

None/Mild/Mod/Severe | Score

Mouth
- ☐ Lichen
- ☐ Hyperkeratotic plaques
- ☐ Xerostomia
- ☐ Mucocele
- ☐ Mucosal Atrophy
- ☐ Pseudomembranes
- ☐ Ulcers
- ☐ Gingivitis
- ☐ Mucositis
- ☐ Erythema
- ☐ Pain
- ☐ Limits oral intake

None/Mild/Mod/Severe | Score

GI Tract
- ☐ Anorexia
- ☐ Nausea
- ☐ Vomiting
- ☐ Diarrhoea
- ☐ Oesophageal web
- ☐ Strictures or stenosis of the oesophagus
- ☐ Weight loss (%)

None/Mild/Mod/Severe | Score

Lung
- ☐ SOB
- ☐ Cough

ET (m)

FEV1

- ☐ Bronchiolitis obliterans
- ☐ BOOP

None/Mild/Mod/Severe | Score

Skin & Nails
- ☐ Poikiloderma
- ☐ Lichen planus
- ☐ Sclerosis
- ☐ Morphea
- ☐ Lichen sclerosus
- ☐ Depigmentation
- ☐ Hypopigmentation
- ☐ Hyperpigmentation
- ☐ Keratosis pilaris
- ☐ Ichthyosis
- ☐ Erythema
- ☐ Macupapular rash
- ☐ Pruritis
- ☐ Dystrophy
- ☐ Ridging
- ☐ Onycholysis
- ☐ Pterygium unguis
- ☐ Nail loss

None Mild Mod Severe

BSA (%) | Score

Joints
- ☐ Joint stiffness or contractures
- ☐ Fasciitis
- ☐ Myositis or polymyositis
- ☐ Arthritis/arthralgia
- ☐ Muscle cramps
- ☐ Reduced ROM
- ☐ Affects activities

None/Mild/Mod/Severe | Score

Genital
- ☐ Lichen planus
- ☐ Vaginal scarring or stenosis
- ☐ Erosions / Fissures / Ulcers

None/Mild/Mod/Severe | Score

Other
- ☐ Ascites
- ☐ Myasthenia Gravis
- ☐ Peicardial Effusion
- ☐ Cardiomyopathy
- ☐ Cardiac conduction defects
- ☐ Coronary artery involvement
- ☐ Peripheral neuropathy
- ☐ Pleural Effusions
- ☐ Nephritic Syndrome

FBC	Hb (g/dl)		WCC ($x10^9$/l)		Ne ($x10^9$/l)		Ly ($x10^9$/l)		Mo ($x10^9$/l)		Pts ($x10^9$/l)	
LFT	Bil (µmol/l)		ALT (IU/l)		Alk P (IU/l)		Score		**Karnofsky Score**			

Figure 2.2: Chronic GvHD Data Collection Form

Chronic GvHD data collection form was designed using the NIH Consensus Conference Classification of Chronic GvHD (Filipovich *et al.,* 2005).

2.2 FLOW CYTOMETRY

2.2.1 SAMPLE COLLECTION AND PREPARATION

Whole blood samples were collected in ethylenediaminetetraacetic acid (EDTA) or lithium heparin (LH) vacutainers (BD Biosciences, Oxford, UK) and kept at room temperature (RT). HSC grafts (1ml in EDTA) were obtained from the Stem Cell Services Laboratory (NHSBT), diluted with phosphate buffered saline (PBS) (Lonza, Slough, UK) to obtain a nucleated cell count between 10-20x10^9/l and stored at 4^{o}C. Buffy coats were obtained from Non-Clinical Issues (NHSBT, Collindale, UK).

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats, whole blood and HSC grafts using Ficoll-Paque gradient centrifugation (GE Healthcare, Little Chalfont, UK). Buffy coats were diluted to 50ml with PBS and carefully layered over 15ml Ficoll-Paque in two 50ml Falcon tubes (BD Biosciences). Whole blood (1-10ml) and HSC grafts (1-10ml) were layered over 5ml Ficoll-Paque in a 15ml Falcon tube (BD Biosciences). The samples were spun at 2000rpm (834g) for 20min at RT with the centrifuge brake off (Sorvall RT7 Plus centrifuge (Thermo Scientific, Hemel Hempstead, UK). PBMC were removed and washed once with PBS to remove Ficoll-Paque (2000rpm, 10min at RT) and once to reduce platelets (1000rpm (210g) for 10min at RT). PBMC were re-suspended in PBS, counted using a haemocytometer (Marienfeld, Lauda-Königshofen, Germany) and Nikon Eclipse E400 microscope (Nikon Ltd, Kingston Upon Thames, UK) or a ViCell XR cell counter (Beckman Coulter, High Wycombe, UK) and diluted with PBS to a concentration of 20x10^6/ml.

2.2.2 ANTIBODY STAINING

The fluorochrome-conjugated anti-human antibodies used in this study were: anti-CD3 PE-Cy7, CD4 FITC, CD14 PE, CD16 PerCP-Cy5.5, CD19 PE, CD20 FITC, CD25 PE, CD25 PE-Cy7, CD56 APC, CD127 PE, CD45 PerCP / CD3 FITC / CD4 APC / CD8 PE Multitest, CD239 (GlyA) PE, IL2 PE, TNFα PE and IFNγ PE (BD Biosciences); anti-CD3 PerCP-Cy5.5, CD8 APC Alexa-Fluor 750, CD45RA PerCP-Cy5.5, CD127 PerCP-Cy5.5, IL17 PE (eBioscience, Hatfield, UK); anti-FOXP3 APC and FcR blocking reagent (Miltenyi Biotech, Bisley, UK). The isotype controls used were IgG1κ FITC, IgG1κ PE, IgG1κ PE, IgG1κ PerCP-Cy5.5, IgG1κ APC (BD Biosciences); IgG1κ PerCP-Cy5.5 (eBioscience); IgG1κ APC (Miltenyi Biotech). Cell viability was assessed using the blue Live/Dead® fixable dead cell stain (Invitrogen, Paisley, UK).

For staining surface antigens using a stain/lyse/wash method, 50µl PBMC ($1x10^6$ cells) or whole blood/HSC graft was stained with 50µl antibody mixture for 30min at 4^oC. The antibody mixture contained the required fluorochrome-conjugated antibodies at twice the final concentration, diluted to 50µl with PBS. For whole blood/HSC grafts, erythrocytes were lysed with 3ml red cell lysis buffer (8.26g NH_4Cl, 1g $KHCO_3$, 0.037g EDTA (all from Sigma-Aldrich, Gillingham, UK) in 1L ddH_20) for 5min at RT. The cells were washed (all washes performed in cold PBS and spun at 1600rpm (510g) unless otherwise stated) for 5min at 4^oC, the supernatant discarded and the cells re-suspended in 200µl PBS for analysis. If red cell lysis was incomplete, residual erythrocytes were lysed in 1ml red cell lysis buffer for 5min at RT and washed as before. When using a stain/lyse/no-wash method, identical staining was used. However, the cells were not washed but suspended in 1ml red cell lysis buffer for 10min at RT, stored at 4^oC and analysed as soon as possible to prevent cell damage.

For intracellular antigens, cells were stained for surface antigens as above and then fixed using 1ml fixation/permeabilization solution (Miltenyi Biotech) for 30min at 4^oC. The cells were washed once with PBS, once with 500µl permeabilization buffer (Miltenyi Biotech) and re-suspended in 50µl permeabilization buffer containing the required fluorochrome-conjugated antibodies. The cells

were stained for 30min at 4°C and then washed with 1ml permeabilization buffer. To minimise background staining, the cells were re-suspended in 1ml permeabilization buffer, allowed to rest for 30min at 4°C, washed a second time and re-suspended in 200µl PBS for analysis.

Whole blood samples/diluted HSC graft samples were also analysed using CD45/CD3/CD4/CD8 Multitest with Trucount tubes (BD Biosciences) as per the manufacturer's instructions. 20µl antibody was added to a Trucount tube followed by 50µl test sample. The cells were stained for 15min at RT and the erythrocytes lysed by the addition of 1ml lysis buffer (BD Biosciences) for 15min. The stained cells were analysed without washing.

To minimise antibody/fluorochrome degradation, all stained samples were kept at 4°C and analysed on the same day.

2.2.3 FLOW CYTOMETER PREPARATION, COMPENSATION AND CONTROLS

Flow cytometry was performed on an LSR II Flow Cytometer (BD Biosciences) with a blue argon-ion 488nm laser, a red helium-neon (HeNe) 635nm laser and a solid-state ultra violet (UV) 355nm laser. To ensure the flow cytometer was functioning correctly and to minimise variation, CST beads (BD Biosciences) were run each morning. For each assay, the optimal photomultiplier tube (PMT) voltages were determined to minimise noise while maximising sensitivity for dim populations and maintaining high resolution (BD Biosciences, 2005). In summary, blank calibration beads (BD Biosciences) were run at PMT voltages from 300v to 750v in 50v increments. For each detector, the % coefficient of variation (CV) (y axis) was plotted against PMT voltage (x axis) and the optimal PMT voltage determined by identifying the voltage at which the %CV plateaued (Figure 2.3).

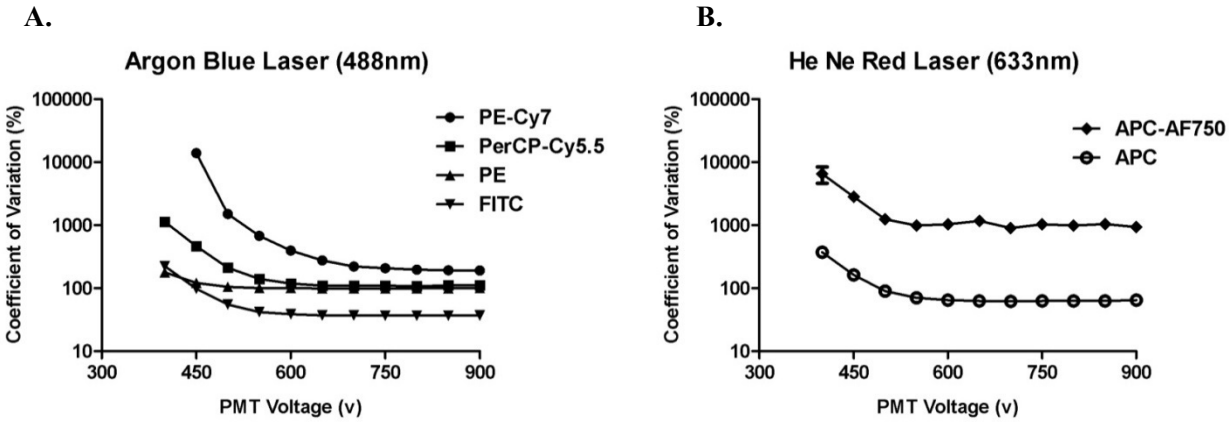


Figure 2.3: Selecting the Optimal PMT Voltage

Blank calibration beads were run at 50v increments (x-axis) and the coefficient of variation (%) for 50,000 events recorded (y-axis). The optimal PMT voltage is the voltage at which the %CV plateaus (BD Biosciences, 2005). **(A)** Argon-ion laser (488nm), **(B)** HeNe laser (635nm).

For multi-colour flow cytometry, spectral compensation must be applied to compensate for the spillover from one fluorochrome into another detector. To calculate compensation, unstained and single stained compensation beads (BD Biosciences) were prepared using conditions identical to those used to stain the samples. Using the optimal PMT voltages, 30,000 events were collected from each single stained or unstained tube and the FACS Diva software (BD Biosciences) used to calculate the compensation values. This compensation was then applied to all experiments using the specific combination of fluorochrome-conjugated antibodies and PMT voltages.

The antibody staining controls used in the flow cytometry were dependent upon the antigens being targeted, the cell population being identified, the antibody/fluorochrome combinations and the degree of spectral spillover (Roederer, 2001). The preferred controls used in this study were:

- **Fluorescence-minus-one (FMO):** When the background staining was due to spillover of emission spectra from other fluorochromes, the negative/positive boundaries were determined using a sample stained with all antibodies except the one of interest (Roederer, 2001).
- **Negative cell population**: For FOXP3 staining, the negative/positive boundary was determined using a population of cells that were negative for the antigen of interest.

Additional staining controls used were:

- **Isotype controls:** When background staining was due to non-specific binding, the non-specific staining was determined using a matched isotype control i.e. identical manufacturer, isotype, fluorochrome and concentration.
- **Fluorescence-minus-one (FMO) plus isotype control:** When background staining was due to spillover and non-specific staining.
- **Unstained control:** To determine autofluorescence signal from the cells.

2.2.4 DATA COLLECTION AND ANALYSIS

All flow cytometry was performed and analysed using FACS Diva software (BD Biosciences). For analysis of the main lymphocyte populations a minimum of 50,000 lymphocytes were collected. For $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell analysis, a minimum of 1000 $FOXP3^{+}$ events were collected (approximately 300,000-500,000 total events).

2.3.1 PBMC ISOLATION

PBMC were isolated from buffy coats supplied by Non-Clinical Issues (NHSBT, Collindale, UK), leucocyte cones supplied by Clinical Issues (NHSBT, Oxford, UK) or HSC grafts supplied by Stem Cell Services Laboratory (NHSBT, Oxford, UK). PBMC were isolated under sterile conditions as per 2.2.1, except that leucocyte cones were diluted to 80ml and layered over 15ml Ficoll-Paque in four 50ml Falcon tubes. For cell culture experiments, PBMC were washed twice to reduce platelets. Viable PBMC were counted using a haemocytometer and Trypan blue solution (0.4%) (1:2-1:20 dilution) (Sigma-Aldrich).

2.3.2 CD4+ T-CELL ISOLATION

$CD4^+$ T cells and naïve $CD4^+$ T cells were separated from PBMC using the $CD4^+$ T-cell isolation kit II (130-091-155, Miltenyi Biotech) and the Naïve $CD4^+$ T-cell isolation kit II (130-094-131, Miltenyi biotech) respectively, as per the manufacturer's instructions. For isolation of $CD4^+$ T cells, up to $5x10^8$ PBMC were suspended in $40\mu l/10^7$ cells autoMACS running buffer. $10\mu l/10^7$ cells biotin-antibody was added and the cells incubated for 10min at 4^oC. $30\mu l/10^7$ cells of running buffer and $20\mu l/10^7$ cells of anti-biotin microbeads were added, mixed and incubated for 15min at 4^oC. The cells were washed in running buffer and centrifuged at 300g for 10min at 4^oC. The supernatant was discarded and the cells re-suspended in 500µl buffer/10^8 cells. Up to 10^8 cells were applied to a pre-rinsed LS separation column (Miltenyi Biotech) positioned within a MACS separator magnet. The unlabelled $CD4^+$ T-cell fraction passed through the column and was collected. The column was rinsed three times with 3ml running buffer and the run through collected into the same tube. The cells were centrifuged at 300g for 10min at 4^oC, re-suspended in PBS and viable cells counted with a haemocytometer and Trypan blue (1:1-1:10). Isolation of naïve $CD4^+$ T

cells was identical except the cells were washed after staining with the biotin-antibody cocktail and re-suspended in 80µl/10^7 cells of running buffer and 20µl/10^7 cells of anti-biotin microbeads.

To deplete the isolated $CD4^+$ T cells of $CD4^+CD25^+FOXP3^+$ Tregs, $CD25^+$ cells were removed using CD25 MicroBeads II (130-092-983, Miltenyi Biotech) as per the manufacturer's instructions. The isolated $CD4^+$ T cells were incubated in 80µl/10^7 cells autoMACS running buffer and 20µl/10^7 cells CD25 MicroBeads for 15min at 4°C. The cells were washed, centrifuged (300g for 10min at 4°C) and re-suspended in 500µl buffer/10^8 cells. Up to 10^8 cells were applied to a pre-rinsed LD separation column (Miltenyi Biotech) positioned within a MACS separator magnet. The unlabelled $CD25^-$ fraction passed through the column and was collected. The column was rinsed twice with 1ml running buffer and the run through collected into the same tube.

$CD4^+CD25^+CD127^{-/dim}$ T cells were separated from PBMC using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T-cell isolation kit II (130-094-775, Miltenyi Biotech) as per the manufacturer's instructions. All non-$CD4^+$ and $CD127^{high}$ cells were first depleted using the biotin-antibody cocktail and anti-biotin microbeads, using the same method as used for $CD4^+$ T-cell isolation, except the cells were separated using LD separation columns. The pre-enriched $CD4^+CD127^{-/dim}$ cells were labelled with CD25 MicroBeads, as above, but separated using MS separation columns (Miltenyi Biotech). To collect the $CD4^+CD25^+CD127^{-/dim}$ T cells, the MS column was removed from the magnet, 1ml buffer added and the plunger pushed into the column to flush out the cells. The isolated $CD4^+CD25^+CD127^{-/dim}$ T cells were washed, re-suspended in PBS and viable cells counted with a haemocytometer and Trypan blue (1:1-1:10).

When sufficient cells were available, the purity of the isolated cell populations was checked by flow cytometry as per 2.2.2-2.2.4.

2.3.3 CD4+ T-CELL CULTURE

Isolated $CD4^+CD25^-$ or naïve $CD4^+CD25^-$ T cells were cultured in 96 well (200µl/well) or 48 well culture plates (500µl/well) (Fisher Scientific, Loughborough, UK) at a starting density of $0.5x10^6$ cells/ml, unless otherwise stated. The cells were cultured in RH10 culture media (RPMI-1640 (Lonza) with 10% heat inactivated human AB serum (NHSBT, Oxford), 2mM Glutamine, 2mM Pyruvate, 100U/ml Penicillin and 0.1mg/ml Streptomycin (all Sigma-Aldrich)) at 37°C and 5% CO_2. Recombinant Il-2 (Novartis, Frimley, UK) was added at 30IU/ml. To activate the T cells, anti-CD3/CD28 Dynabeads were added at cell to bead ratios of 1:1 to 1000:1 (Invitrogen) for three to seven days. On days three, five and seven, the culture media was refreshed by removing half of the media and adding an equal volume of pre-warmed fresh media. If stated, cell cultures were also divided 1:2. TGFβ (0-10ng/ml) (R&D Systems) was added on day zero and Azacitidine (0-10µM) (Sigma-Aldrich) was added daily for the first three days unless otherwise stated. Following culture, the anti-CD3/CD28 Dynabeads were removed and the cells either analysed immediately or rested for seven days in RH10/Il-2 only. Cell counts were measured using a haemocytometer and Trypan blue solution (1:1-1:10 dilution). Cultured cells were stained for flow cytometry with anti-CD4 FITC, anti-CD25 PE and anti-FOXP3 APC (staining volume of 50µl/test) as per 2.2.2-2.2.4. 10µl human FcR blocking reagent was added prior to the anti-FOXP3 antibody to minimise background staining.

2.3.4 CFSE PROLIFERATION ASSAY

Isolated $CD4^+CD25^-$ T cells were washed in PBS, spun at 500g for 10 minutes at RT and re-suspended in warm PBS (37°C) to a concentration of $2.5x10^6$/ml in a 50ml Falcon tube. Carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) was added at a final concentration of 0.5µM and mixed promptly to ensure the CFSE was evenly dispersed. The cells were placed at 37°C for 7min with regular agitation. An equal volume of warm foetal calf serum (FCS) (Sigma-Aldrich) was added and the tube filled with warm PBS. The cells were spun at 500g

for 10 minutes at 37°C, the supernatant discarded, the CFSE labelled cells re-suspended in pre-warmed RH10 culture media and allowed to rest for two hours at 37°C.

CFSE labelled T-cell cultures were set up in 96 well plates as per 2.3.3. Cells were activated for three to five days with anti-CD3/CD28 Dynabeads. Unused CFSE labelled T cells and unstained unlabelled T cells were retained for compensation and negative controls. At the end of culture, the cells were harvested, the anti-CD3/CD28 Dynabeads removed, cells washed in PBS and fixed and permeabilized as per 2.2.2. The cells were re-suspended in permeabilization buffer and stained with FcR blocking reagent, anti-CD4 PE and anti-FOXP3 APC (staining volume 50µl) for 30min at 4°C. The cells were washed twice with 500µl permeabilization buffer and re-suspended in 200µl PBS for analysis. Additional tubes prepared were single stained controls for spectral compensation, FMO controls and non-stimulated stained cells.

2.3.5 CYTOKINE STAINING

To assess cytokine production, cells were suspended in RH10 culture media and stimulated with 50ng/ml phorbol myristate acetate (PMA), 1µM Ionomycin and 5µg/ml Brefeldin A (all Sigma-Aldrich) for four hours at 37°C. The stimulated cells were harvested, washed in PBS and fixed and permeabilized as per 2.2.2. The cells were stained using FcR blocking reagent, anti-CD4 FITC, anti-FOXP3 APC and anti-IL2 PE or TNFα PE or IFNγ PE or IL17 PE (staining volume 50µl) for 30min at 4°C. The cells were washed twice with 500µl permeabilization buffer and re-suspended in 200µl PBS for analysis. The antibody staining controls used were FMO controls, FMO plus IC controls and stained non-stimulated cells.

2.3.6 SUPPRESSION ASSAY

To assess suppressive function of putative Tregs, an *in vitro* proliferation assay was used which measures the ability of $CD4^+CD25^+CD127^{-/dim}$ T cells (putative Tregs) to suppress anti-CD3/CD28 driven proliferation of autologous $CD4^+CD25^-$ T cells. $CD4^+CD25^-$ T cells and $CD4^+CD25^+CD127^{-/dim}$ T cells were isolated from PBMC as per Methods 2.3.2. $CD4^+CD25^-$ T cells were labelled with 0.5µM CFSE as per 2.3.4 and re-suspended in RH10 at $0.625x10^6$/ml. $5x10^4$ CFSE-labelled $CD4^+CD25^-$ T cells (80µl) were added to triplicate wells of a 96-well round-bottom plate, pre-coated with 5µg/ml anti-human CD3 antibody (Biolegend) (5µg/ml anti-CD3 antibody in 0.05M carbonate-bicarbonate buffer pH 9.0 (Sigma-Aldrich), kept overnight at 4°C, washed with PBS). $CD4^+CD25^+CD127^{-/dim}$ T cells were serially diluted (1 in 2), using RH10 media, starting at $5x10^5$/ml. 100µl of each dilution was added to triplicate wells containing the CFSE-labelled $CD4^+CD25^-$ T cells to obtain final $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:1 to 1:32. Control wells were prepared with $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:0, 0:1 and 0:2. rIL2 (Novartis) and anti-human CD28 antibody (Biolegend), at final concentrations of 10IU/ml and 1µg/ml respectively, was added and the assay cultured at 37°C for three to five days. Unlabelled $CD4^+CD25^-$ T cells and non-stimulated CFSE-labelled $CD4^+CD25^-$ T cells were retained to produce compensation controls and FMO/unstained controls.

On the day of analysis, the cultured cells were washed in PBS and suspended in 500µl PBS containing 1:1000 dilution of blue Live/Dead fixable stain for 5min at RT in the dark. The cells were re-washed in PBS, fixed, permeabilized and stained with FcR blocking reagent, anti-CD4 PE and anti-FOXP3 APC (staining volume 50ul) for 30min at 4°C. The cells were washed twice with 500µl permeabilization buffer and re-suspended in 200µl PBS for analysis. The antibody staining controls used were single stained controls, unstained negative control, FMO controls and non-stimulated CFSE-labelled $CD4^+CD25^-$ T cells. The percentage suppression of proliferation was calculated using the formula 100 x 1-(Proportion of $CFSE^{low}$ cells in the wells containing $CD4^+CD25^-$ T cells plus $CD4^+CD25^+CD127^{-/dim}$ T cells / Proportion of $CFSE^{low}$ cells in the wells with $CD4^+CD25^-$ T cells only).

2.4.1 DNA EXTRACTION

Genomic DNA was isolated from 100µl whole blood or $5x10^6$ cells using the DNeasy® Blood and Tissue Kit (Qiagen, Crawley, UK) as per the manufacturer's instructions. 20µl proteinase K was added to 200µl diluted whole blood/cells. 200µl buffer AL was added, vortexed and incubated for 10min at 56°C. 200µl 96-100% ethanol (Sigma-Aldrich) was added, vortexed and placed into a DNeasy Mini spin column inside a collection tube. The column was centrifuged at 6000g for 1min (all spins 6000g at RT unless stated) and the flow through discarded. 500µl buffer AW1 was added, centrifuged for 1min and the flow through discarded. 500µl buffer AW2 was added and centrifuged for 3min at 20,000g. Finally, the column was placed into a 1.5ml eppendorf tube, 100µl of AE buffer applied to the column membrane, incubated for 1min at RT and the DNA eluted by centrifuging for 1min. The extracted DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo-Scientific) and stored at -20°C. To assess DNA quality, 5µl DNA was mixed with 1µl x6 MassRuler loading dye (Fermentas, St. Leon-Rot, Germany) and run on a 1% agarose gel (Agarose (Sigma-Aldrich), Tris/Boric Acid/EDTA (TBE) buffer (BioRad, Hemel Hempstead, UK), 1:10,000 GelRed (Biotium, Hayward, U.S.A.)) with GeneRuler™ 100bp DNA ladder (Fermentas).

2.4.2 BISULPHITE CONVERSION

500ng genomic DNA was bisulphite converted using the EZ DNA Methylation Gold Kit (Zymo Research Corp, Orange, USA) as per the manufacturer's protocol. 50µl DNA was added to 100µl CT conversion agent (single vial of CT conversion agent was reconstituted in 600µl dH_20, 50µl M-dissolving buffer, 300µl M-dilution buffer). The mix was heated in a T3000 thermocycler (Biometra, Goettingen, Germany) at 98°C for 10min, 64°C for 150min and 4°C storage for up to 20 hours. The mix was placed into a Zymo-Spin™ IC column containing 600µl M-binding buffer,

placed in a collection tube and centrifuged at 10000g for 30s (all spins 10000g). The flow through was discarded, 200µl M-wash buffer added and spun for 30s. 200µl M-desulphonation buffer was added to the column for 20min at RT before centrifuging for 30s. The column was washed twice with 200µl M-wash buffer and spun for 30s each. Finally, the column was placed into a 1.5ml eppendorf, 40µl M-elution buffer added and the DNA eluted by centrifuging for 30s. Bisulphite treated DNA was quantified using a Nanodrop with an absorption coefficient at 260nm of 40µg/ml (bisulphite treated DNA is single stranded).

2.4.3 PCR PRIMERS AND PROBES

Bisulphite specific (BS) and methylation specific (MS) primers and probes used for PCR amplification are shown in Table 2.1 and Appendix I. The BS primers for FOXP3 were published by Baron and colleagues (2007). The BS primers for CD3 were designed using Primer 3 (Copyright Untergasser A., Nijveen H., Rozen S. and Skaletsky H. 2006-2011). All other primers and probes were published by Sehouli and colleagues (2011). Probes were labelled with 5' 6-FAM (5-Carboxyfluorescein) and 3' black hole quencher. Primers and probes were obtained from ThermoFisher Scientific GmbH, Ulm, Germany.

2.4.4 PCR AMPLIFICATION

PCR amplification was performed using HotStarTaq DNA Polymerase (Qiagen). 25µl reaction mixture containing x1 PCR buffer (Qiagen), 200µM dNTP (Qiagen), 0.2-0.5µM forward and reverse primers, 1U HotStarTaq polymerase (Qiagen), 50-200ng template DNA, 1.5-4.0mM $MgCl_2$ (Qiagen) and PCR grade water (Ambion, Paisley, UK) was prepared and placed into a 200µl PCR tube (Eppendorf, Stevenage, UK). Amplification was performed in a T3000 thermocycler (Biometra) with an activation step 95^{o}C for 15min; 30-40 cycles (95^{o}C for 1min, 40-66^{o}C for 45s; 72^{o}C for 1min); final extension 72^{o}C for 10min. Amplified DNA was cleaned using QIAquick PCR

purification (Qiagen) (all buffers in kit). 25µl DNA was added to 125µl buffer PB followed by 10µl 3M sodium acetate (Sigma-Aldrich). The solution was pipetted into a QIAquick column, placed into a collection tube and centrifuged at 14000g for 60s (all spins 14000g at RT). The flow through was discarded, 0.75ml buffer PE added and the column and spun twice for 30s. To elute the DNA, the column was placed into a 1.5ml eppendorf tube, 50µl EB buffer added and the column spun for 1min.

Amplicon	Specificity	Forward	Reverse
FOXP3	BS	TGTTTGGGGGTAGAGGATTT	TATCACCCCACCTAAACCAA
	BS & MS^U	GTTTTTGATTTGTTTAGATTTTTTTGTT	CCTCTTCTCTTCCTCCATAATATCA
	BS & MS^U (Pr)	ATGGTGGTTGGATGTGTTGGGTT	
	BS & MS^M	GTTTTCGATTTGTTTAGATTTTTTCGTT	CCTCTTCTCTTCCTCCGTAATATCG
	BS & MS^M (Pr)	ATGGCGGTCGGATGCGTC	
CD3	BS	GGTAGAGAATATGGAAAAGGTGGT	CCCAACCTCCCACTAACAAA
	BS & MS^U	TTTAAATATTGTTATATTTTGA	AAAAATCTAACTACTACAACTTAC
	BS & MS^U (Pr)	GAGAAGTTGTTGGTTTTATAGTGTAAG	
	BS & MS^M	TTAAATATTGTTATATTTCGA	AAATCTAACTACTACGACTTAC
	BS & MS^M (Pr)	TCGTCGGTTTTATAGCGT	
GAPDH	BS	TGGGGGTAGGGTAGTTG	AAACCCACTTCTTTAATTTACC
	BS & MS^U	GGTTTTTGGTATTGTAGGTTTT	CCAATTACAACATAACAACCA
	BS & MS^U (Pr)	TGTTTGGATGTTGTGTTTGTGGTAGAGTG	

Table 2.1: PCR Primers and Probes

PCR primers and probes for the *FOXP3*, *CD3* and *GAPDH* loci. Bisulphite specific (BS), methylation specific (unmethylated) (MS^U), methylation specific (methylated) (MS^M), Probe (Pr).

2.4.5 DNA SEQUENCING

DNA sequencing was performed by the DNA Sequencing Service (Weatherall Institute of Molecular Medicine (WIMM), Oxford, UK). The primers used for sequencing were the forward and reverse primers used in the PCR amplification or the WIMM Sequencing Service stock primers (M13 forward (cgccagggttttcccagtcacgac), M13 reverse (gtgagcggataacaatttcacacagg), T3 (cgaaattaaccctcactaaaggga), T7 (bluescript) (gtaatacgactcactatagggc)). Results were analysed with Sequence Scanner v1.0 (Copyright Applied Biosystems, 2005).

2.4.6 CLONING

Cloning PCR products was performed using TOPO TA Cloning Kit (Invitrogen). 1µl PCR product was mixed with 1µl salt solution, 1µl TOPO® vector (Invitrogen) and 3µl PCR water, incubated for 30min at RT and placed on ice. 2µl cloning reaction was added to one vial of OneShot ® Chemically Competent E.Coli (Invitrogen) and incubated on ice for 30min. The cells were heat-shocked at 42^{o}C for 30s and placed back onto ice. 250µl pre-warmed S.O.C. medium (Invitrogen) was added and incubated for one hour at 37^{o}C in a 200rpm shaker. 10-100µl cells were spread onto pre-warmed LB agar (Sigma-Aldrich) plates containing 50µg/ml kanamycin (Sigma-Aldrich) and incubated overnight at 37^{o}C. Individual colonies were picked and placed into 3ml 2TY (16g tryptone, 10g yeast extract, 5g sodium chloride, 1L dH_20 (all from Sigma-Aldrich)) containing 50µg/ml kanamycin. The cultures were incubated overnight at 37^{o}C in a shaker (200rpm).

Plasmid DNA was isolated using QIAprep Miniprep (Qiagen). 1.4ml cells were placed into a 1.5ml eppendorf tube and centrifuged at 200g for 5min at 4^{o}C. The supernatant was aspirated and the cells re-suspended in 250µl buffer P1. 250µl buffer P2 was added and mixed. 250µl buffer N3 was added, mixed and centrifuged at 10000g for 10min at RT (all spins were performed at 10000g at RT). The supernatant was removed, placed into a QIAprep spin column in a collection tube and centrifuged for 1 min. The flow through was discarded, 0.5ml buffer PB added and centrifuged for 1min. The flow through was again discarded, 0.75ml buffer PE added and centrifuged twice for

1min. To elute the DNA, the column was placed into an eppendorf tube, 50µl EB buffer added and rested for 60s before centrifuging for 60s. Eluted DNA was quantified on a Nanodrop spectrophotometer.

To produce larger quantities of DNA, 100µl of the starter culture was added to 50ml 2TY containing 50µg/ml kanamycin in a sterile 500ml conical flask and incubated overnight at 37^{o}C on a 200rpm shaker. The culture was poured into a 50ml Falcon tube and centrifuged at 3200g for 30min at 4^{o}C. The supernatant was discarded and the cell pellet stored at -20^{o}C. DNA was later extracted using the QIAprep Maxiprep (Qiagen). To summarise, the cell pellet was re-suspended in 10ml buffer P1. 10ml buffer P2 was added and mixed before adding 10ml buffer P3 and placed onto ice for 20min. The tube was spun at 20000g for 30min at 4^{o}C and the supernatant poured into a pre-equilibrated (10ml QBT buffer) Q500 DNA extraction column. The column was washed twice with 30ml buffer QC and the DNA eluted with 15ml buffer QF. DNA was precipitated by adding 10.5ml isopropanolol (Sigma-Aldrich) and the tube centrifuged at 3500g for 90min at 4^{o}C. The supernatant was decanted and 5ml 70% ethanol (Sigma-Aldrich) added. The tube was centrifuged at 3500g for 90min at 4^{o}C, the supernatant decanted and the tube allowed to dry. 500µl EB buffer was added and the DNA carefully mixed. The extracted DNA was quantified using Nanodrop spectrophotometer and stored at -20^{o}C.

2.4.7 METHYLATION-SPECIFIC REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Methylation-specific real-time quantitative polymerase chain reaction (MSRT-qPCR) was performed by Hannah Tudor (Oxford BRC Molecular Diagnostic Service, John Radcliffe Hospital, Oxford, UK). In summary, the stock plasmid controls were linearised with PST1 restriction endonuclease (CTGCAG) (New England Biolabs, Hitchin, UK). Plasmid standards were diluted to produce final concentrations of 300000, 30000, 3000, 300 and 30 template copies per reaction.

MSRT-qPCR reactions were set-up using 10µl 2 x Epitect Methylight mastermix (Qiagen), 0.8µl (8pmol) forward and reverse MS primers, 0.4µl (4pmol) MS-probe, 1µl bisulphite-treated DNA/plasmid, 7µl RNase free water (Qiagen). All samples and standards were run in duplicate. RT-PCR reactions were performed on a Rotor-Gene Q real-time PCR cycler (Qiagen). *FOXP3* CpG, *FOXP3* TpG and *GAPDH* TpG were run with cycling conditions 95^{o}C for 10min and 50 cycles (95^{o}C for 15s, 61^{o}C for 60s). *CD3* CpG and *CD3* TpG were run with cycling conditions 95^{o}C for 10min and 50 cycles (95^{o}C for 15s, 53^{o}C for 60s). The threshold used when analysing the traces was 0.08.

2.5 STATISTICS AND GRAPHS

Statistics were performed using IBM SPSS Statistics 20 (Copyright IBM Corporation 1989, 2011), GraphPad Prism5 (Copyright GraphPad Software Inc 1992-2007) and R 2.14.0 (Copyright R Foundation for Statistical Computing, Vienna, Austria, 2011). For all tests, $P<0.05$ was considered significant (* <0.05, ** <0.01, ***<0.001, ****<0.0001). Graphs were produced using GraphPad Prism5 (Copyright GraphPad Software Inc 1992-2007). Box and whiskers plots show the median, 1st and 3rd quartile, 5th and 95th centile. Cumulative incidence and survival curves were plotted using R 2.14.0.

2.5.1 GENERAL STATISTICAL TESTS

Data were checked and explored for missing points, errors or outliers. Categorical variables were tested between groups using either the Fisher's exact test or the $\chi 2$ test. The distribution of continuous data was assessed for normality using histograms and the D'Agostino-Pearson normality test. For normally distributed data, parametric tests were applied. Continuous data from two groups were compared using the independent t-test or the paired t-test. For the independent t-test, homogeneity of variance was tested using Levene's Test for Equality of Variance and equal variances were assumed with $P>0.05$. Data from more than two groups were compared using the analysis of variance (ANOVA) test and post-hoc tests were performed by pairwise comparisons with Bonferroni correction. When normal distribution could not be assumed, that data were transformed using either $\log_{10}$, $\sqrt{}$ or $\log_{10}(x/(1-x))$ transformation. If transformation corrected the distribution, the parametric tests were applied. However, if transformation did not correct the distribution or if transformation could not be applied, non-parametric tests were used. Continuous data from two groups were compared using the Mann-Whitney test or the Wilcoxon signed-rank test. Data from more than two groups were compared using the Kruskall-Wallis test.

Correlations between two continuous variables were analysed using the Pearson's correlation coefficient or the Spearman's correlation coefficient. To analyse the relationships between one or more independent variables with a continuous dependent variable, regression analysis was performed. When performing regression analysis, several assumptions were tested (Field, 2005):

- Linearity: Linear relationship between independent and dependent variable. This was checked by plots of standardised residuals versus standardised predicted and partial plots of the residuals of the dependent variable and each of the independent variables.
- Independence: All dependent variables must come from separate person/source.
- Normally distributed errors: The residuals in the model should be normally distributed. This was checked by histograms of the residuals and probability plots (P-P).
- Independent errors: The residual terms should be uncorrelated. This was tested by the Durbin-Watson test.
- No multicollinearity: Should not have perfect linearity between two or more independent variables. This was tested using the variance inflation factor (VIF) and tolerance collinearity diagnostics.
- Homoscedasticity**:** The variance of the residuals should be constant. This was checked by looking at plots of the standardised residuals versus standardised predicted.

In addition, the data were also checked for outlier or extreme cases that may over influence the model. As suggested by Field (2005), extreme cases were considered if:

- More than 5% cases had standardized residuals >2, more than 1% >2.5 or any case >3.
- Cook's distance >1
- Leverage values > 2 x Average leverage (i.e. (No. of independent variables+1)/sample size)
- DFBeta >1
- Covariance ratio (CVR) values above or below the acceptable CVR value (i.e. 1 ± 3 x Average leverage)

2.5.2 TIME DEPENDENT VARIABLES

Overall survival (OS) and relapse-free survival (RFS) were analysed using Kaplan-Meier estimation. Engraftment, GvHD, CMV activation, relapse and NRM were analysed using cumulative incidence with competing risks. The competing risks for engraftment, aGvHD, cGvHD, CMV reactivation, relapse and NRM were death without engraftment, death without aGvHD, death without cGvHD, death without CMV reactivation, death without relapse and relapse respectively. For patients receiving two transplants, follow-up data from the first transplant were censored on day zero of the second transplant. For patients receiving DLI, GvHD data were censored on the day of receiving the DLI. Variables considered in univariate analysis were recipient and donor age, sex and CMV IgG status; disease (Leukaemia v Lymphoproliferative v Other); disease status (CR v PR v AD); conditioning (RIC v MAC); Donor (Sibling v Unrelated); Source (PBSCH v BM); T-cell depletion (Alemtuzumab); HLA-mismatch; female-to-male transplant. Continuous variables were categorised with the median as a cut-off. Stem cell graft variables considered in univariate analysis were $CD34^+$, $CD3^+$ (T-cell), $CD19^+$ (B-cell), $CD3^-CD56^+$ (NK-cell), $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell (putative Tregs) dose and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). Continuous graft variables were categorised with the cut-off being the boundary between the 1st and 2nd quartile (see 4.3.4).

Multivariate analysis for OS and RFS was tested by Cox Proportional Hazards. Engraftment, GvHD, CMV activation, relapse and NRM were tested using the method devised by Fine and Gray (1999). Variables with $P<0.1$ in univariate analysis were included in the multivariate analysis and the model developed using a backward approach. To validate the model, the excluded variables were re-introduced one at a time to ensure they did not significantly add to the model. In addition, the model was also checked for interaction effects between the significant variables. Analysis of the time dependent variables and the production of curves were performed using R 2.14.0.

Chapter 3

Quantification of Regulatory T cells

3. QUANTIFICATION OF REGULATORY T CELLS

3.1 INTRODUCTION

To perform studies of regulatory T cells (Tregs) in alloHSCT, it is essential to have accurate and reproducible methods to quantify Tregs. Quantification of human Tregs remains challenging due to their paucity in the peripheral blood (PB) and lack of any currently identified Treg specific surface markers (Rieger *et al.,* 2006). The discrepancy in the results between different studies measuring Tregs in GvHD may be due to different methodologies, use of different antigens to define Tregs and alternative ways to present the data (Clark *et al.,* 2004; Rezvani *et al.,* 2006; Rieger *et al.,* 2006; Arimoto *et al.,* 2007). At present, a common technique to measure Tregs is multi-colour flow cytometry, staining for a combination of antigens including CD4, CD25 and intracellular FOXP3 (Rieger *et al.,* 2006; Pabst *et al.,* 2007; Magenau *et al.,* 2010). This method has high-sensitivity, can identify $CD4^+CD25^+FOXP3^+$ T cells with reasonable specificity by staining cells for more than one antigen and can produce absolute and relative counts. The work presented in this chapter describes how in-house multi-colour flow cytometry assays to measure $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (putative Tregs) and other lymphocyte populations were developed and tested. It will also test the hypothesis that sample collection and handling can influence $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell measurements and therefore consistent methods are required throughout the clinical study.

Although flow cytometry is a commonly used method to measure Tregs, there are several limitations. Sample preparation, antibody staining and running the cells through a flow cytometer is time consuming and expensive. This is particularly relevant when testing large numbers of samples using multiple fluorochrome-conjugated monoclonal antibodies. The rarer the cell of interest, the greater the number of total events that must be analysed and the longer it takes to run each sample. There is also concern relating to the specificity of CD25 and FOXP3 expression. As CD25 and

FOXP3 are up-regulated in activated human T cells, it remains uncertain whether all of the $CD4^+CD25^+FOXP3^+$ T cells are naturally occurring Tregs (nTregs) (Allan *et al.,* 2007; Pillai *et al.,* 2007; Wang *et al.,* 2007). Following the identification of a demethylated *FOXP3* TSDR enhancer specific to $CD4^+CD25^+FOXP3^+$ nTregs, several groups have proposed using methylation-specific PCR assays to quantify Tregs (Baron *et al.,* 2007; Sehouli *et al.,* 2011). These techniques appear specific for $CD4^+CD25^+FOXP3^+$ nTregs and allow batch processing of multiple samples using small quantities of DNA. Therefore, the second part of this chapter describes the development and testing of an in-house methylation-specific real-time qPCR (MSRT-qPCR) assay to measure putative Tregs and T cells and tests how the results compare to multi-colour flow cytometry.

3.2 SPECIFIC MATERIALS AND METHODS

3.2.1 FLOW CYTOMETRY

Flow cytometry assays were developed as described in 2.2. The fluorochrome-conjugated antibodies used were anti-CD3 PE-Cy7, CD4 FITC, CD14 PE, CD15 FITC, CD16 PerCP-Cy5.5, CD19 PE, CD20 FITC, CD25 PE, CD25 PE-Cy7, CD56 APC, CD127 PE, CD45 PerCP / CD3 FITC / CD4 APC / CD8 PE Multitest; CD239 PE (BD Biosciences); anti-CD3 PerCP-Cy5.5, CD8 APC Alexa-Fluor 750, CD127 PerCP-Cy5.5 (eBiosciences); anti-FOXP3 APC (Miltenyi Biotech). The isotype controls (IC) used were IgG1κ PE, IgG1κ FITC (BD Biosciences); IgG1κ PerCP-Cy5.5 (eBiosciences); IgG1κ APC (Miltenyi Biotech). Antibody titrations were performed by staining $1x10^6$ PBMC (50µl) with 50µl diluted antibody at 1 in 2 serial dilutions, starting at twice the supplier's recommended concentration.

The flow cytometer, spectral compensation and controls were prepared as per 2.2.3 and all data collected and analysed as per 2.2.4.

3.2.2 SUPPRESSION ASSAY

A full description of the *in vitro* proliferation assay method used to assess suppressive function of putative Tregs is provided in Methods 2.3.6. In summary, this assay uses fluorescent carboxyfluorescein succinimidyl ester (CFSE) cell staining (0.5µM) to assess anti-CD3/CD28 (5µg/ml plate bound anti-CD3; 1µg/ml soluble anti-CD28) driven proliferation of $CD4^+CD25^-$ T cells. Upon entry into cells, acetate groups are cleaved from the CFSE dye by intracellular esterases producing a fluorescent CFSE that binds to intracellular amines. During cell division, there is halving of the fluorescence intensity of the CFSE dye (Lyons, 2000).

In this *in vitro* suppression assay, anti-CD3/CD28 driven proliferation of CFSE-labelled $CD4^+CD25^-$ T cells is performed in the presence of serial dilutions of isolated autologous

$CD4^+CD25^+CD127^{-/dim}$ T cells (putative Tregs). If the $CD4^+CD25^+CD127^{-/dim}$ T cells possess 'regulatory' suppressive function, they will reduce the anti-CD3/CD28 driven proliferation of the CFSE-labelled $CD4^+CD25^-$ T cells, as determined by the proportion of $CD4^+CD25^-$ T cells with low CFSE fluorescence intensity.

3.2.3 METHYLATION-SPECIFIC REAL-TIME QPCR

The methods used to develop the MSRT-qPCR are fully described in Methods 2.4. Fully unmethylated DNA (Qiagen) and fully methylated DNA (Millipore, Watford, UK) were used to prepare the plasmid standards and to test the specificity of the BS MS primers.

Specific cell populations used to test the MSRT-qPCR assay were isolated from PBMC using Methods 2.3.1-2.3.2. The magnetic bead isolation kits used were $CD4^+CD25^+CD127^{dim/-}$ Regulatory T-cell, $CD4^+$ T-cell, CD3 MicroBeads, CD8 MicroBeads, CD14 MicroBeads, CD15 MicroBeads, CD20 MicroBeads, CD25 MicroBeads and CD56 MicroBeads (Miltenyi Biotech).

3.3 $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T-CELL FLOW CYTOMETRY ASSAY

To quantify $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (putative Tregs) in blood samples and HSC grafts, a multi-colour flow cytometry assay was developed. This section describes the design and testing of this assay, including the selection of antigens, antibodies and fluorochromes, the gating strategies used and testing the assay with clinical samples.

3.3.1 SELECTING ANTIGENS AND FLUOROCHROMES

Accurate detection of Tregs remains challenging since there is currently no known surface marker specifically expressed on Tregs alone. Therefore, in this assay, a combination of antigens was used i.e. CD3 (+), CD4 (+), CD8 (-), CD25 (+), FOXP3 (+) and CD127 (-/dim) (Hicheri *et al.,* 2008; Peters *et al.,* 2008). CD3, CD4 and CD8 were chosen to identify the parent $CD4^+$ T-cell population. CD25 and FOXP3 are both expressed in Tregs and commonly used antigens to detect Tregs (Yagi *et al.,* 2004; Roncador *et al.,* 2005; Zorn *et al.,* 2005; Sakaguchi *et al.,* 2006). CD127 was added because its expression inversely correlates with FOXP3 and potentially allows discrimination between regulatory T cells ($CD127^{dim/-}$) and activated T cells ($CD127^+$) (Liu *et al.,* 2006; Seddiki *et al.,* 2006b). As this assay was performed on a flow cytometer with Argon and HeNe lasers only (UV excitable fluorochromes were not available for these antigens), detecting more than six antigens in a single panel was unsatisfactory because the overlap of emission spectra from seven fluorochromes caused loss of sensitivity. This was a particular concern for antigens that were weakly expressed (FOXP3) or whose strength of expression formed a continuous distribution from negative to positive (CD25 and CD127). Within this thesis, unless otherwise stated, putative Tregs will therefore be phenotypically defined as **$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells**. It is acknowledged that whilst this may be an imperfect phenotypic definition of regulatory T cells, it is considered a valid approach for identifying putative Tregs and thus will serve as a useful starting point for this type of analysis.

Selection of the fluorochromes and the specific antibody-fluorochrome combinations used was based upon the principals defined by Maecker and colleagues (2004).

A) Choose the brightest fluorochromes available.

B) Choose fluorochromes to minimise spectral spillover.

C) Use the brightest fluorochromes on the antibodies directed at the weakly expressed antigens.

D) Avoid spillover from bright populations into detectors requiring high sensitivity.

E) Choose stable fluorochromes and avoid tandem degradation.

At the time these assays were developed, the brightest fluorochromes available with least spectral overlap for this flow cytometer were FITC, PE, PerCP-Cy5,5 and PE-Cy7 (Argon laser) and APC and APC-Alexa Fluor 750 (HeNe laser). Several combinations of antibody-fluorochrome pairings were tested (Table 3.1). Although the brightest fluorochromes (PE/APC/PE-Cy7) were reserved for the weakest antigens (CD127/FOXP3/CD25), spectral spillover from the PerCP-Cy5.5 bright cells (CD3) into the PE-Cy7 detector caused loss of sensitivity in the PE-Cy7 channel (CD25) (Figure 3.1). This wide distribution or 'spreading' of the PerCP-Cy5.5 bright cells in measurements recorded in the PE-Cy7 channel occurs due to measurement errors within the flow cytometer and is most noticeable in bright populations with significant spillover (Roederer, 2001). The panel was therefore revised to ensure PE-$Cy7^{+}$ events could be separated from the PE-$Cy7^{-}$PerCP$Cy5.5^{+}$ events. The final combination selected was CD3 PE-Cy7, CD4 FITC, CD8 APC-AF 750, CD25 PE, FOXP3 APC and CD127 PerCP-Cy5.5 (Table 3.1).

Fluorochrome	Antigen/Antibody	
	Original Panel	Revised Panel
PE*	CD127#	CD25#
FITC	CD4	CD4
PerCP-Cy5.5	CD3	CD127#
PE-Cy7*	CD25#	CD3
APC*	FOXP3#	FOXP3#
APC AF-750	CD8	CD8

Table 3.1: $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell Flow Cytometry Panel

Antigens, fluorochromes and antibody-fluorochrome pairings used in the flow cytometry assay. (*) Brightest fluorochromes; (#) weakly expressed antigens. Arrows show direction of the most significant spillover (e.g. from FITC into PE detector).

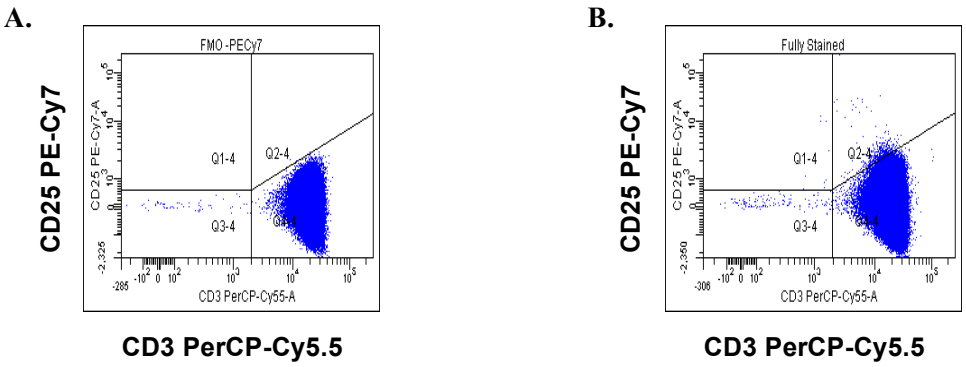


Figure 3.1: Reduced Sensitivity in CD25 PE-Cy7 due to 'Spreading'

PBMC were isolated from buffy coats using Ficoll-Paque separation and stained with the original $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell flow cytometry panel as shown in Table 3.1. CD3 PerCP-Cy5.5 (x-axis) and CD25 PE-Cy7 (y-axis) staining is shown for $CD3^{+}$ cells **(A)** Fluorescence-minus-one (FMO) control (no anti-CD25 PE-Cy7) shows a wide distribution or "spreading" of the bright $CD3^{+}$ cells in measurements recorded in the PE-Cy7 channel after appropriate compensation (median PE-Cy7 signal is zero). **(B)** When anti-CD25 PE-Cy7 is added, $CD3^{+}CD25^{dim/+}$ events cannot be distinguished from the $CD3^{+}CD25^{-}$ cells.

3.3.2 ANTIBODY TITRATION

Having selected the panel, each antibody was titrated to find the optimal concentration. Although each was supplied at a pre-titrated recommended concentration, they were tested to:

A) Ensure the antibody did not have high non-specific background staining.
B) Ensure the antibody produced a good signal-to-noise ratio with a high stain index, quantified by D/W where D is the difference between the positive and negative populations and W is two standard deviations (SD) of the negative population (Maecker et al., 2004).
C) Check whether the volume could be reduced to:
    - Reduce the total volume of all antibodies to less than 50µl.
    - Minimise costs.

The optimal antibody concentration was determined by staining $1x10^6$ PBMC with 1 in 2 serial dilutions of the antibody, starting at twice the supplier's recommended concentration. For each concentration, the percentage of positive stained cells, the median fluorescence intensity (MFI) of the negative and positive populations and the SD of the negative population was determined. For antigens with distinct separate groups (CD3, CD4 and CD8), the negative/positive boundary was set according to the populations observed (Figure 3.2 & 3.5). For antigens showing a continuous negative to positive spread of expression (CD25 and CD127), the negative/positive boundary was established using the matched isotype control at a 0.2% cut off (Figure 3.3 & 3.6). For FOXP3, the isotype control proved unreliable. Therefore, the negative/positive boundary was determined by double staining with CD20 and gating the $CD20^+FOXP3^-$ population (Figure 3.4 & 3.6). The final working concentrations selected are shown below in Table 3.2.

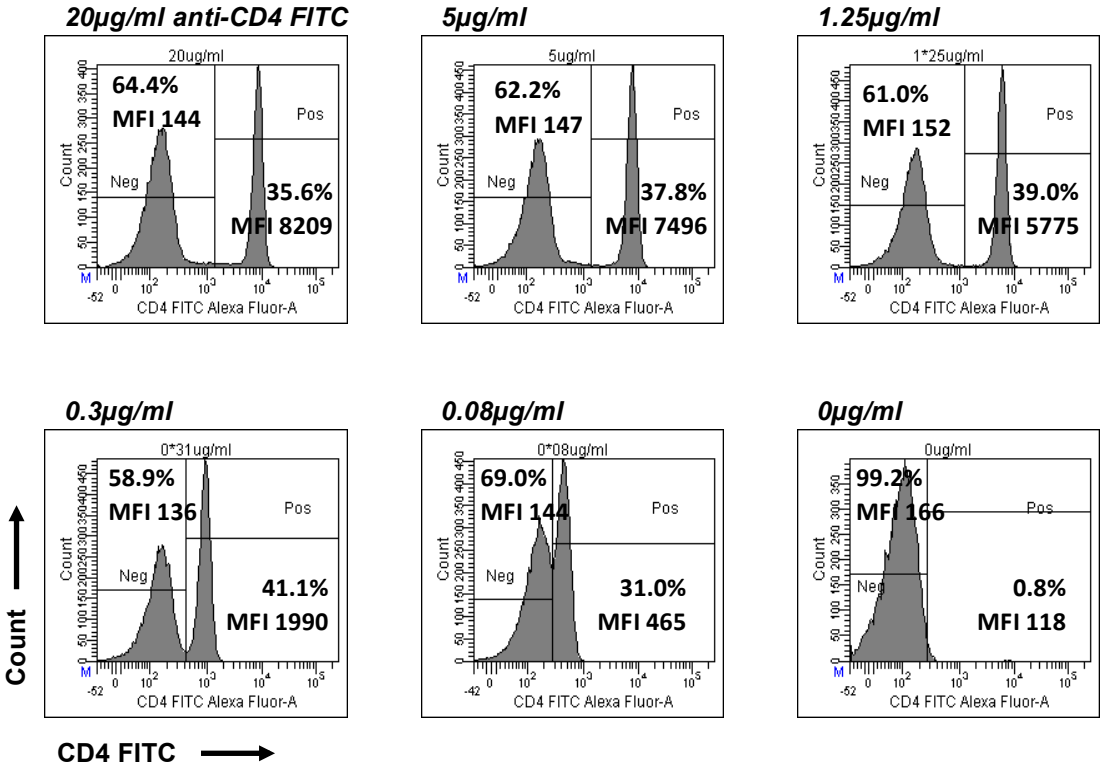


Figure 3.2: Antibody Titration for Antigens with Distinct Negative/Positive Populations

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with 1 in 2 serial dilutions of anti-CD4 FITC antibody (0-20µg/ml). Lymphocytes were first gated by FSS/SSC and the CD4 negative/positive populations gated as shown. Flow plots show the percentage of lymphocytes and median fluorescence intensity (MFI) within the CD4 negative/positive populations. Only alternate anti-CD4 FITC concentrations tested are shown.

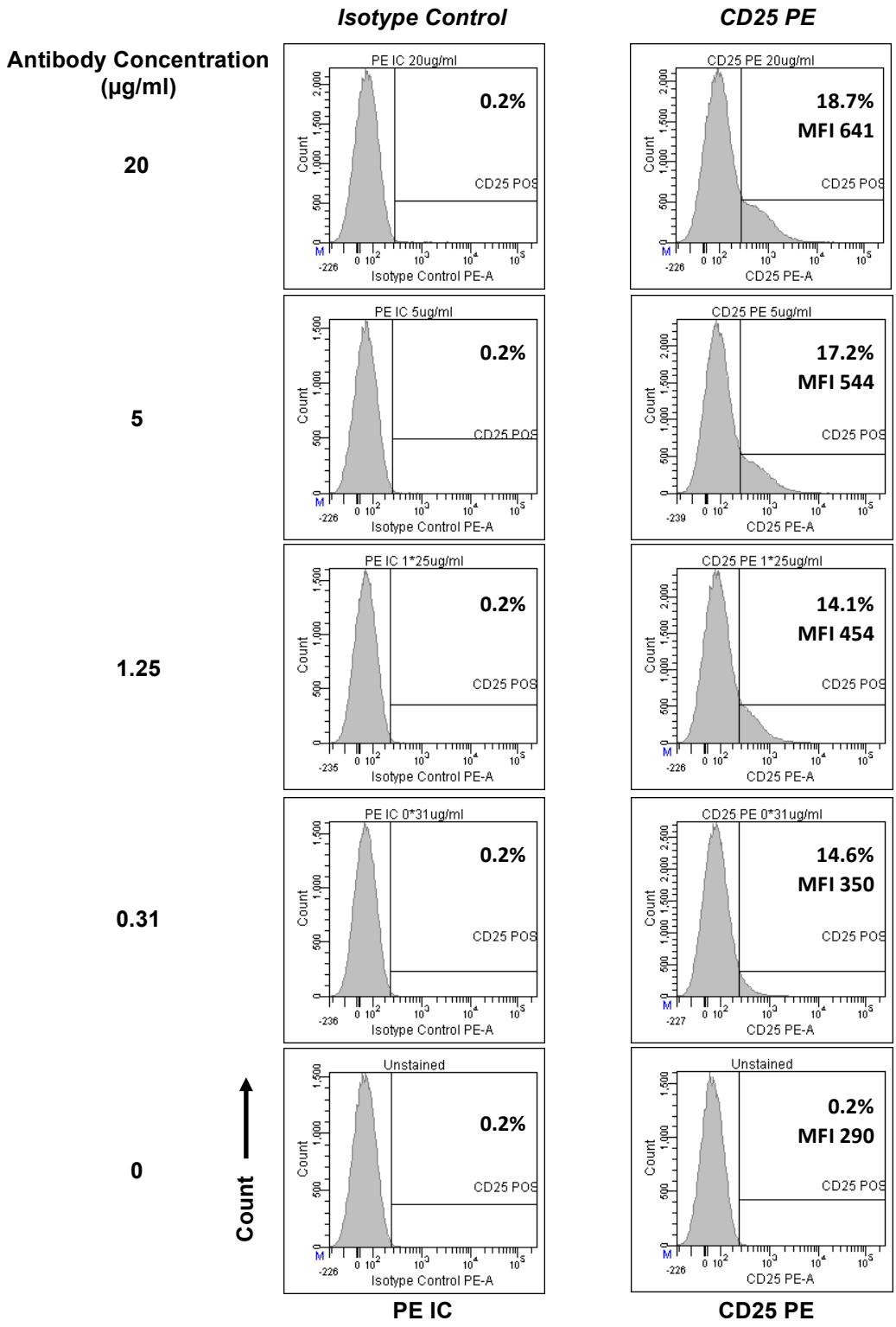


Figure 3.3: Antibody Titration for Antigens with Continuous Spread of Expression

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with 1 in 2 serial dilutions of anti-CD25 PE or matched isotype control (0-20µg/ml). Lymphocytes were first gated by FSS/SSC and the CD25 negative/positive boundary set using the isotype control stained cells (0.2% positive). Flow plots show the percentage of lymphocytes and median fluorescence intensity (MFI) in the positive gate. Only alternate concentrations tested are shown.

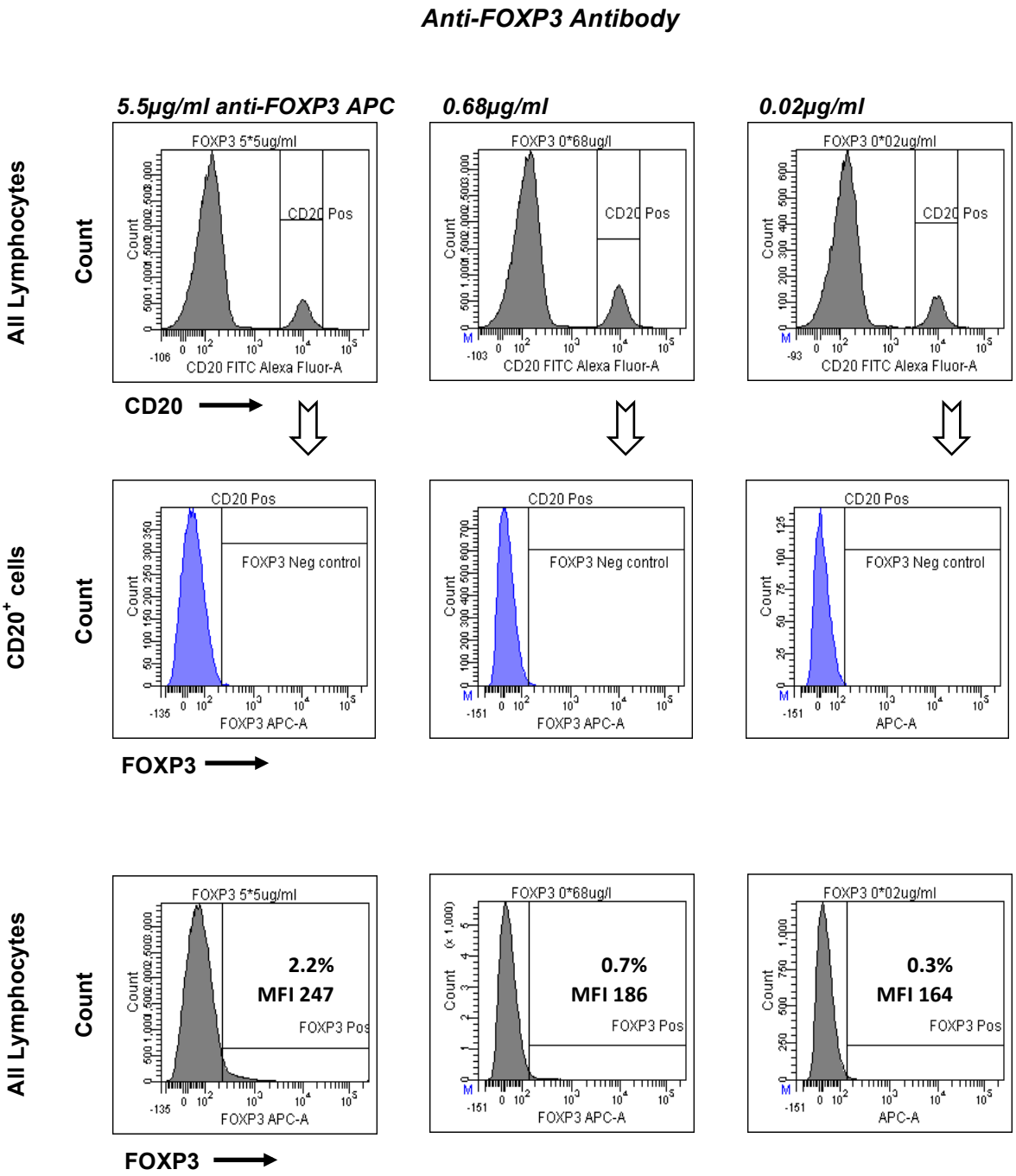


Figure 3.4: Anti-FOXP3 APC Antibody Titration

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with 1 in 2 serial dilutions of anti-FOXP3 APC (0-11µg/ml) and 5µg/ml anti-CD20 FITC. Lymphocytes were first gated by FSS/SSC and the FOXP3 negative/positive boundary determined using $CD20^+FOXP3^-$ cells (0.2% positive) (top and middle rows). Flow plots (bottom row) show the percentage of lymphocytes positive for FOXP3 and their median fluorescence intensity (MFI).

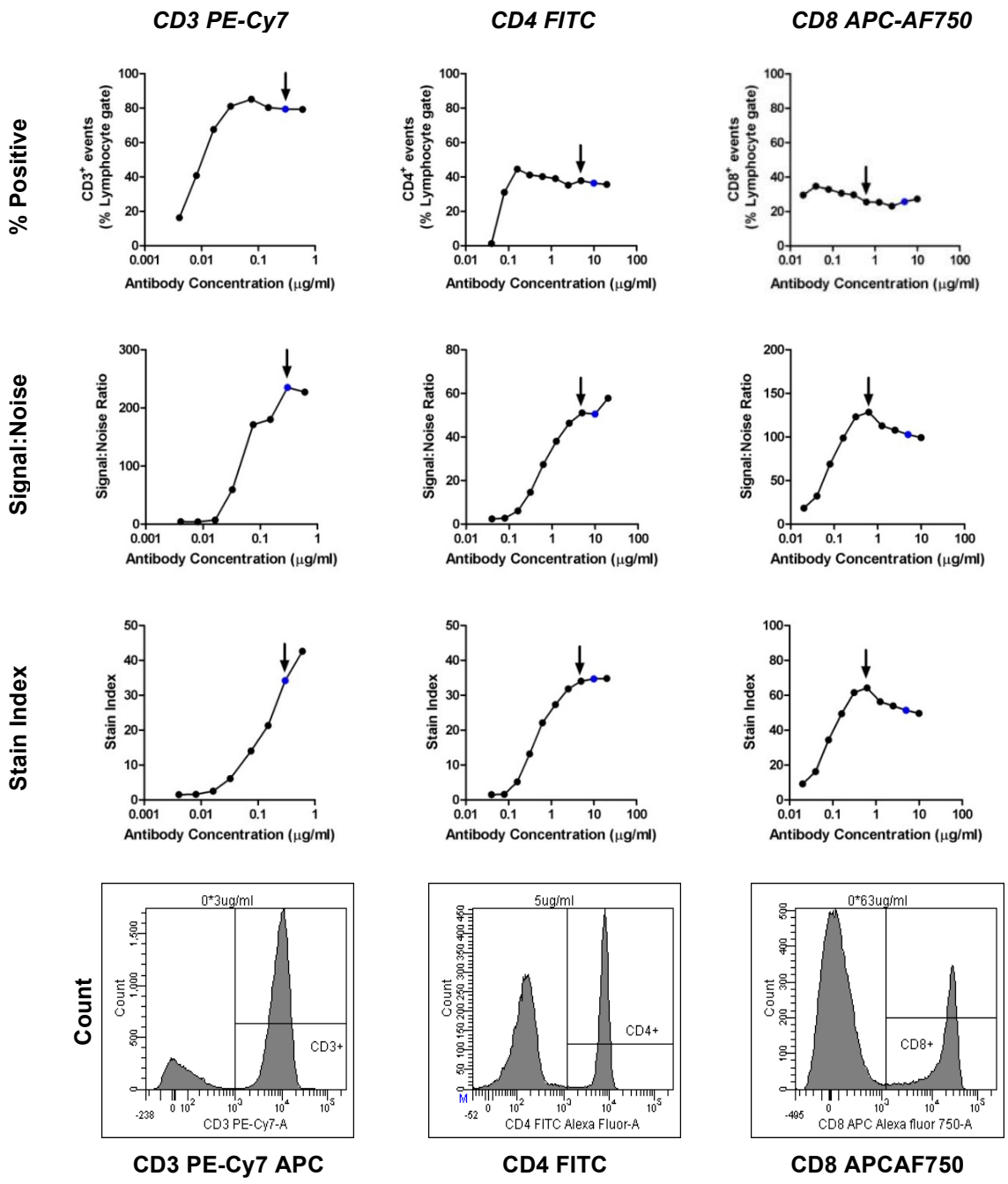


Figure 3.5: Anti-CD3 PE-Cy7, CD4 FITC and CD8 APC-AF750 Titration

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with anti-CD3 PE-Cy7 (0-0.6µg/ml), anti-CD4 FITC (0-20µg/ml) or anti-CD8-APC AF750 (0-10µg/ml). Antibody concentration (x axis) against the % positive cells (top), signal-to-noise ratio (middle) and stain index (bottom). Supplier recommended concentration (•); Concentration used in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell assay (↓). Flow plots show the staining pattern at the concentration used in the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry assay.

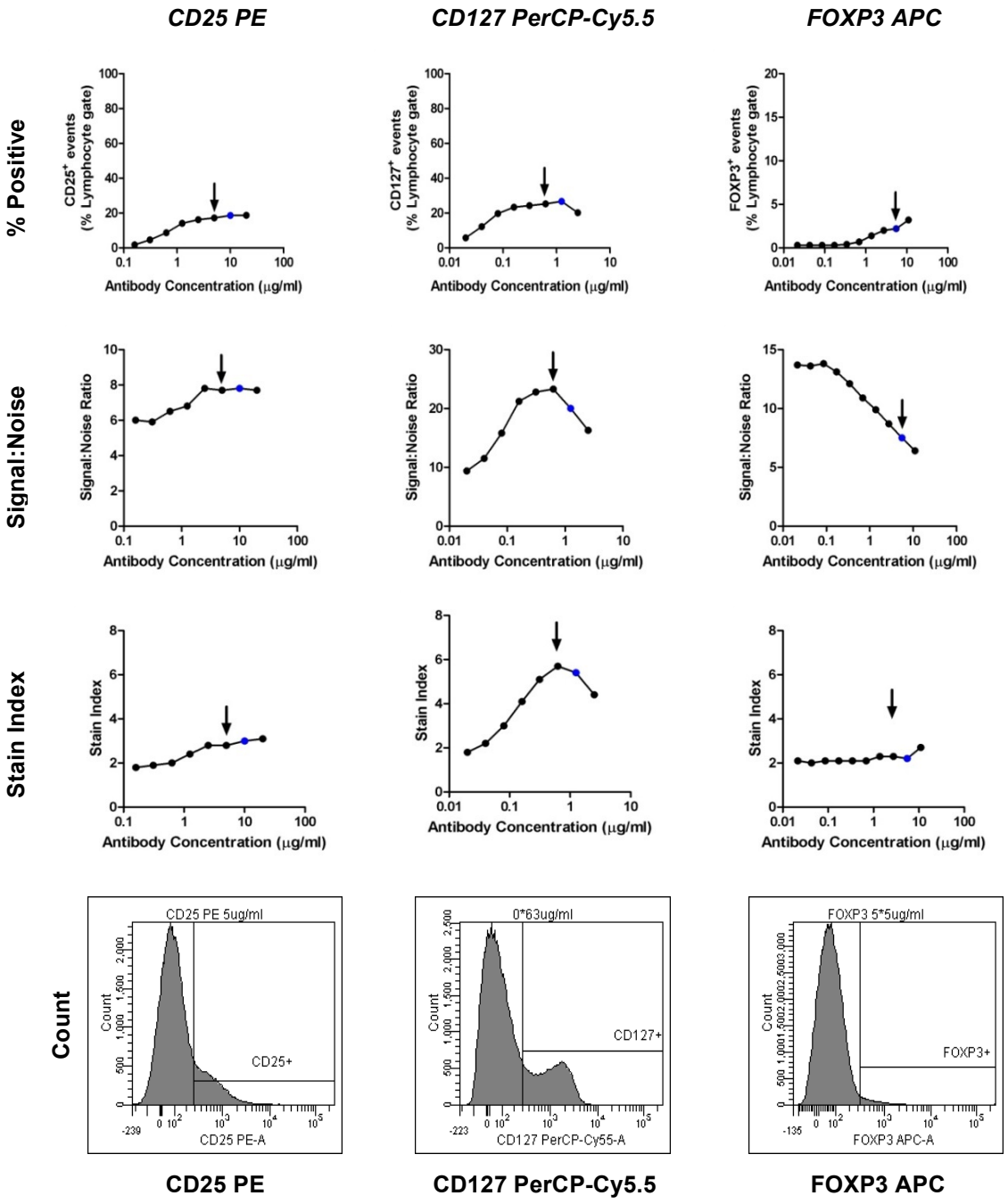


Figure 3.6: Anti-CD25 PE, CD127 PerCP-Cy5.5 and FOXP3 APC Titration

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with anti-CD25 PE (0-20µg/ml), anti-CD127 PerCP-Cy5.5 (0-2.5µg/ml) or anti-FOXP3 APC (0-10µg/ml). Antibody concentration (x axis) against the % positive cells (top), signal-to-noise ratio (middle) and stain index (bottom). Supplier recommended concentration (•); Concentration used in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell assay (↓). Flow plots show the staining pattern at the concentration used in the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry assay.

Antibody	Recommended concentration (µg/ml)	Final concentration used (µg/ml)	Volume per test (µl)
CD3 PE-Cy7	0.30	0.30	5
CD4 FITC	10.00	5.00	10
CD8 APC-AF 750	5.00	0.63	2.5
CD25 PE	10.00	5.00	10
CD127 PerCP-Cy5.5	1.25	0.63	10
FOXP3 APC	5.00	5.00	5

Table 3.2: Selected Antibody Concentrations for the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell Flow Cytometry Assay

For each fluorochrome-conjugated antibody, the manufacturer's recommended working concentration (µg/ml), the selected concentration used in this project (µg/ml) and the equivalent volume used per test (µl) for a total staining volume of 100µl are shown.

3.3.3 CHECKING SENSITIVITY

To confirm that multi-colour staining did not significantly reduce the sensitivity to detect each antigen, $1x10^6$ PBMC were stained with the six-colour panel and compared to single stained controls. The percentage of the total lymphocytes positive for each antigen using the two methods is shown in Figures 3.7 & 3.8. For CD3, CD4, CD8 and CD127 single colour staining, the positive cells were gated as shown in Figure 3.8. For CD25 single staining, the negative/positive boundary was determined using a matched isotype control. For FOXP3 staining, the negative/positive boundary was determined using $CD20^+FOXP3^-$ cells as above. With the six-colour panel, the CD3, CD4, CD8, CD25 and CD127 negative/positive boundaries were defined using FMO controls to take into account the 'spreading' of the negative population due to spectral spillover (Maecker and Trotter, 2006). For FOXP3, the FMO control underestimated the negative/positive boundary due to background intracellular staining. Although this was minimised with repeated washing, a more consistent FOXP3 negative/positive boundary was set by gating on $CD3^+CD4^-CD8^-$ population (McIver *et al.,* 2008; Lee *et al.,* 2011). For all antigens tested, there was good consistency between the single and multi-colour staining. Although the multi-colour panel showed a slight loss of sensitivity for $CD25^{dim}$ cells (13.4% v 15.2% lymphocytes, P=0.03), this was not felt to be a significant problem when quantifying $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells as most are $CD25^{high}$.

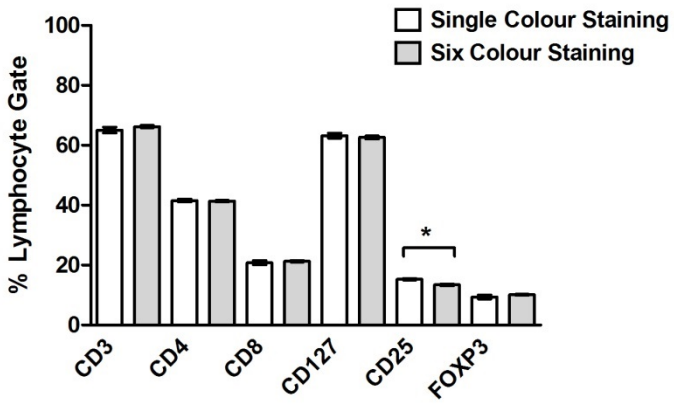


Figure 3.7: Comparing Single-Colour Staining versus Multi-Colour Staining

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with single antibodies or the six-colour $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5.5). Lymphocytes were gated using FSC/SSC. The figure shows the percentage of total lymphocytes staining positive for each antigen using single-colour staining (empty bars) and the six-colour panel (grey bars). $*P<0.05$

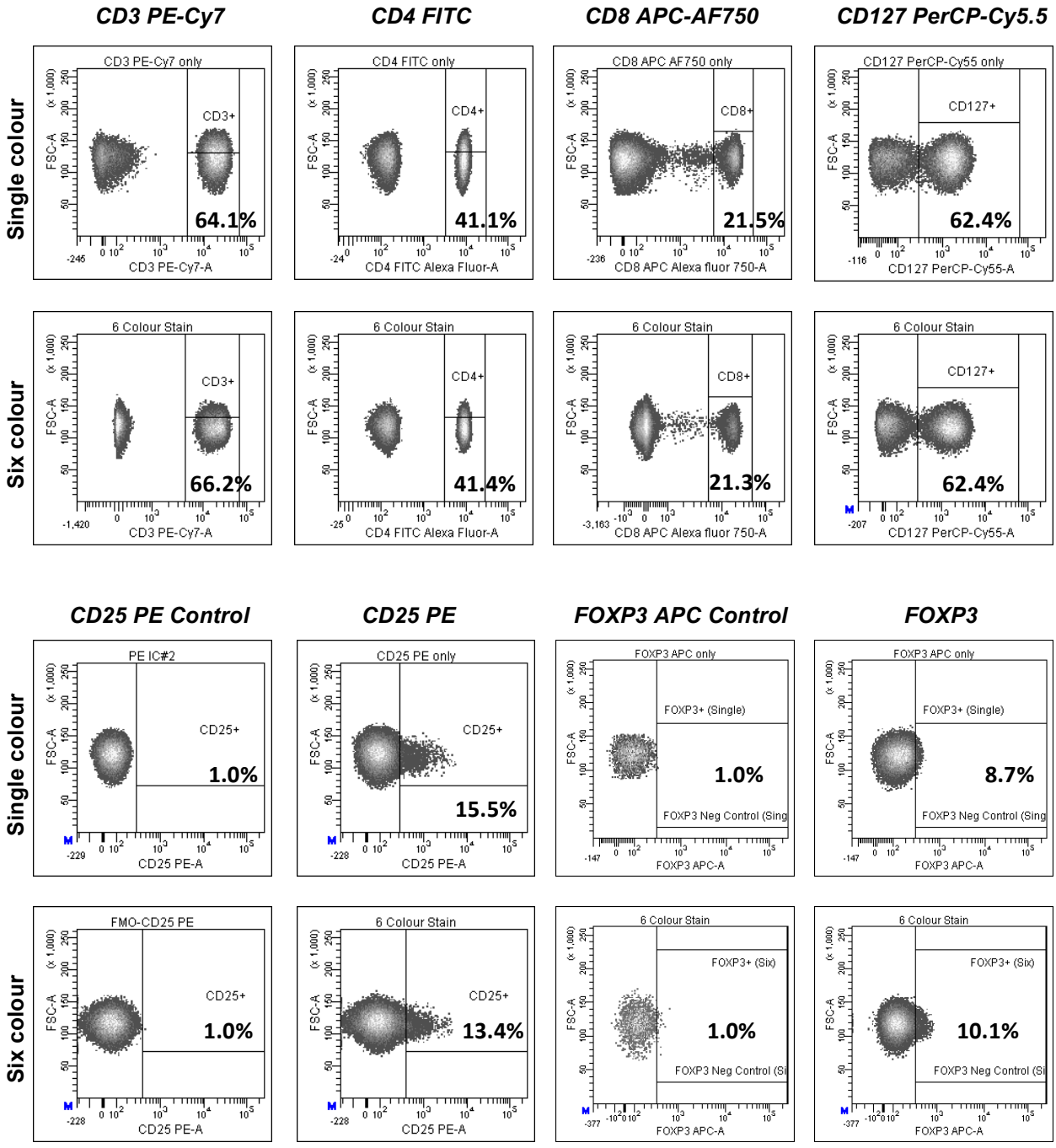


Figure 3.8: Comparing Single-Colour Staining versus Multi-Colour Staining

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained were stained with single antibodies or the six-colour $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5). Lymphocytes were first gated using FSC/SSC. Negative/positive boundaries for CD25PE were established using an isotype control (single-colour) and FMO control (six-colour). Negative/positive boundaries for FOXP3 were established using FOXP3 negative populations: $CD20^+$ (single-colour) and $CD3^+CD4^-CD8^-$ (six-colour).

3.3.4 FLOW CYTOMETRY ANALYSIS

The method used to analyse the multi-colour flow cytometry data is shown below in Figures 3.9 & 3.10. $CD3^+$ cells were first gated using CD3, FSC (size) and SSC (granularity). $CD3^+$ cells were analysed for CD4 and CD8 expression and the $CD3^+CD4^+CD8^-$ cells analysed for CD25 and FOXP3 expression. $CD3^+CD4^+CD25^+FOXP3^+$ cells were gated and analysed for expression of CD127. Putative Tregs in this assay were defined as $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and expressed as the percentage of the total $CD4^+$ T cells. Absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cell counts were calculated by multiplying the $CD25^+FOXP3^+CD127^{-/dim}$ cells (expressed as a percentage of the total $CD4^+$ T cells) by the absolute $CD4^+$ T-cell count (Chapter 3.4). CD3, CD4, CD8, CD25 and CD127 negative/positive boundaries were established using FMO controls. The FOXP3 negative/positive boundary was set using $CD3^+CD4^-CD8^-$ cells.

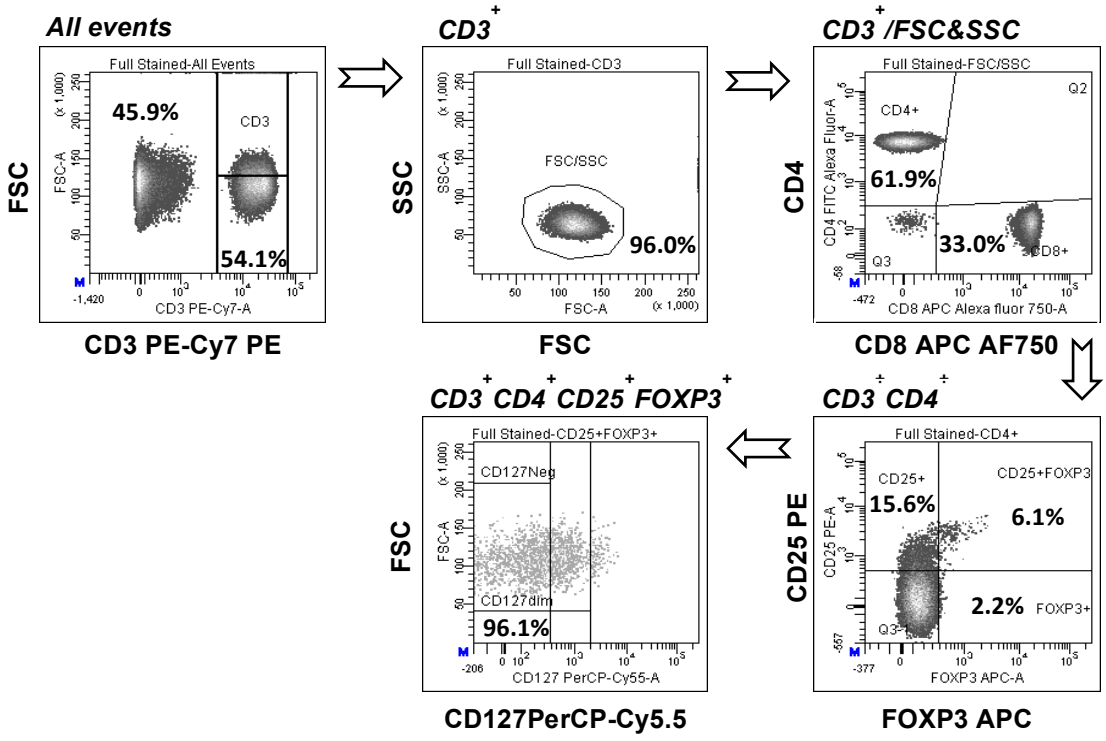


Figure 3.9: $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell Flow Cytometry Assay

Ficoll-Paque isolated PBMC ($1x10^6$/test) were stained with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5.5). $CD3^+$ cells were gated by CD3/FSC/SSC. $CD3^+CD4^+CD8^-$ cells were analysed for FOXP3/CD25 expression. $CD3^+CD4^+CD25^+FOXP3^+$ cells were analysed for CD127.

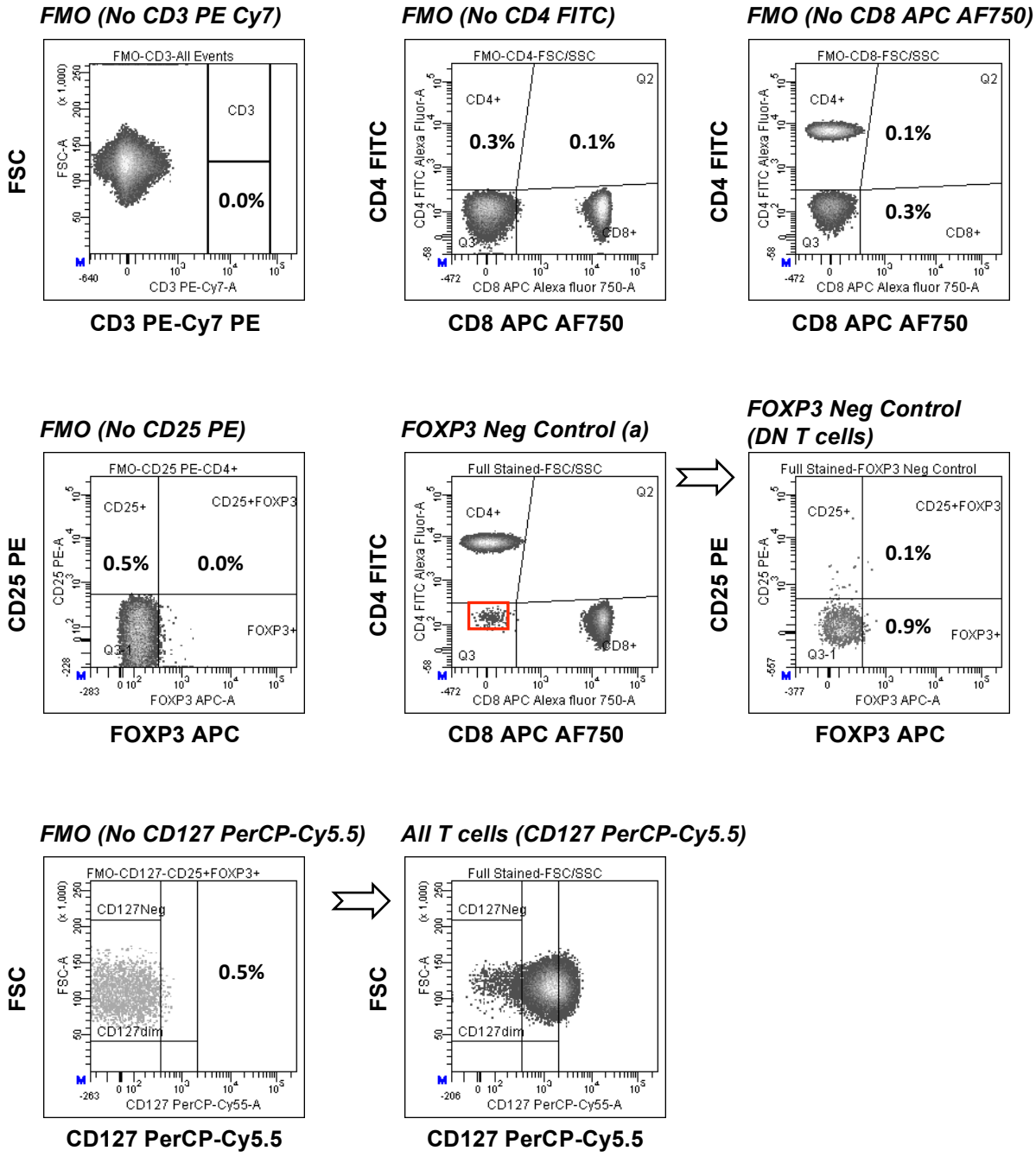


Figure 3.10: Staining Controls for CD4⁺CD25⁺FOXP3⁺CD127$^{-/dim}$ T-cell Flow Cytometry

Ficoll-Paque isolated PBMC (1x10^6/test) were stained with the CD4$^+$CD25$^+$FOXP3$^+$CD127$^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5.5). Negative/positive boundaries for CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE and CD127 PerCP-Cy5.5 staining were established using FMO controls. FOXP3 APC negative/positive boundary was set using FOXP3 negative population CD3$^+$CD4$^-$CD8$^-$ (red box).

3.3.5 ESTIMATING VARIANCE IN THE $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/DIM}$ T-CELL FLOW CYTOMETRY ASSAY

Before commencing the clinical study, the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell assay was tested on samples obtained from healthy volunteers. To estimate the variance in the assay, PBMC were isolated from whole blood samples (three healthy volunteers) and each PBMC isolation stained in triplicate (Figure 3.11A). On the same day, PBMC were also isolated and stained from triplicate blood samples, taken at the same time, from the same donor (three healthy volunteers) (Figure 3.11B). The percentage of lymphocytes (FSC/SSC), positive for the each antigen was measured and the coefficient of variation calculated (Figure 3.11C). The mean %CV for CD3, CD4, CD8, CD25 and CD127 was < 5% for replicates tested from both within and between PBMC isolations. For FOXP3, the mean %CV was higher (variation between samples was 10.2 ± 0.8%), reflecting the low percentage of lymphocytes expressing FOXP3 and the greater variability in staining intracellular antigens due to cell permeabilization and washing.

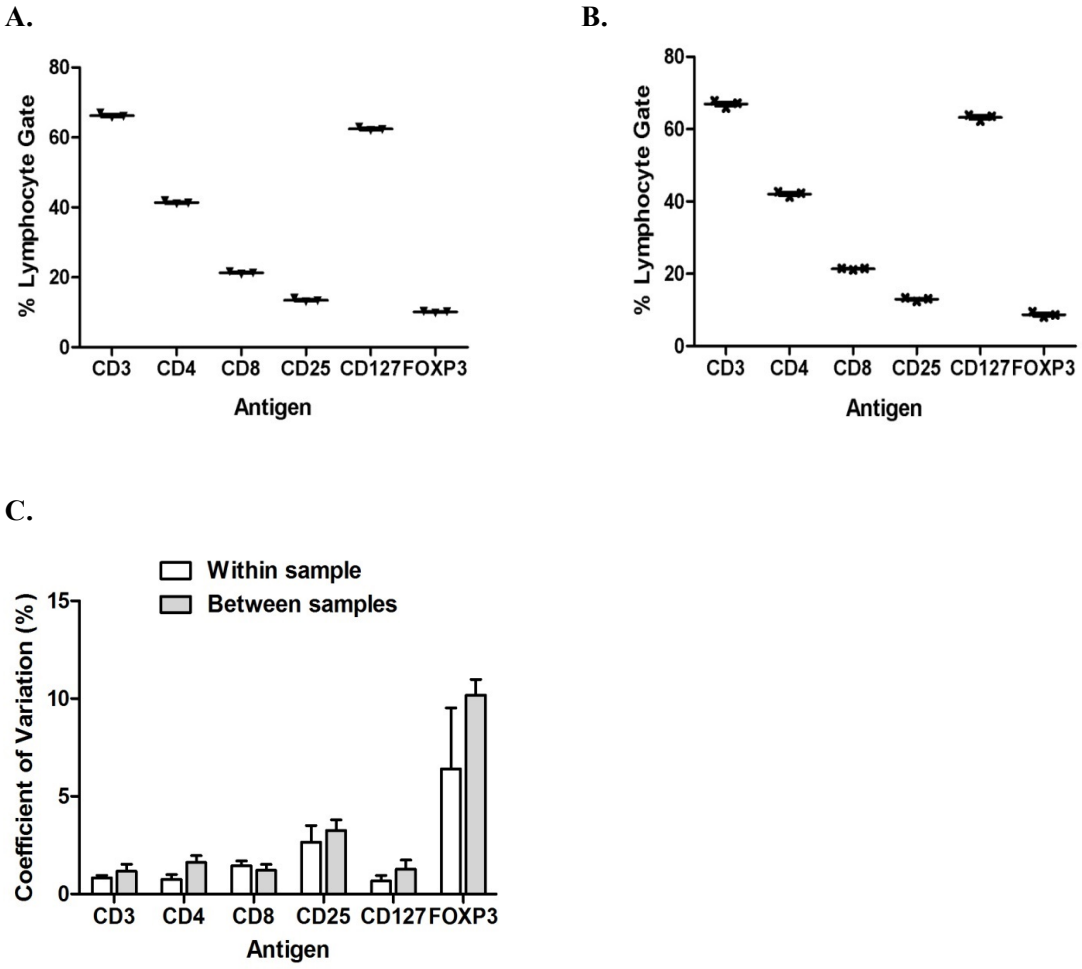


Figure 3.11: Variation within the Assay – Staining Antigens

PBMC were isolated using Ficoll-Paque separation from triplicate peripheral blood samples (taken at the same time) obtained from three healthy volunteers. $1x10^6$ PBMC/test were stained were stained with the six-colour $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5.5). Lymphocytes were gated using FSC/SSC. Figures show the percentage of lymphocytes positive for each antigen from **(A)** Single PBMC sample stained in triplicate; **(B)** Triplicate PBMC isolations from a single donor**.** Results from a single representative donor are shown. **(C)** The mean %CV ± SEM from all three donors for replicates stained from within a PBMC sample (empty bars) and between triplicate PBMC samples (grey bars).

To determine the variability of the assay in measuring individual cell types, each cell was expressed as a percentage of its parent cell population (Figure 3.12). The mean %CV for $CD3^+$ cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells was < 4% for replicates tested within and between PBMC isolations. The variance for the $CD4^+CD25^+FOXP3^+$ T cells was higher, with a mean %CV of 5.8 ± 2.9%, probably reflecting the higher variability in the intracellular FOXP3 staining. For all cells, the mean variance was higher for the replicates measured from triplicate PBMC isolations. However, a mean %CV of <10% for all cells was felt to be acceptable as clinically significant differences in cell counts would be expected to be much greater than 10% given the wide variation in reported T-cell and $CD4^+CD25^+FOXP3^+$ Treg counts between individuals post-alloHSCT (Matthews *et al.,* 2009; Magenau *et al.,* 2010).

Alternative methods of cell preparation and antibody staining were tested. However, when the assay was performed by directly staining 50-100µl whole blood, insufficient number of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were reliably detected (<100 events), particularly in lymphopenic post-transplant samples. Directly staining 500µl-1ml whole blood was unfeasible due to the high costs of the fluorochrome-conjugated monoclonal antibodies. Performing red cell lysis on 10ml of whole blood and re-suspending the cells in 50µl PBS resulted in cell clumping.

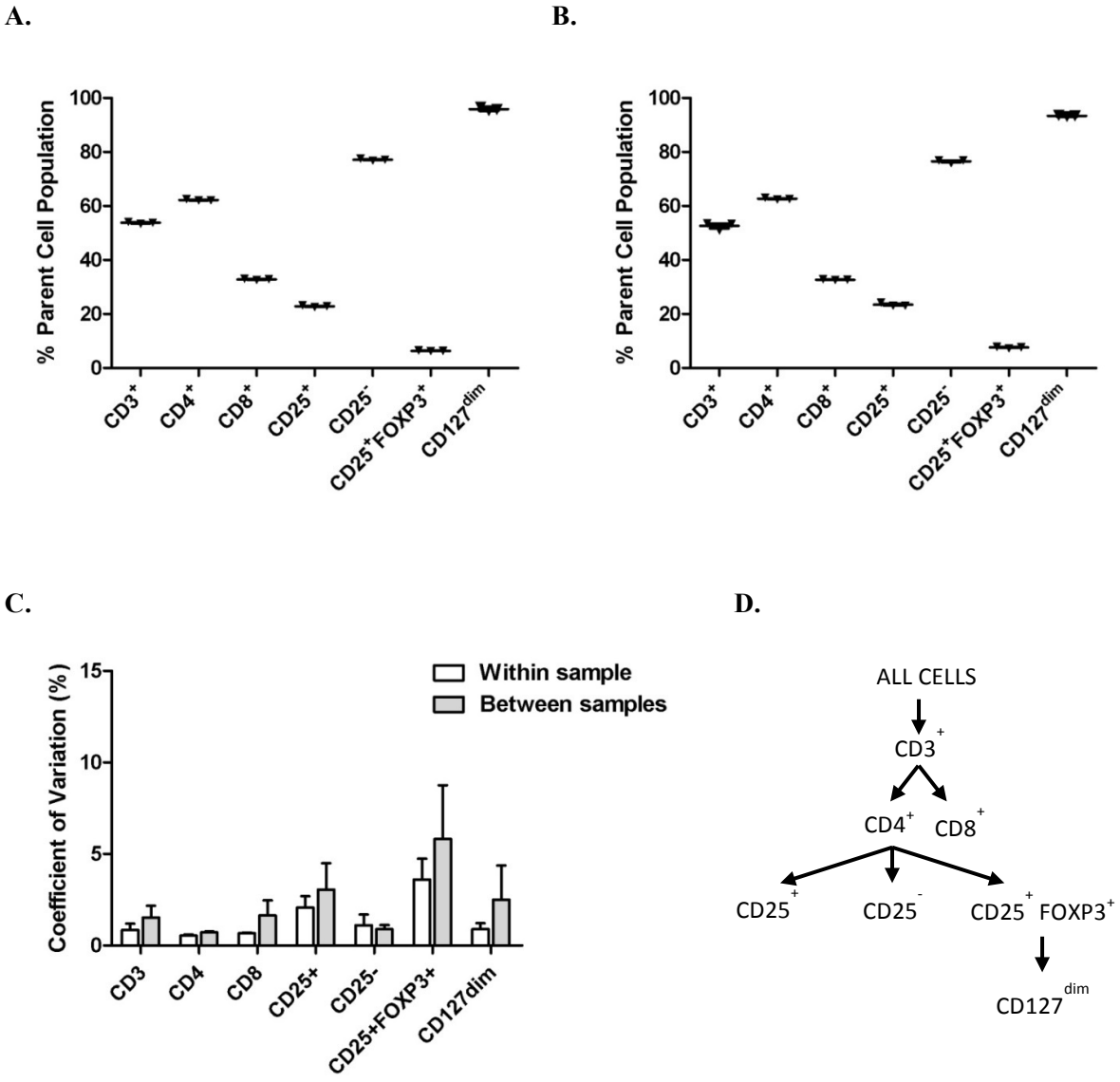


Figure 3.12: Variation within the Assay: Quantifying Cells

PBMC were isolated using Ficoll-Paque separation from triplicate peripheral blood samples (taken at the same time) obtained from three healthy volunteers. $1x10^6$ PBMC/test were stained were stained with the six-colour $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5.5). Figures show the proportion of cells (% parent population) from **(A)** Single PBMC sample stained in triplicate; **(B)** Triplicate PBMC isolations from a single donor**.** Results from a single representative donor are shown. **(C)** The mean %CV ± SEM from all three donors for replicates stained from within a PBMC sample (empty bars) and between triplicate PBMC samples (grey bars). (**D**) Schematic to demonstrate the origin of each cell population.

3.3.6 EFFECT OF SAMPLE ANTI-COAGULANT AND STORAGE TIME

As the clinical samples could be collected at different times and from different hospitals, it was important to check the effect of the sample anti-coagulant and storage time on the quantification of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Paired whole blood samples (10ml) were collected from healthy donors in EDTA and LH (*N*=13). Although the total lymphocyte counts, as measured on an automated Sysmex XE2100 FBC analyser, was not significantly different (2.09 v $2.07x10^9$/l (*P*=0.77)), the blood collected in LH produced slightly higher $CD4^+$ counts (%$CD3^+$ cells) than blood collected in EDTA (57.2 v 54.0%, *P*=0.03; Figure 3.13A). Conversely, the LH collected blood produced significantly lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) than the blood collected in EDTA (4.44 v 5.70%, *P*<0.0001; Figure 3.13B). Although it is unclear whether this difference is due to the effect of the anti-coagulant on the cells, the PBMC isolation or the antigen/antibody binding, it confirmed that the samples should always be collected in the same anti-coagulant. As EDTA blood samples were being collected for FBC analysis, EDTA was also chosen for the flow cytometry samples.

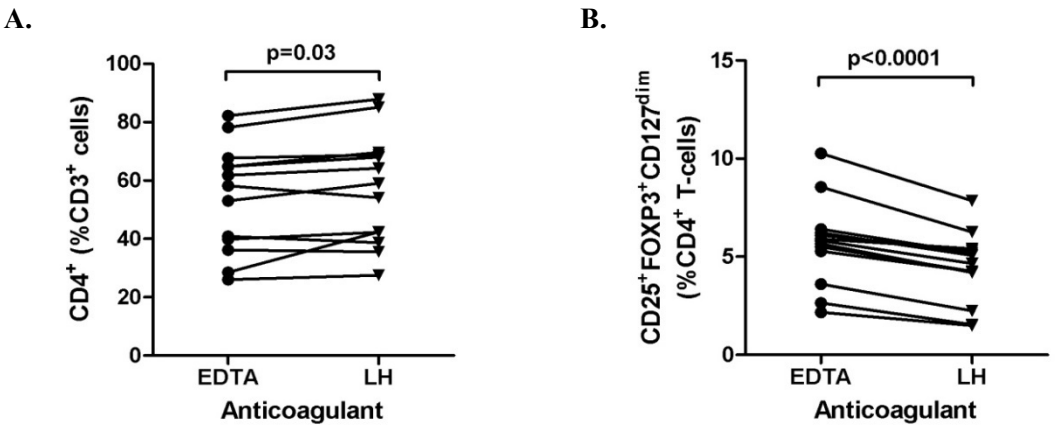


Figure 3.13: Effect of Sample Anti-coagulant

Paired whole blood samples (EDTA and LH) were collected from healthy volunteers (*N*=13). Ficoll-Paque isolated PBMC ($1x10^6$/test) were stained with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC, CD127 PerCP-Cy5.5). Figure shows the effect of the sample anti-coagulant on quantification of **(A)** $CD4^+$ cells (%$CD3^+$ cells) and **(B)** $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). Data were analysed using paired t-test.

To determine the effect of the variable transit time with which the blood samples may reach the laboratory, triplicate samples were collected from healthy volunteer donors (*N*=9) and analysed after 2, 24 and 48 hours storage at RT. The automated FBC analyser was unable to produce an automated count for two of the nine samples kept for 48 hours. In addition, all of the 48 hour samples showed poor red cell separation using Ficoll-Paque and therefore, were unacceptable for further analysis. When comparing the paired 2 hour and 24 hour samples, there was no significant difference detected in either the total lymphocyte count (2.03 v 1.95x10^9/l, *P*=0.37) or the relative $CD4^+$ T-cell count (%$CD3^+$ cells) (48.9 v 50.9%, *P*=0.08; Figure 3.14A). However, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were significantly lower in the 24 hour samples compared to the fresh samples (5.25% v 6.29%, *P*=0.002; Figure 3.14B). It is unclear whether the time delay affects the actual number $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the sample, the antigens being detected or the processing and staining of the PBMC. However, it was concluded that all samples should be analysed on the day of collection.

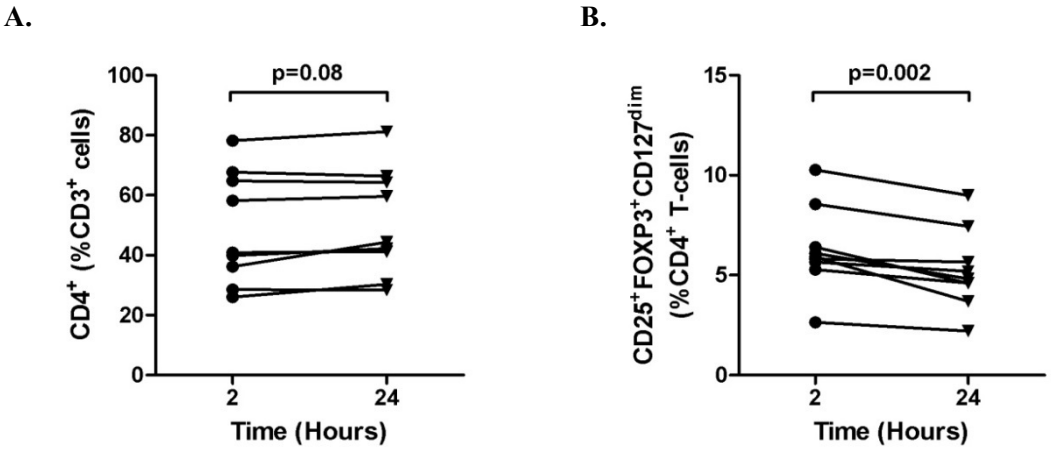


Figure 3.14: Effect of Sample Storage Time

Paired EDTA whole blood samples (stored for two hours or 24 hours at RT) were collected from healthy volunteers (*N*=9). Ficoll-Paque isolated PBMC (1x10^6/test) were stained with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF750, CD25 PE, FOXP3 APC and CD127 PerCP-Cy5.5). Figure shows the effect of storage time on quantification of **(A)** $CD4^+$ cells (%$CD3^+$ cells) and **(B)** $CD25^+FOXP3^+CD127^{-/dim}$ T cells (%$CD4^+$ T cells). Data were analysed using paired t-test.

3.4 LYMPHOCYTE FLOW CYTOMETRY ASSAY

To quantify the main lymphocyte populations (T, B and NK cells), a second six-colour flow cytometry assay was devised. However, as these cells are more abundant than $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and because all the antigens being detected are expressed on the cell surface, sufficient cells could be analysed by directly staining 50µl of whole blood. A method comprising antibody staining followed by lysis of red cells and a final wash (stain/lyse/wash) was employed. Staining whole blood avoids the possibility that Ficoll-Paque separation may alter the ratio of T, $CD4^{+}$ T, $CD8^{+}$ T, B and NK cells and/or affect antibody binding to the antigen (Ashmore *et al.,* 1989; Tamul *et al.,* 1995). The work in this section describes the development and testing of the lymphocyte flow cytometry panel.

3.4.1 SELECTING THE ANTIGENS AND FLUOROCHROMES

The six antigens selected to measure the main lymphocyte populations were CD3, CD4 and CD8 (T cells), CD19 (B cells) and CD16 and CD56 (NK cells). As before, the six fluorochromes used were FITC, PE, PerCP-Cy5.5, PE-Cy7, APC and APC Alexa-Flour 750. To maximise efficiency and minimise costs, the same antibody-fluorochrome paring was used for CD3 (PE-Cy7), CD4 (FITC) and CD8 (APC-AF750) as used in the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell flow cytometry panel. The remaining antibody/ fluorochrome combinations used were CD19 PE, CD56 APC and CD16 PerCP-Cy5.5. All three new antibodies were titrated (Figure 3.15) and the final concentrations selected are shown in Table 3.3.

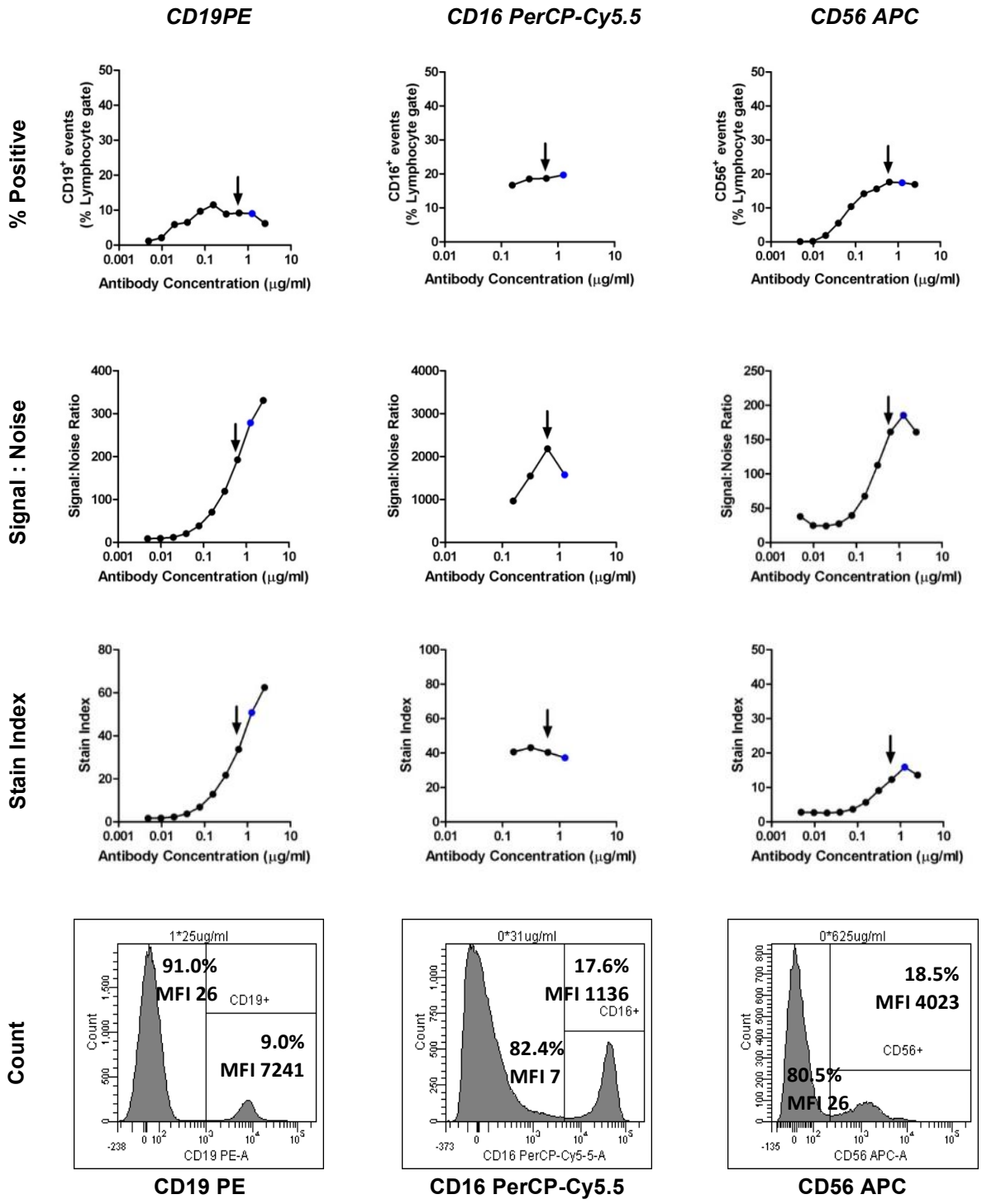


Figure 3.15: Anti-CD19 PE, CD16 PerCP-Cy5.5 and CD56 APC Titration

PBMC were isolated from buffy coats using Ficoll-Paque separation. $1x10^6$ PBMC/test were stained with anti-CD19 PE (0-2.5µg/ml), anti-CD16 PerCP-Cy5.5 (0-2.5µg/ml) or anti-CD56-APC (0-2.5µg/ml). Antibody concentration (x axis) against the % positive cells (top), signal-to-noise ratio (middle) and stain index (bottom). Supplier recommended concentration (•); Concentration used in lymphocyte assay (↓). Flow plots show the staining pattern at the concentration used in the lymphocyte assay.

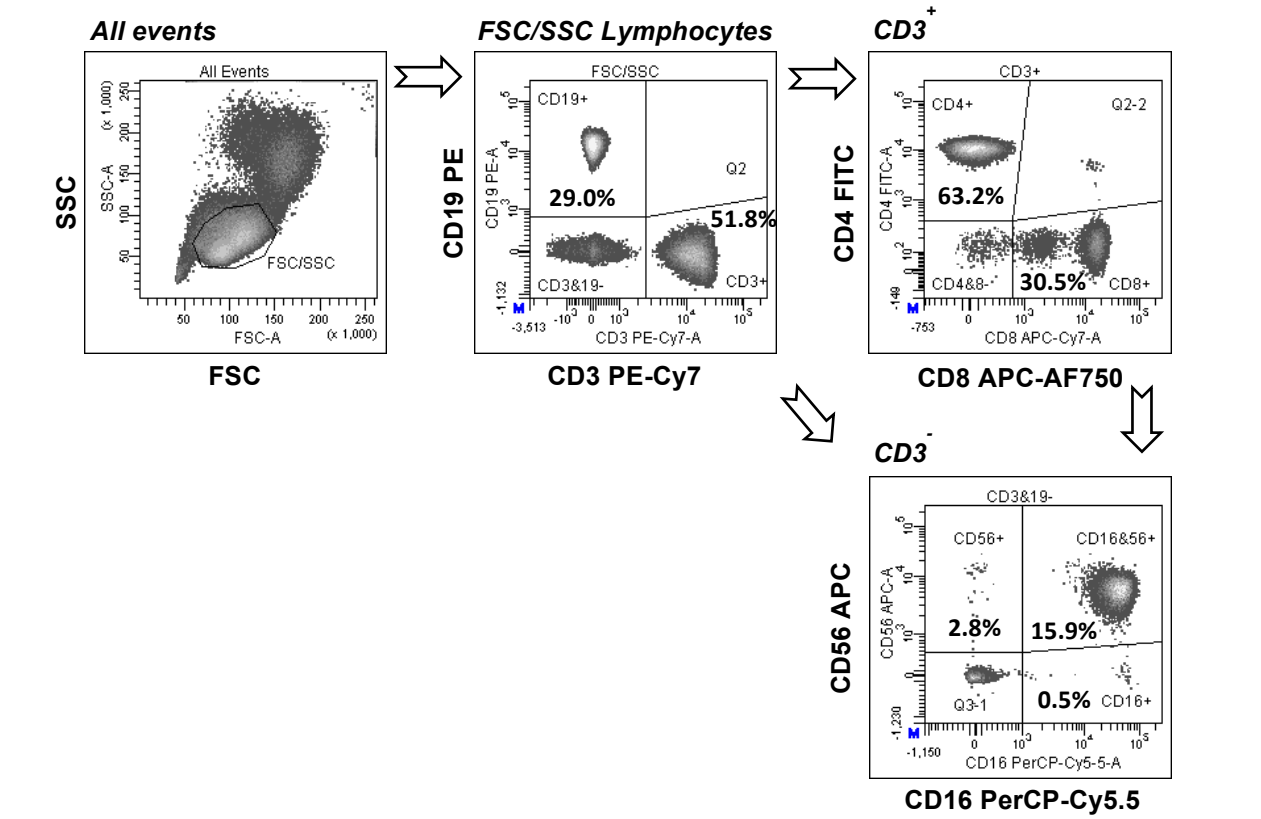
Antibody	Recommended concentration (µg/ml)	Concentration used (µg/ml)	Volume per test (µl)
CD3 PE-Cy7	0.30	0.30	5
CD4 FITC	10.00	5.00	10
CD8 APC-AF 750	5.00	0.63	2.5
CD19 PE	1.25	0.63	10
CD16 PerCP-Cy5.5	1.25	0.63	10
CD56 APC	1.25	0.63	10

Table 3.3: Selected Antibody Concentrations for Lymphocyte Flow Cytometry Assay

For each fluorochrome-conjugated antibody, the manufacturer's recommended working concentration (µg/ml), the selected concentration used in this project (µg/ml) and the equivalent volume used per test (µl) for a total staining volume of 100µl are shown.

3.4.2 FLOW CYTOMETRY ANALYSIS

The gating sequence used to analyse the flow cytometry data to quantify the lymphocyte populations is shown in Figure 3.16. Lymphocytes were first gated using FSC/SSC and analysed for expression of CD19 and CD3. $CD3^-CD19^+$ (B cells) and $CD3^+CD19^-$ (T cells) events were expressed as a percentage of the total lymphocytes. T cells were analysed for their expression of CD4 and CD8. $CD3^+CD4^+CD8^-$ (T-helper cells) and $CD3^+CD4^-CD8^+$ (T-cytotoxic cells) were also expressed as a percentage of the total lymphocytes. Finally, the $CD3^-CD19^-$ were analysed for their expression of CD56 and CD16 and $CD3^-CD56^+$ (NK cells) were expressed as percentage of the total lymphocytes. The negative controls used to ascertain the negative/positive boundaries were FMO controls. To calculate the absolute counts, the value for each cell type (% lymphocytes) was multiplied by the total lymphocyte count obtained from an automated WBC differential count.



Controls:

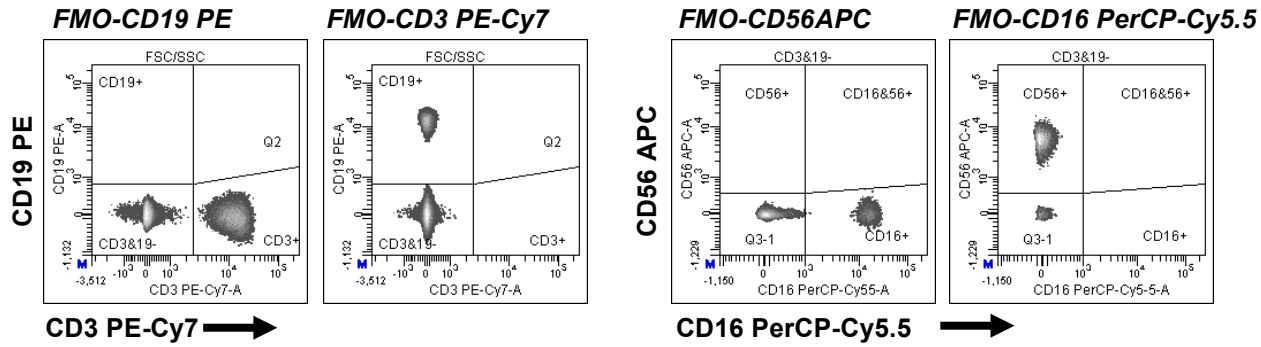


Figure 3.16: Lymphocyte Flow Cytometry Analysis

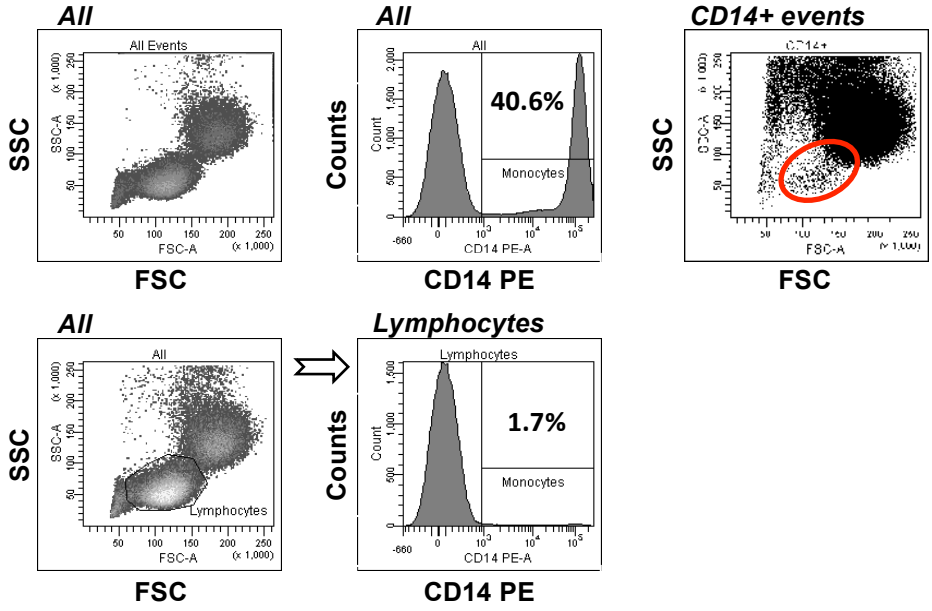
50µl whole blood (EDTA) was stained with the lymphocyte flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD19 PE, CD16 PerCP-Cy5.5, CD56 APC) using a stain/lyse/wash method. Lymphocytes were gated using FSC/SSC and analysed for expression of CD19 and CD3 ($CD3^+$ cells, $CD19^+$ cells). $CD3^+$ cells were analysed for their expression of CD4 and CD8 ($CD3^+CD4^+$ and $CD3^+CD8^+$ cells). The $CD3^-CD19^-$ cells were analysed for expression of CD56 and CD16 ($CD3^-CD56^+$ cells). Bottom row shows the FMO controls used to set negative/positive boundaries for CD3, CD19, CD56 and CD16 respectively.

3.4.3 POTENTIAL PROBLEMS WITH THE LYMPHOCYTE FLOW CYTOMETRY PANEL

A) POTENTIAL ERRORS

During use of the lymphocyte flow cytometry panel, several potential errors were noted resulting from the use of FSC/SSC only to gate lymphocytes. First, non-lymphocytes could have been present within the FSC/SSC gate. To demonstrate this, whole blood was stained with anti-CD14 (monocytes) or anti-Glycophorin A (erythrocytes). As shown in Figure 3.17, most of the $CD14^+$ cells had a higher FSC/SSC profile than the lymphocytes. However, 1.56% (0.80-2.20%, *N*=3) of the events in the initial FSC/SSC gate also expressed CD14. While this could be due to the CD14 antigen being present on a small population of lymphocytes or non-specific binding, it could also be due to monocytes being present within the lymphocyte gate. Similarly, when stained with Glycophorin A (GlyA), some GlyA positive cells were still present following red cell lysis. GlyA positive events accounted for 5.50% (0.20-15.3%, *N*=3) of the lymphocyte gate. This wide variation could be due to the differing effectiveness of the red cell lysis between samples. Non-lymphocytes in the FSC/SSC gate would cause under-estimation of the absolute counts. However, addition of another antibody to assist with initial gating of the lymphocytes and to exclude platelets/erythrocytes from the initial gate, e.g. CD45 Common Leucocyte Antigen (CLA), was not possible due to the overlap of emission spectra and loss of sensitivity when using seven or more fluorochromes.

A. CD14 PE



B. Glycophorin A PE

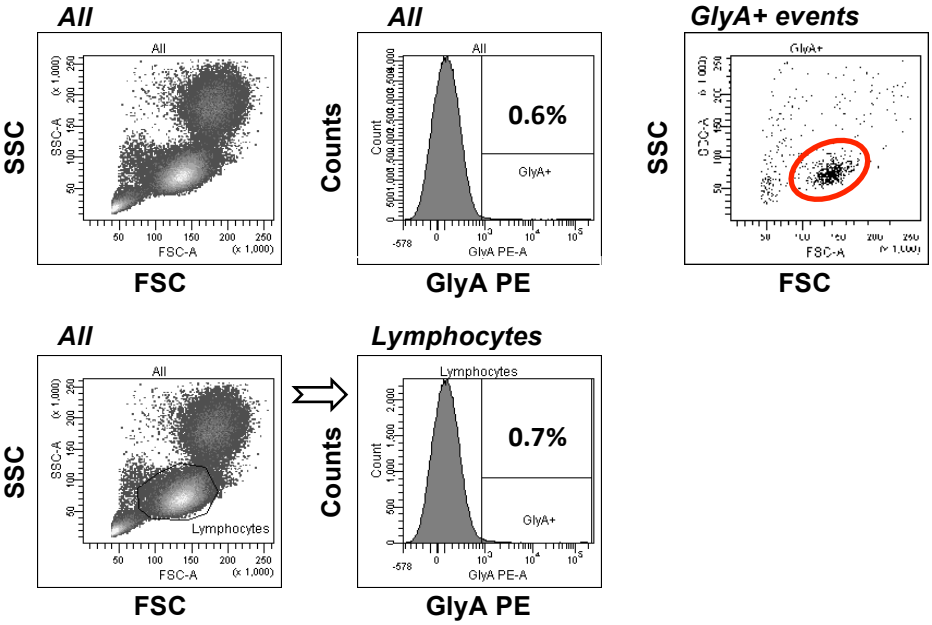


Figure 3.17: Non-Lymphocytes in the FSC/SSC Lymphocyte Gate

50µl whole blood (EDTA) from a healthy volunteer donor was stained with **(A)** anti-CD14 PE: Flow plots show CD14 expression for all cells (top row) and for the lymphocyte FSC/SSC gate (bottom row); **(B)** anti-GlyA PE: Flow plots show GlyA expression for all cells (top row) and for the lymphocyte FSC/SSC gate (bottom row). The red oval shows the approximate location of the lymphocyte FSC/SSC gate demonstrating that $CD14^+$ and $GlyA^+$ events can be present within this region. Figure shows the flow plots from one of the three donor samples tested.

In contrast, lymphocytes could be excluded from analysis if they were outside the boundary of the initial FSC/SSC gate. To demonstrate this, $CD3^+$, $CD19^+$ or $CD3^+CD56^-$ cells were first identified without using FSC/SSC gating. When back gated onto a FSC/SSC plot, the proportion of cells that would be excluded using the original FSC/SSC gating can be observed (Figure 3.18). From a random selection of post-alloHSCT blood samples (*N*=22), 10.2% (2.30-42.3%), 18.0% (2.50%-95.9%) and 10.6% (2.20-54.5%) of the $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cells respectively were outside the original FSC/SSC gate. There was wide variability between samples and the extent to which this occurred appeared random, potentially limiting the power of the assay. Using a larger FSC/SSC gate increased the percentage of these events included but also increased the number of non-lymphocytes. Further analysis of the flow cytometry plots identified that some lymphocytes had higher FSC/SSC profiles than the main population (Figure 3.18). It was hypothesised that this could have been due to cell clumping during washing and centrifugation. Samples were therefore tested in duplicate using antibody staining and red cell lysis, with and without a final washing step. As demonstrated in Figure 3.19, $CD3^+$ cells that had been washed after staining had higher FSC and were positive for CD3 and CD16, in keeping with possible cell clumping. In view of these errors, further methods to analyse the flow cytometry data were explored.

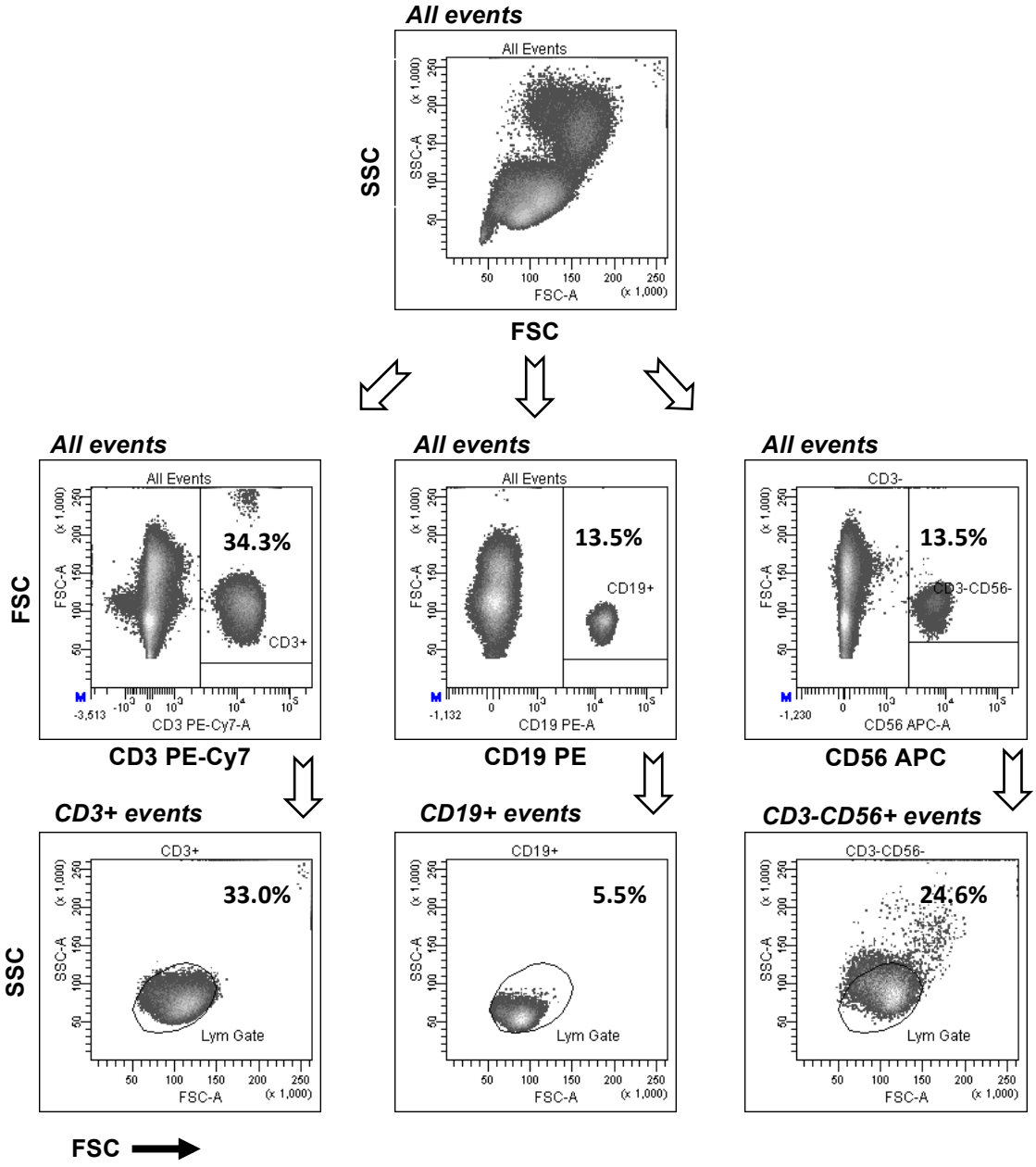


Figure 3.18: Lymphocytes Excluded by the FSC/SSC Gate

50µl whole blood (EDTA) from a healthy volunteer donor was stained with the six-colour lymphocyte flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD19 PE, CD16 PerCP-Cy5.5 and CD56 APC). FSC/SSC profile for all cells (top row). $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cells were identified without prior use of the FSC/SSC lymphocyte gate (middle row). Back-gating of $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ events onto FSS/SSC plots demonstrates the proportions of these cells that would be excluded from further analysis by using a FSC/SSC lymphocyte gate (bottom row).

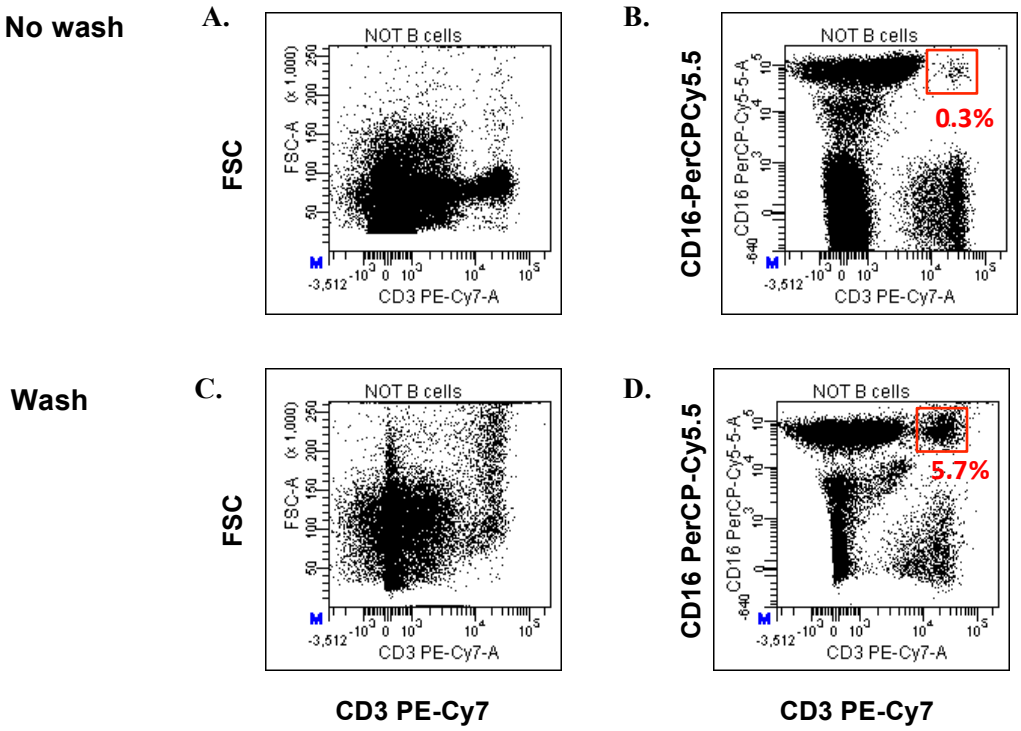


Figure 3.19: Changes Caused by Washing

50µl whole blood (EDTA) from a healthy volunteer donor was stained with the six-colour lymphocyte flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD19 PE, CD16 PerCP-Cy5.5, CD56 APC) using (**A-B**) Stain/lyse/no wash method; (**C-D**) Stain/lyse/wash method. **(A&C)** Plots show FSC/CD3 PE-Cy7; (**B&D)** Plots show CD16 PerCP-Cy5.5/CD3 PE-Cy7.

B) REVISED METHOD OF ANALYSING THE FLOW CYTOMETRY DATA

In view of these potential errors, an alternative method of analysing the data was developed in which gating the lymphocytes using an initial FSC/SSC was no longer required. This method was based on the principle of the lymphosum i.e. T + B + NK cells (Schenker *et al.,* 1993). Each of the three main lymphocyte populations was identified as shown in Figure 3.20. B cells were first identified by their clear expression of CD19 ($CD19^+$). The $CD19^-$ events were analysed for CD3 expression and the T cells measured ($CD3^+$). Finally, the $CD19^-CD3^-$ events were analysed for expression of CD56 and the NK cells recorded ($CD3^-CD56^+$). The B, T and NK cell counts were added to form the total lymphosum count and each cell type was expressed as a percentage of this

value. Absolute counts were calculated by multiplying the %lymphosum by the total lymphocyte count obtained from the automated FBC analyser. A limitation of this method is that it would exclude lymphocytes not detected by anti-CD3, anti-CD19 or anti-CD56 e.g. γδ T cells. However, it was anticipated this would not have a significant effect on the results as these cells represent only a small proportion of the total lymphocytes in PB and HSC grafts (<5% T cells) (Riccieri *et al.,* 2005; Pabst *et al.,* 2007).

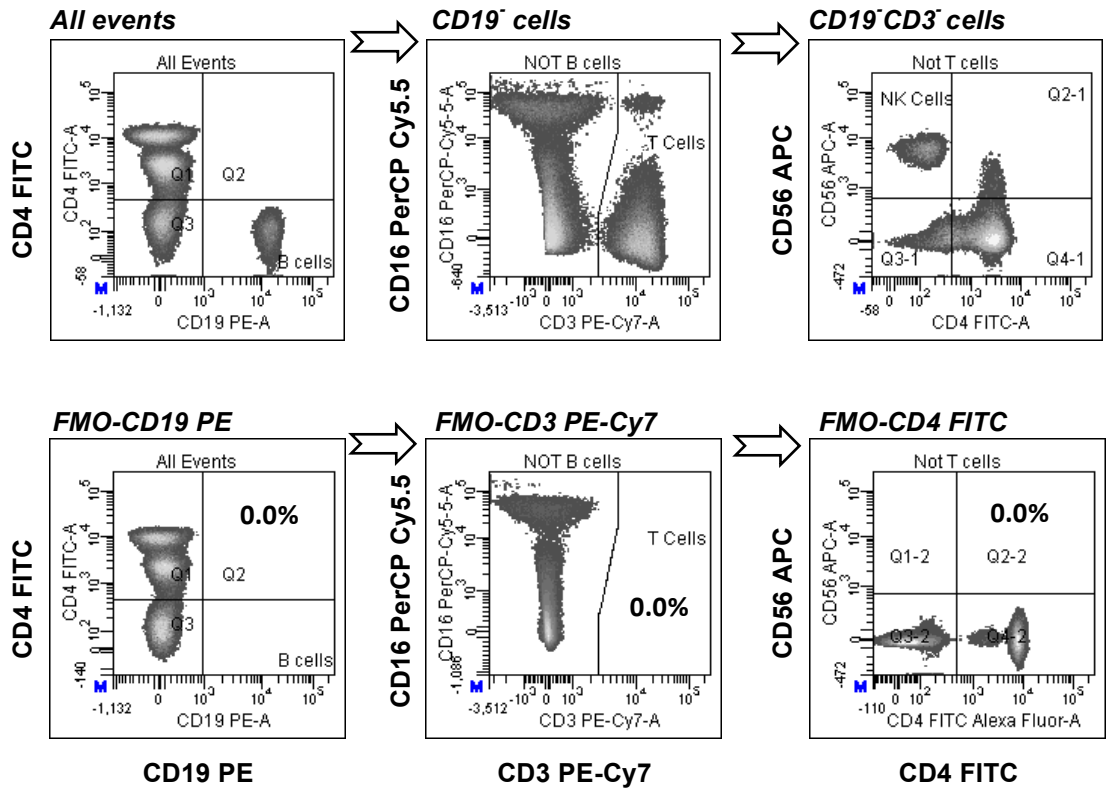


Figure 3.20: Analysis of the Lymphocyte Flow Cytometry Data using Lymphosum

50µl whole blood (EDTA) from a healthy volunteer donor was stained with the six-colour lymphocyte flow cytometry panel. Stained cells were first analysed for their expression of CD19 ($CD19^+$ B cells). The $CD19^-$ events were analysed for CD3 and the T cells identified ($CD3^+$). The $CD3^-$ events were analysed for their expression of CD56 and the NK cells recorded ($CD3^-CD56^+$). The B, T and NK cell counts were added to form the total lymphosum count and each cell type was expressed as a percentage of the lymphosum. Top row shows stained cells; Bottom row shows staining FMO controls.

3.4.4 TESTING THE LYMPHOCYTE FLOW CYTOMETRY PANEL

To test the accuracy of the lymphocyte flow cytometry panel and to ascertain if the errors with FSC/SSC gating would affect the results, approximately 50% of the clinical study samples were run in parallel with a commercially available assay i.e. CD45/CD3/CD4/CD8 Multitest with Trucount tubes (BD Biosciences). This was a single-stage assay in which lymphocytes are first gated using CD45 (common leucocyte antigen)/SSC and the T cells identified by their expression of CD3, CD4 and CD8. Absolute counts were calculated by comparing the number events against a reference bead standard within each tube (Figure 3.21). The advantages of this assay are: (A) CD45/SSC reduces the probability of non-lymphocytes within the primary gate (excludes CD45 negative events, e.g. platelets and erythrocytes, while separating lymphocytes from other leucocyte populations using SSC); (B) Absolute counts are calculated by comparison against a reference bead population; (C) Uses a lyse/stain/no wash method; (D) Rapid (30min). This assay was not used for the entire project as it did not quantify B or NK cells. The associated 6-colour CD45/CD3/CD4/CD8/CD19/CD16/CD56 Multitest with Trucount tubes (BD Biosciences) was too expensive to be used for all the samples in this study.

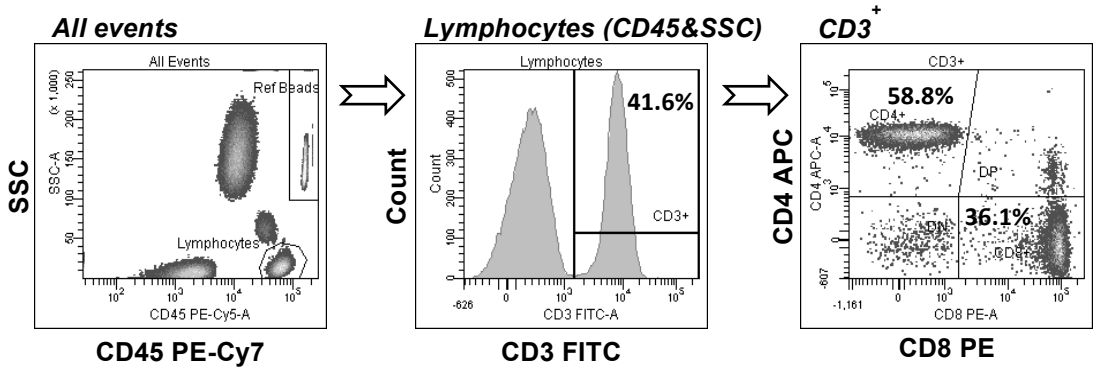


Figure 3.21: CD45/CD3/CD4/CD8 Multitest with Trucount Tubes

50µl whole blood (EDTA) was stained with CD45/CD3/CD4/CD8 Multitest using Trucount tubes (BD Biosciences). Lymphocytes were first gated using SSC/CD45 expression. T cells were then identified by their expression of CD3, CD4 and CD8 as shown. Absolute cell counts were calculated by comparing the cell number against the reference bead standard within each tube. Flow plots shown are from a single representative sample.

The absolute $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cell counts obtained from the lymphocyte flow cytometry panel, using both methods of data analysis, were compared to the counts obtained using the Trucount assay. Only those samples whose Sysmex total lymphocyte count and Trucount total lymphocyte count were within ± 10% were used in this analysis (*N*=243). Despite concerns with FSS/SSC gating in the original method of analysis, there was good correlation between the Trucount assay and lymphocyte assay for all three counts i.e. CD3 ρ =0.94, *P*<0.0001; CD4 ρ =0.87, *P*<0.0001; CD8 ρ =0.93, *P*<0.0001 (Figure 3.22A-C). However, using the 'lymphosum' analysis increased this correlation even closer for CD3 ρ =0.98, *P*<0.0001; CD4 ρ =0.96, *P*<0.0001; CD8 ρ =0.98, *P*<0.0001 (Figure 3.22D-F). Based upon these findings, the absolute counts obtained from the six colour flow cytometry assay were felt to be acceptable and the flow cytometry data were analysed using the modified lymphosum analysis.

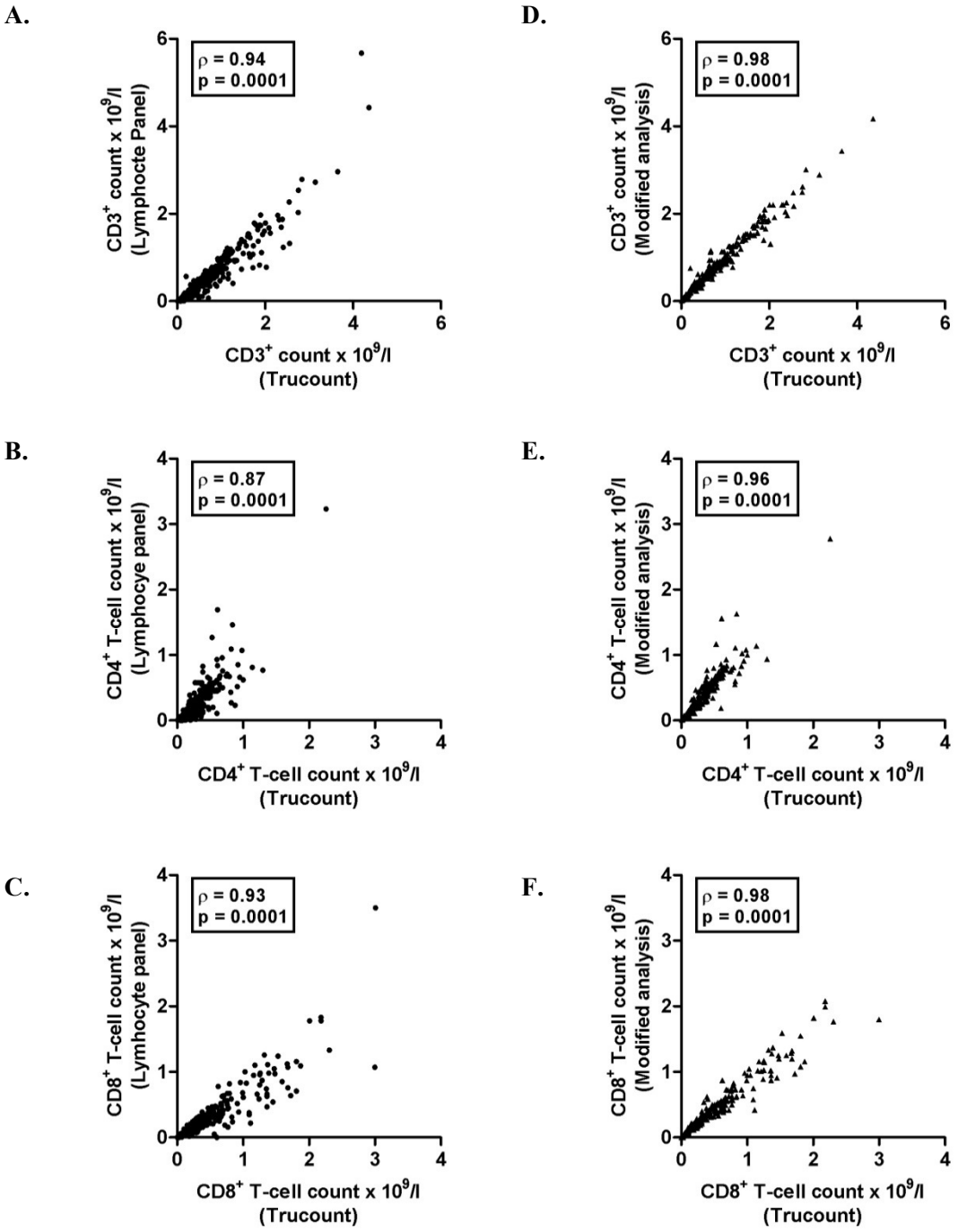


Figure 3.22: Correlation between Lymphocyte Flow Cytometry Assay and Trucount Assay

50µl whole blood (EDTA) from 243 post-alloHSCT was stained in duplicate with the lymphocyte flow cytometry panel (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD19 PE, CD16 PerCP-Cy5.5 and CD56 APC) and the CD45/CD3/CD4/CD8 Multitest using Trucount tubes (BD Biosciences). Figure shows **(A&D)** $CD3^+$; **(B&E)** $CD4^+$ T-cell**; (C&F)** $CD8^+$ T-cell counts produced by the two methods. The lymphocyte flow cytometry data were analysed using **(A-C)** FSC/SSC gating of lymphocytes and **(D-F)** Lymphosum analysis.

3.5 SUPPRESSIVE FUNCTION

Having established flow cytometry methods to quantitatively measure $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (putative Tregs), it was necessary to test whether these cells have suppressive function. However, Treg suppressive function tests remain controversial since *in vitro* suppression may not reflect *in vivo* function (Sakaguchi *et al.,* 2010). In addition, although intracellular FOXP3 can be used to identify putative Tregs in the flow cytometry assay, it cannot be used to isolate viable Tregs for functional tests. Taking these points into consideration, an *in vitro* suppression assay was set up using a putative Treg rich product, isolated using CD4, CD25 and CD127 magnetic bead isolation (Miltenyi Biotech).

3.5.1 $CD4^+CD25^+CD127^{-/DIM}$ T-CELL AND $CD4^+CD25^-$ T-CELL ISOLATION

$CD4^+CD25^+CD127^{-/dim}$ T cells and autologous $CD4^+CD25^-$ T cells were isolated from buffy coats as described in Methods 2.3.1-2.3.2. A representative cell isolation procedure is shown in Figure 3.23. Starting cell purities were assessed by flow cytometry. The median $CD3^+$ cell purity was 91.4% of all events (70.0-97.0, *N*=9 isolations, each from a different buffy coat) and 95.4% (73.4-96.2%, *N*=3 isolations, each from a different buffy coat) in the isolated $CD4^+CD25^-$ T-cell and $CD4^+CD25^+CD127^{-/dim}$ T-cell populations respectively (Figure 3.24). The median $CD4^+CD25^-$ cell purity was 93.8% of $CD3^+$ cells (86.9-97.4%, *N*=9 isolations) and the median $CD4^+CD25^+CD127^{dim}$ cell purity was 78.3% of $CD3^+$ cells (69.8-86.7, *N*=3 isolations). 2.5% (0.5-4.3%, *N*=9 isolations) of the $CD4^+CD25^-$ T-cell populations were $FOXP3^+$. Only three isolation procedures produced sufficient $CD4^+CD25^+CD127^{dim}$ T cells to check starting cell purity and set up the experiment.

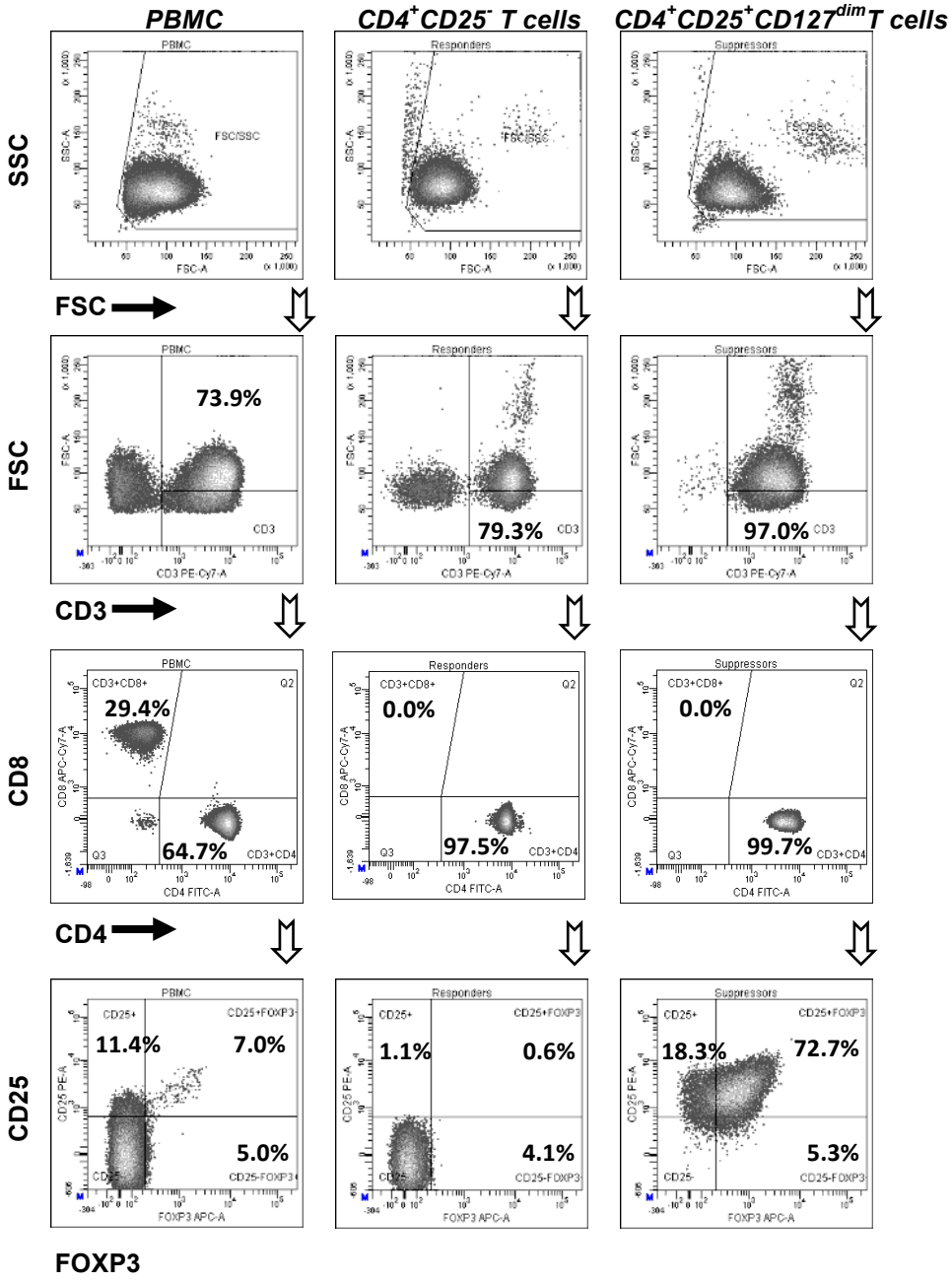


Figure 3.23: Isolation of CD4+CD25- T Cells and CD4+CD25+CD127-/dim T Cells for the *in vitro* Suppression Assay

PBMC were isolated from buffy coats using Ficoll-Paque separation. $CD4^+CD25^-$ T cells were isolated using the magnetic bead $CD4^+$ T-cell isolation kit II (Miltenyi Biotech) and CD25 MicroBeads (Miltenyi Biotech). $CD4^+CD25^+CD127^{-/dim}$ T cells were isolated using the magnetic bead $CD4^+CD25^+CD127^{dim/-}$ Regulatory T-cell isolation kit II (Miltenyi Biotech). Figure shows flow cytometry plots for the PBMC, isolated $CD4^+CD25^-$ T cells and isolated $CD4^+CD25^+CD127^{-/dim}$ T cells from a single representative isolation. $CD3^+$ cells were gated by FSC/SSC/CD3 (first and second rows). $CD3^+$ cells were then assessed for CD4 and CD8 expression (third row). The $CD3^+CD4^+$ cells were assessed for their expression of CD25 and FOXP3 (bottom row).

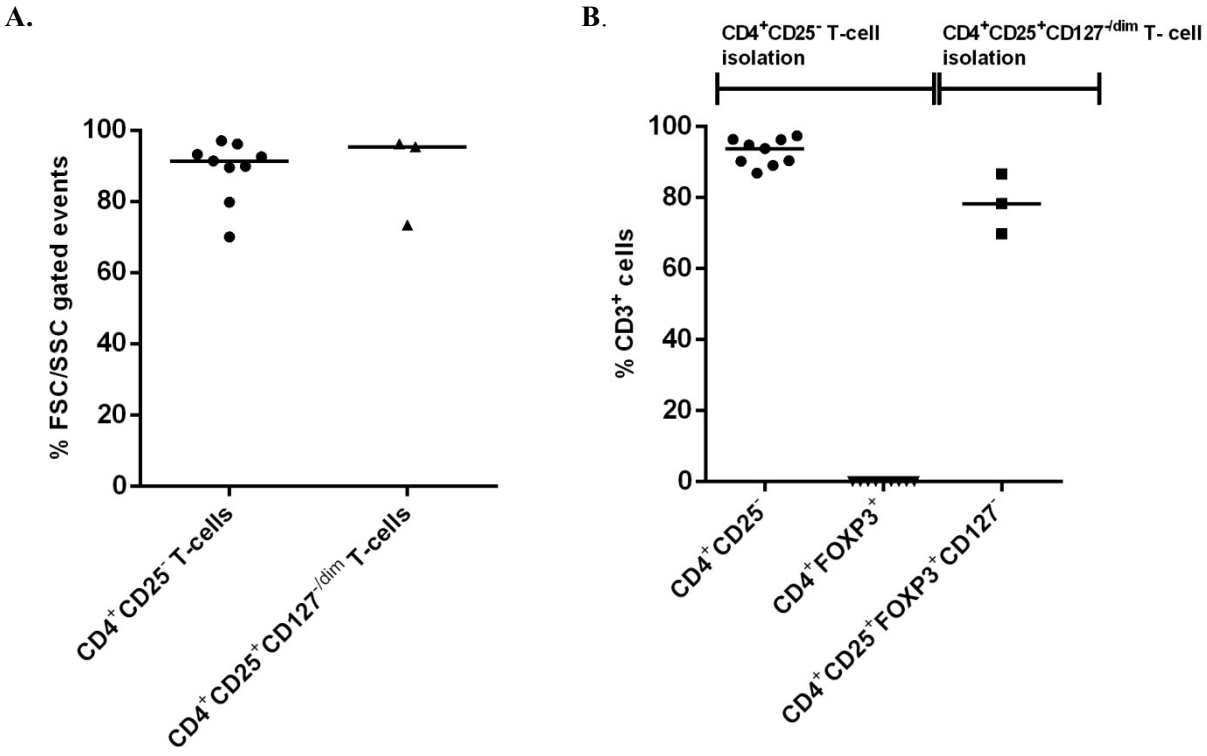


Figure 3.24: Purity of Isolated CD4+CD25- T-Cell and CD4+CD25+CD127-/dim T-Cell Populations

PBMC were isolated from buffy coats using Ficoll-Paque separation. $CD4^+CD25^-$ T cells were isolated using the magnetic bead $CD4^+$ T-cell isolation kit II (Miltenyi Biotech) and CD25 MicroBeads (Miltenyi Biotech). $CD4^+CD25^+CD127^{-/dim}$ T cells were isolated using the magnetic bead $CD4^+CD25^+CD127^{dim/-}$ Regulatory T-cell isolation kit II (Miltenyi Biotech). Figure summarises the purity (as assessed by flow cytometry) of the isolated $CD4^+CD25^-$ T-cell and $CD4^+CD25^+CD127^{-/dim}$ T-cell populations from all the cell isolation procedures (*N*=9, each performed on PBMC from a different buffy coat). The isolated cell populations are expressed as **(A)** Percentage of all cells (gated by FSC/SSC); **(B)** Percentage of $CD3^+$ cells (gated by FSC/SSC/CD3). Horizontal lines show the median values. *(Due to limited cell numbers, only three isolation procedures provided sufficient $CD4^+CD25^+CD127^{-/dim}$ T cells to test the starting cell purity as well as set up the suppression assay).*

3.5.2 SUPPRESSION ASSAY

In vitro suppression assays were set up at $CD4^+CD25^+CD127^{-/dim}$ T-cell (putative Tregs) to $CD4^+CD25^-$ T-cell ratios from 1:1 to 1:32 as per Methods 2.3.6. In these assays, $CD4^+CD25^+CD127^{-/dim}$ T cells suppressed anti-CD3/CD28 driven proliferation of $CD4^+CD25^-$ T cells by 33.0 ± 7.7%, 14.1 ± 5.6% and 5.8 ± 4.0% at ratios of 1:1, 1:2 and 1:4 respectively (Figure 3.25 & 3.26). Although some suppression was seen by the addition of unlabelled $CD4^+CD25^-$ T cells ($CD4^+CD25^+CD127^{-/dim}$ T cells to $CD4^+CD25^-$ T cells 0:2), possibly due to the increased starting cell density, this was significantly lower than the suppression caused by the same number of $CD4^+CD25^+CD127^{-/dim}$ T cells ($CD4^+CD25^+CD127^{-/dim}$ T cells to $CD4^+CD25^-$ T cells 1:1) (6.5 v 33.4%, $P<0.001$).

In summary, these data show that $CD4^+CD25^+CD127^{-/dim}$ T cells (putative Tregs) are able to suppress anti-CD3/CD28 driven proliferation of autologous $CD4^+CD25^-$ T cells.

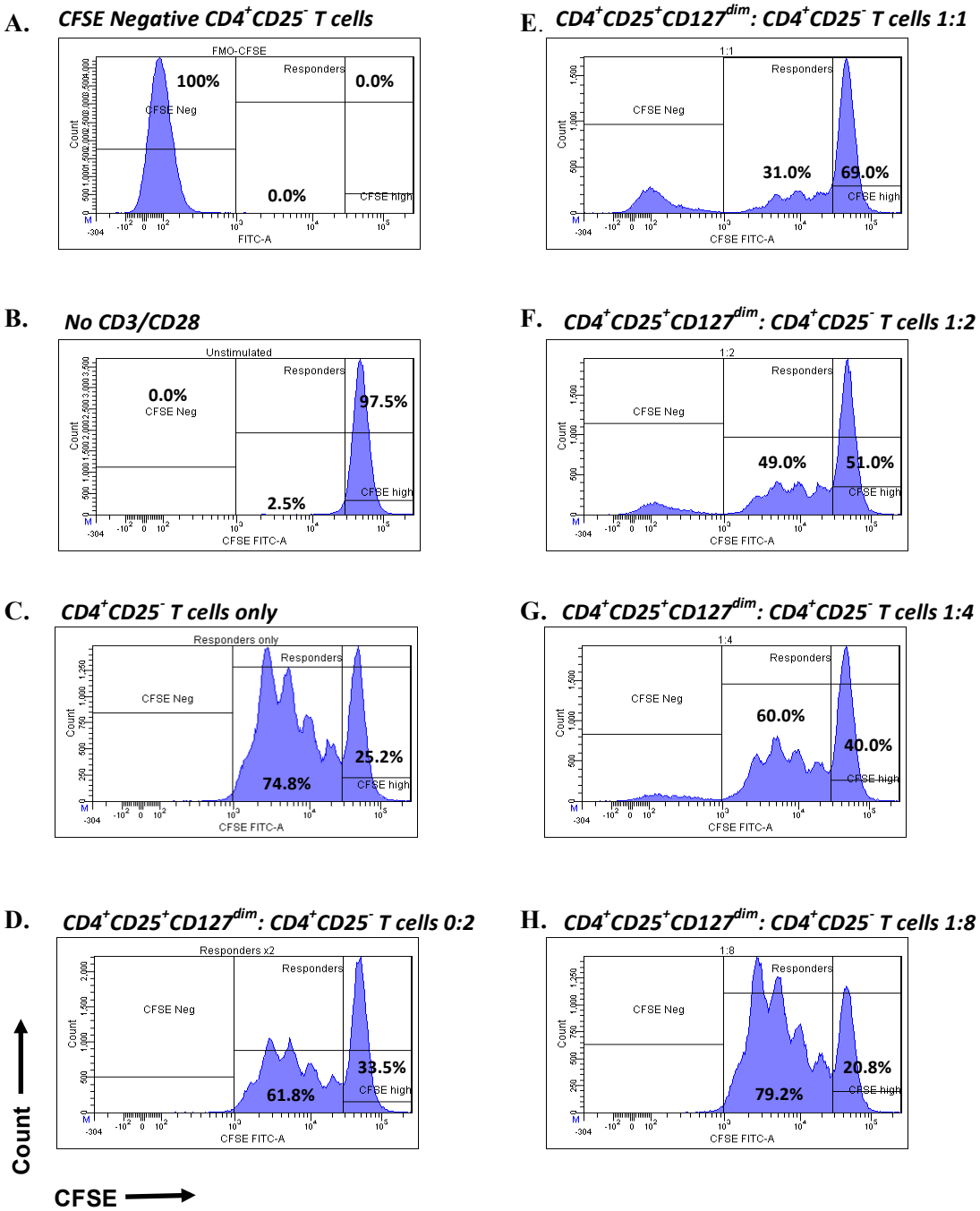


Figure 3.25: Anti-CD3/CD28 Driven Proliferation Assay

$5x10^4$ CFSE-labelled (0.5µM) $CD4^+CD25^-$ T cells (96.3% T cells were $CD4^+CD25^-$) were activated for five days in 96-well U-bottomed plates with plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1µg/ml) in the presence or absence of autologous $CD4^+CD25^+CD127^{-/dim}$ T cells (78.5% T cells were $CD4^+FOXP3^+CD127^{-/dim}$). Flow plots show CFSE staining for **(A)** Unlabelled $CD4^+CD25^-$ T cells (No CFSE); **(B)** No anti-CD3/CD28 stimulation; **(C)** $CD4^+CD25^+CD127^{-/dim}$ T cells only; **(D)** $CD4^+CD25^+CD127^{-/dim}$ T cells x 2; **(E-H)** $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:1 to 1:8 respectively. Data shown are from a single experiment that is representative of the five performed (each used cells isolated from different buffy coats).

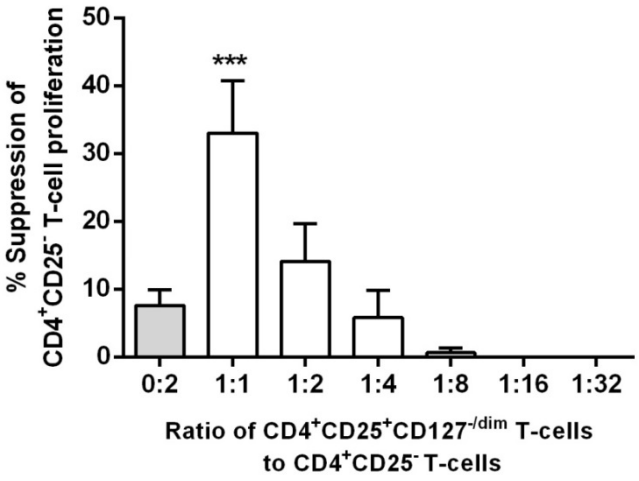


Figure 3.26: Anti-CD3/CD28 Driven Proliferation Assay

$5x10^4$ CFSE-labelled (0.5µM) $CD4^+CD25^-$ T cells were activated for five days in 96-well U-bottomed plates with plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1µg/ml) in the presence or absence of autologous $CD4^+CD25^+CD127^{-/dim}$ T cells ($CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:1 to 1:32). Figure shows the mean %suppression (i.e. suppression of anti-CD3/C28 driven proliferation of $CD4^+CD25^-$ T cells) from five separate experiments (each experiment was performed using cells isolated from different buffy coats. Data were analysed using repeated-measures ANOVA with post-hoc Bonferroni tests with all $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios compared to control group i.e. 0:2.

3.6 METHYLATION-SPECIFIC PCR

3.6.1 PRINCIPLE OF THE DNA METHYLATION-SPECIFIC REAL-TIME QPCR ASSAY

Methylation of DNA CpG dinucleotides is a fundamental epigenetic mechanism regulating gene expression. Approximately 20% of CpG dinucleotides in the human genome are unmethylated and are commonly located in CpG islands within the gene promoter or regulatory regions (Deaton and Bird, 2011). The addition of a methyl group to the 5 position of the cytosine pyrimidine ring generally reduces or silences gene expression. Identification of unmethylated CpG islands specific to particular cells allows detection of these cells (Baron *et al.,* 2006).

Unmethylated CpG islands specific to T cells and $CD4^+CD25^+FOXP3^+$ Tregs have been described in the *CD3* gene and *FOXP3* gene respectively (Baron *et al.,* 2007; Sehouli *et al.,* 2011). Similarly, an unmethylated CpG island within all cells has been described in the *GAPDH* gene (Sehouli *et al.,* 2011). To identify the methylation status at these loci, DNA can be bisulphite treated, converting unmethylated cytosines to uracil while leaving methylated cytosines unchanged. PCR amplification of the bisulphite-treated DNA generates a TpG in the position of the original unmethylated CpG and a CpG in the position of the methylated CpG. All other unmethylated cytosines in the gene will also be converted to uracil and replaced with thymidine during PCR amplification. PCR primers and probes can, therefore, be designed to differentiate between an unmethylated CpG and a methylated CpG. These oligonucleotides are bisulphite-specific (BS) (complementary to the bisulphite treated DNA but not genomic DNA) and strand-specific (forward and reverse DNA strands are no longer complementary). If they include one or more CpG dinucleotides, they will also be methylation-specific (MS) (complementary to either a TpG (unmethylated CpG) or a CpG (methylated CpG)) (Herman *et al.,* 1996). In this study, the TpG-specific oligonucleotides will be complementary to the bisulphite-treated DNA from $CD4^+CD25^+FOXP3^+$ Tregs and T cells while the CpG-specific oligonucleotides will be complementary to the bisulphite-treated DNA from non-

Tregs and non-T cells respectively. The TpG to TpG+CpG ratio is a measure of nTregs or T cells as a proportion of the total cell number (Sehouli *et al.,* 2011).

3.6.2 PRODUCING METHYLATED AND UNMETHYLATED PLASMID STANDARDS

To develop a MSRT-qPCR assay to measure T cells and $CD4^+CD25^+FOXP3^+$ nTregs, methylated and unmethylated plasmid standards for each gene loci being tested (*TSDR*, *CD3* and *GAPDH*) were produced and tested.

A) AMPLIFICATION OF THE TARGET REGIONS USING BISULPHITE SPECIFIC PRIMERS

Using bisulphite-treated genomic DNA, the PCR cycling conditions published by Sehouli and colleagues (2011) were tested. The BS primers used were as specified in 2.4.3. For each pair of primers, a PCR temperature gradient was performed (annealing temperatures (AT) range 48-64^{o}C) followed by a magnesium gradient (1.5-4.0mM) (Figure 3.27). The optimal PCR conditions selected were annealing temperatures of 57^{o}C, 59^{o}C and 53^{o}C for *FOXP3*, *CD3* and *GAPDH* respectively with a magnesium concentration of 2mM. To confirm the specificity of the BS primers, PCR amplification was performed on both genomic and bisulphite-treated DNA (PBMC DNA, unmethylated and methylated DNA) (Figure 3.28). The BS primers only amplified products of correct size (i.e. corresponding to the target region) when using bisulphite-treated DNA. When using genomic DNA, the target regions were not significantly amplified. Taken together, these findings confirm that the BS primers used were specific for bisulphite-treated DNA at the loci of interest but were not methylation specific.

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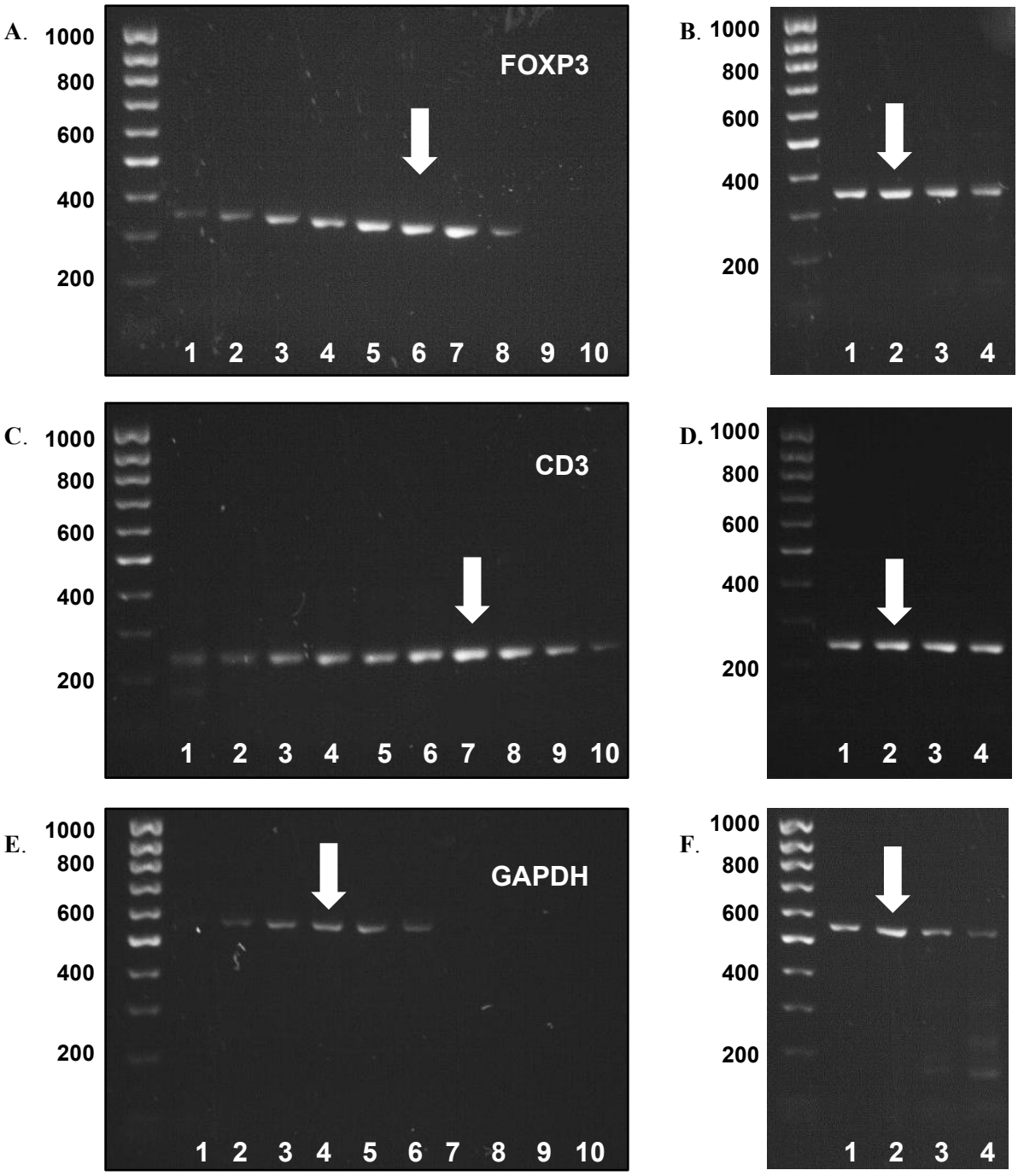


Figure 3.27: PCR Temperature and Magnesium Gradients

(A&B) *FOXP3* (336bp) **(C&D)** *CD3* (233bp) **(E&F)** *GAPDH* (538bp) targets were amplified by PCR using 1U HotStarTaq DNA polymerase (Qiagen) (cycling conditions: 95°C for 15min; 40 cycles (95°C for 1min, 40-66°C for 45s, 72°C for 1min); 72°C for 10min) from bisulphite-treated genomic DNA (healthy volunteer PBMC) using 0.5µM bisulphite-specific primers**. (A/C/E)** Annealing temperature gradient (1=48.0, 2=49.2, 3=51.0, 4=53.0, 5=55.0, 6=57.1, 7=59.1, 8=61.1, 9=62.9, 10=64.0°C). **(B/D/F)** Magnesium gradient (1=1.5, 2=2.0, 3=3.0, 4=4.0mM). DNA ladder shows base pairs. Selected PCR conditions indicate by the **(↓)**.

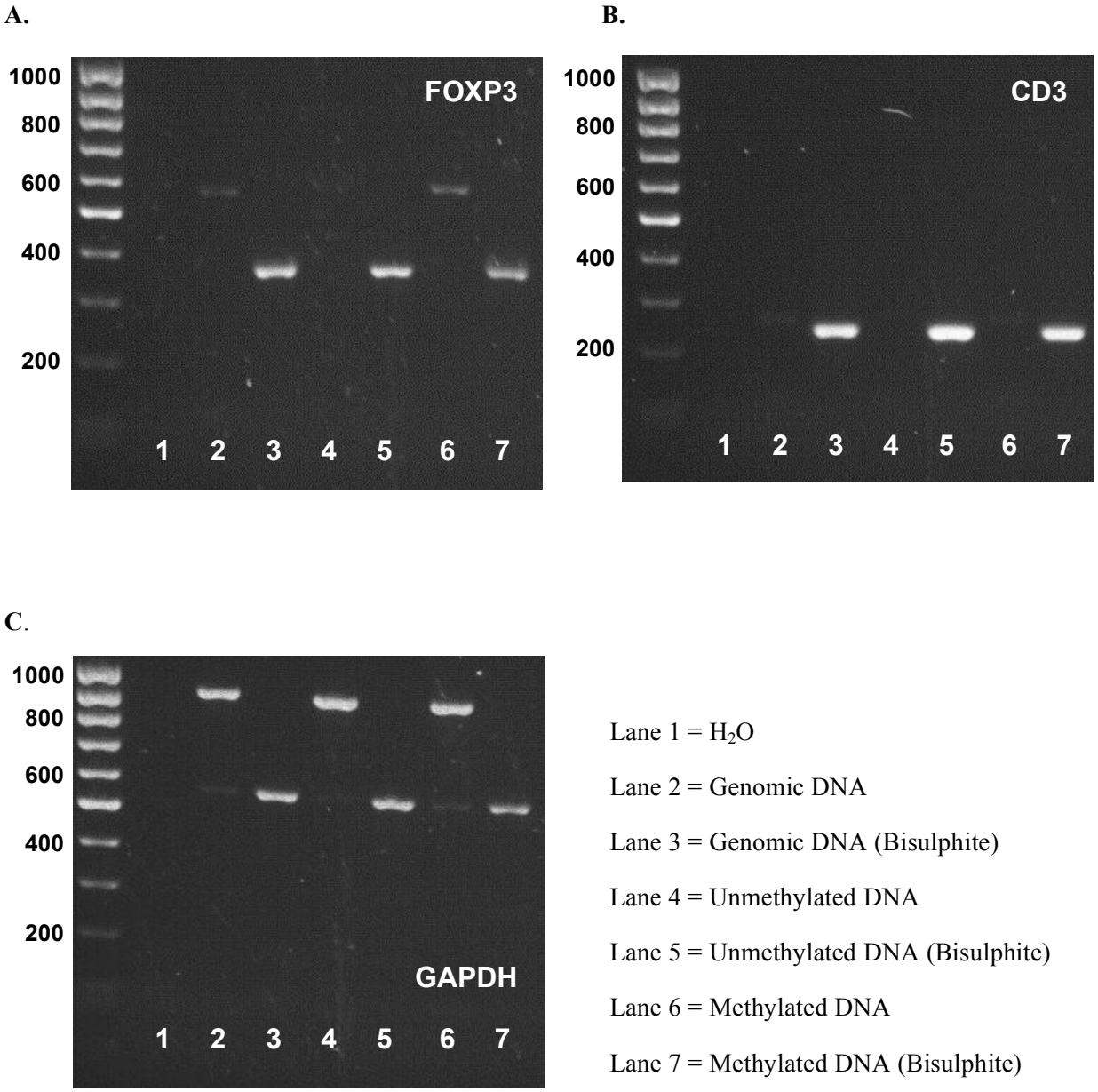


Figure 3.28: Checking Specificity of Bisulphite-Specific Primers

(A) *FOXP3* (336bp), **(B)** *CD3* (233bp), **(C)** *GAPDH* (538bp) target regions were amplified by PCR using 1U HotStarTaq DNA polymerase (Qiagen) (cycling conditions: 95°C for 15min; 40 cycles (95°C for 1min, 57°C (FOXP3) / 59°C (CD3) / 53°C (GAPDH) for 45s, 72°C for 1min); 72°C for 10min) using 0.5µM bisulphite-specific primers. The template DNA used in this experiment was untreated (Lanes 2/4/6) or bisulphite-treated (Lanes 3/5/7) genomic DNA (healthy volunteer PBMC) (Lanes 2&3), unmethylated DNA (Qiagen) (Lanes 4&5) and fully methylated DNA (Millipore) (Lanes 6&7). Lane 1 contains no DNA (H_20 and PCR mix). DNA ladder shows base pairs.

B) CLONING AND SEQUENCING OF RT-QPCR STANDARDS

The PCR products from the bisulphite-treated methylated and unmethylated DNA were cleaned, inserted into plasmid vectors and used to transform E Coli cells as described in Methods 2.4.6. The transformed cells were plated, individual colonies selected and grown in culture. The plasmid DNA was then isolated and sequenced. For *FOXP3* and *CD3*, two colonies with identical DNA sequences to the reference region were selected and grown in large cultures i.e. one for the unmethylated sequence and one for the methylated sequence. For *GAPDH,* only a colony matching the unmethylated sequence was selected (Sehouli *et al.,* 2011). The DNA sequences matched at the binding sites for the BS MS primers and probes used in the MSRT-qPCR assay. At the end of culture, the plasmid DNA was isolated, quantified, the DNA sequence re-checked. To maintain consistency within the MSRT-qPCR assay, sufficient plasmid DNA was produced to use in all subsequent experiments.

3.6.3 BISULPHITE-SPECIFIC METHYLATION-SPECIFIC REAL-TIME QPCR

The BS MS primers and probes used in the MSRT-qPCR assay were as published by Sehouli and colleagues (2011) (Table 2.1). Using serial dilutions of the plasmid standards, the BS MS primers and probes were tested. These were used to generate standard curves to quantify unmethylated *FOXP3* (nTregs), methylated *FOXP3* (non-Tregs), unmethylated *CD3* (T cells), methylated *CD3* (non-T cells) and unmethylated *GAPDH* (all cells) as described in Methods 2.4.7 (Figure 3.29).

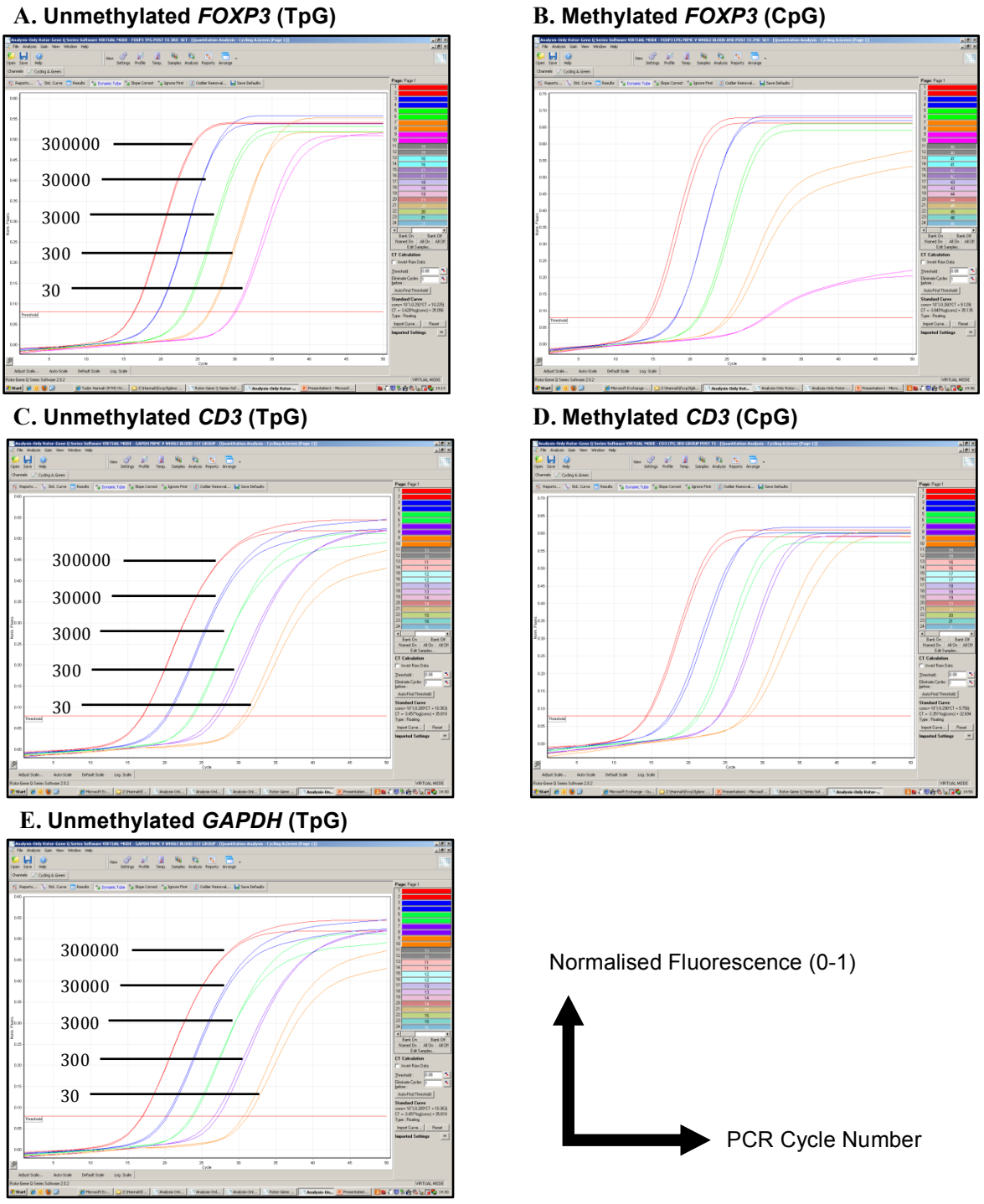


Figure 3.29: *FOXP3, CD3* and *GAPDH* MSRT-qPCR Standard Curves

(A) Unmethylated *FOXP3;* **(B)** Methylated *FOXP3;* **(C)** Unmethylated *CD3;* **(D)** Methylated *CD3;* **(E)** Unmethylated *GAPDH* plasmid standards were analysed using the MSRT-qPCR assay. Cycling conditions: 95°C for 10min; 50 cycles (95°C for 15s, 61°C (FOXP3/GAPDH) / 53°C (CD3) for 60s, 72°C for 1min). Template copy numbers per reaction were 300000, 30000, 3000, 300 and 30. Curves shown are representative from a single MSRT-qPCR run. *Plots supplied by Hannah Tudor (Oxford BRC Molecular Diagnostic Service, Oxford, UK).*

3.6.4 TESTING THE METHYLATION-SPECIFIC REAL-TIME QPCR ASSAY

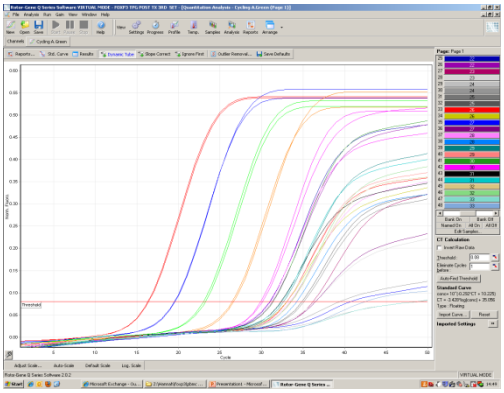
To test the hypothesis that the unmethylated *FOXP3* TSDR and *CD3* locus used in this assay are specific to putative Tregs and T cells respectively, DNA from sorted cells was tested using the MSRT-qPCR assay. In addition, to test the sensitivity and accuracy of the MSRT-qPCR assay, the results were compared to the proportion of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and T cells in each isolated cell population as identified by flow cytometry. T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+$) and $CD8^+$ T cells ($CD3^+CD8^+$), putative Tregs ($CD4^+CD25^{high}CD127^{-/dim}$ T cells), B cells ($CD20^+$), NK cells ($CD3^-CD56^+$), monocytes ($CD14^+$) and granulocytes ($CD15^+$) were isolated using antibody-coated magnetic beads (Methods 2.3.1.-2.3.2). Genomic DNA from each cell population was isolated and bisulphite treated (Methods 2.4.1-2.4.2). The efficiency of the bisulphite conversion was determined by PCR amplification, cloning and sequencing the *FOXP3* TSDR and calculating the number of non-converted single cytosine nucleotides. The conversion efficiency in this study was always greater than 99.5%. The MSRT-qPCR assay was run as per Methods 2.4.7. Representative examples are shown in Figure 3.30.

As shown in Table 3.4 only the isolated T-cell populations contained a high proportion of cells (>90%) with an unmethylated (TpG) *CD3* locus (median 99.2% (82.0-99.8%)). Some methylated copies were detected in the isolated T-cell samples. Due to impurities following magnetic bead sorting, the number of methylated *CD3* copies approximated the number of contaminating non-T cells. Conversely, the non-T-cell samples contained low proportions of unmethylated *CD3* (median 5.9% (2.7-26.6%)). The samples that contained higher amounts of unmethylated *CD3* (>10%) also contained high proportions of contaminating T cells as measured by flow cytometry (5.7-30.6%). Overall, when measuring T cells, there was a strong correlation between the MSRT-qPCR assay and flow cytometry (ρ=0.85, P<0.0001; Figure 3.31A). With regards to FOXP3, only the isolated $CD4^+CD25^{high}CD127^{-/dim}$ T-cell sample with high purity (88% $FOXP3^+$) contained a high proportion of cells (>80%) with an unmethylated *FOXP3* TSDR. As with CD3, the fraction of *FOXP3* CpG variants detected in this sample was similar to the proportion of contaminating non-$CD4^+CD25^{high}CD127^{-/dim}$ T cells as determined by flow cytometry (12.1%). In keeping with the

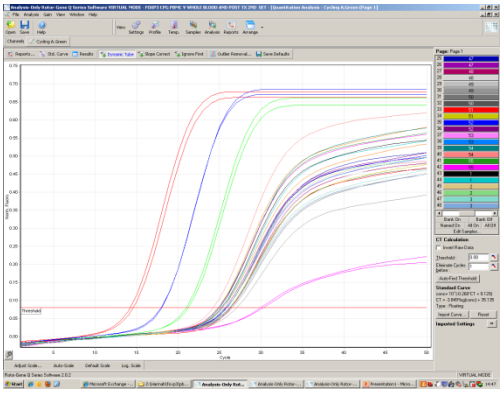
hypothesis, all the other cell populations contained very small proportions of unmethylated *FOXP3* TSDR (median 0.0% (0.0-0.6%)). The proportion of $FOXP3^+$ cells detected by the MSRT-qPCR assay and by flow cytometry also showed a strong correlation (ρ=0.86, P<0.0001; Figure 3.31B). Taken together, these results indicate that unmethylated *CD3* and *FOXP3* TSDR show good specificity for T cells and putative Tregs respectively and the MSRT-qPCR assay provides an alternative method to quantify these cell populations.

When calculating the *FOXP3/CD3* ratio, two corrections were applied with the MSRT-qPCR data. First, as *FOXP3* is on the X-chromosome but *CD3* and *GAPDH* are on chromosomes 11 and 12 respectively, the number of *FOXP3* TpG copies was corrected by a factor of two as these cells were isolated from male donors. Second, as the total number of copies (i.e. TpG+CpG) differed between the *FOXP3*, *CD3* and *GAPDH* assays, the number of *FOXP3* TpG and *CD3* TpG copies were both corrected to *GAPDH*. On comparison of the flow cytometry and MSRT-qPCR methods, there was no significant correlation obtained with the *FOXP3/CD3* ratios (ρ=0.20, P=0.35; Figure 3.31C). However, this should be viewed with caution considering the samples analysed. First, the majority of the tested samples were non-T-cell samples with very low *FOXP3/CD3* ratios (<0.2) and were therefore not representative of the entire range (0-1). Second, as many of the isolated samples contained very few T cells, small changes in FOXP3 events produced large effects on the *FOXP3/CD3* ratios using either method. The lack of a significant correlation for the *FOXP3/CD3* ratios is probably due the combined effect of the increased errors that occur within both assays when absolute numbers (FOXP3 and CD3) are expressed as ratios.

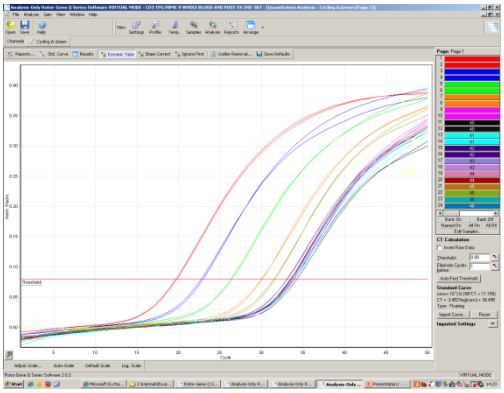
A. Unmethylated *FOXP3* (TpG)



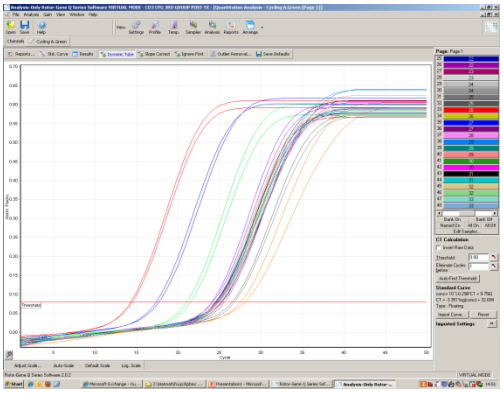
B. Methylated *FOXP3* (CpG)



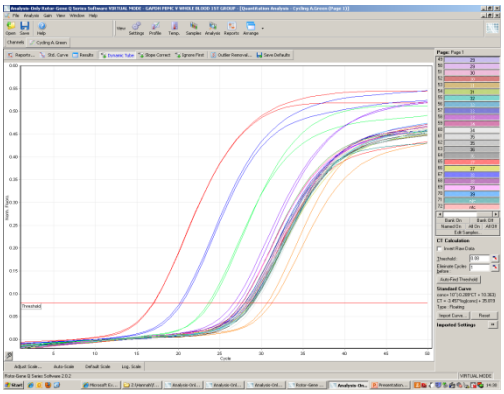
C. Unmethylated *CD3* (TpG): T cells



D. Methylated *CD3* (CpG) Non-T cells



E. Unmethylated *GAPDH* (TpG): All cells



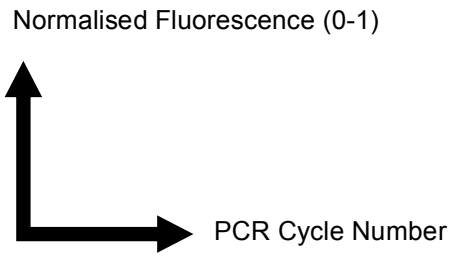


Figure 3.30: MSRT-qPCR Assay

Bisulphite-treated genomic DNA isolated from magnetic bead sorted cell populations was analysed by the MSRT-qPCR assay using bisulphite-specific methylation-specific primers (8pmol) and probes (4pmol) for *FOXP3*, *CD3* and *GAPDH* as per Table 2.1. Cycling conditions: 95°C for 10min; 50 cycles (95°C for 15s, 61°C (FOXP3/GAPDH) / 53°C (CD3) for 60s, 72°C for 1min). All samples were run in duplicate. Figures shows representative MSRT-qPCR traces from a single run for **(A)** Unmethylated *FOXP3;* **(B)** Methylated *FOXP3;* **(C)** Unmethylated *CD3;* **(D)** Methylated *CD3;* **(E)** Unmethylated *GAPDH. Plots supplied by Hannah Tudor (Oxford BRC Molecular Diagnostic Service, Oxford, UK).*

Cell Type	Purity	CD3					FOXP3					GAPD	Flow	PCR
		Flow	TpG	CpG	C & T	TpG	Flow	TpG	CpG	C+T	TpG	TpG	FOXP3 /CD3	FOXP3 /CD3
		%	Copies	Copies	Copies	%	%	Copies	Copies	Copies	%	Copies		
$CD3^+CD4^+CD25^+$ $CD127^{-/dim}$	89.4	99.3	2119	5	2124	99.8	87.9	1736	213	1949	89.1	1689	0.885	0.893
	21.8	81.8	644	142	785	82.0	21.8	304	1245	1549	19.6	1226	0.267	0.239
$CD3^+$	99.7	99.4	868	3	871	99.7	2.4	38	979	1017	3.7	713	0.024	0.037
	97.4	97.4	1764	32	1795	98.2	6.6	100	2497	2597	3.9	2245	0.068	0.039
	91.8	91.8	1988	16	2003	99.2	1.5	109	2605	2714	4.0	2615	0.016	0.040
	96.2	96.2	1976	33	2008	98.4	5.1	105	2761	2865	3.6	2144	0.053	0.037
$CD3^+CD4^+$	99.0	99.1	558	6	564	99.0	2.1	25	660	685	3.6	504	0.021	0.037
$CD3^+CD4^+CD25^-$	97.1	97.3	198	1	199	99.5	0.6	1	354	355	0.1	227	0.006	0.001
$CD3^+CD8^+$	99.5	99.5	2077	8	2084	99.6	0.8	13	3058	3071	0.4	2491	0.008	0.004
$CD3^-$	81.9	18.0	77	342	419	18.4	0.5	2	1142	1144	0.2	987	0.028	0.010
	94.3	5.7	55	482	536	10.2	0.5	4	2040	2044	0.2	1727	0.088	0.019
	86.8	13.2	141	515	656	21.4	0.6	4	2098	2101	0.2	1360	0.045	0.008
$CD20^+$	Not tested	9.6	78	626	704	11.0	0.6	15	2418	2432	0.6	1605	0.063	0.054
	Not tested	3.7	90	957	1047	8.6	0.6	1	3522	3523	0.0	3820	0.162	0.002
	91.2	3.5	14	346	360	3.8	0.7	6	1102	1108	0.5	756	0.200	0.144
$CD3^-CD56^+$	78.3	5.0	20	394	414	4.8	0.4	1	1506	1506	0.0	1152	0.080	0.007
	Not tested	3.9	83	351	434	19.1	0.4	0	3345	3345	0.0	2517	0.103	0.000
	73.6	7.5	10	345	354	2.7	0.4	1	1058	1058	0.0	780	0.053	0.018
$CD14^+$	Not tested	5.9	27	540	567	4.8	0.4	1	2072	2073	0.0	1233	0.068	0.005
	Not tested	6.8	57	988	1045	5.5	0.9	1	3114	3114	0.0	2189	0.132	0.003
	82.6	10.4	18	281	299	5.9	0.6	0	810	810	0.0	767	0.058	0.000
$CD15^+$	78.8	7.1	30	612	642	4.7	0.4	0	2939	2939	0.0	1144	0.056	0.000
	Not tested	8.9	45	737	782	5.8	0.2	1	2501	2501	0.0	2685	0.022	0.003
	83.8	30.6	149	412	561	26.6	0.7	3	1838	1841	0.2	1361	0.023	0.006
PBMC	N/A	65.7	597	265	862	69.3	1.2	21	2082	2103	1.0	979	0.018	0.014

(On previous page) **Table 3.4: Testing the Methylation-Specific RT-qPCR assay**

$CD3^+$, $CD3^+CD4^+$, $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{high}CD127^{-/dim}$, $CD3^+CD8^+$, $CD3^-$, $CD20^+$, $CD3^-CD56^+$, $CD14^+$ and $CD15^+$ cells were isolated from healthy donor PBMC (from leucocyte cones) using antibody-coated magnetic MicroBeads (Miltenyi Biotech). The sorted cell populations (*N*=25) were analysed for $CD3^+$ and $FOXP3^+$ cells using both the MSRT-qPCR assay and flow cytometry. Table shows the % positive cells by flow cytometry; the copy number for the unmethylated variant (TpG), methylated variant (CpG), combined total (TpG+CpG) and % unmethylated TpG (TpG/(TpG+CpG) x100). For GAPDH, the copy number of the unmethylated variant (TpG) is shown. The table also shows the corrected FOXP3/CD3 ratios.

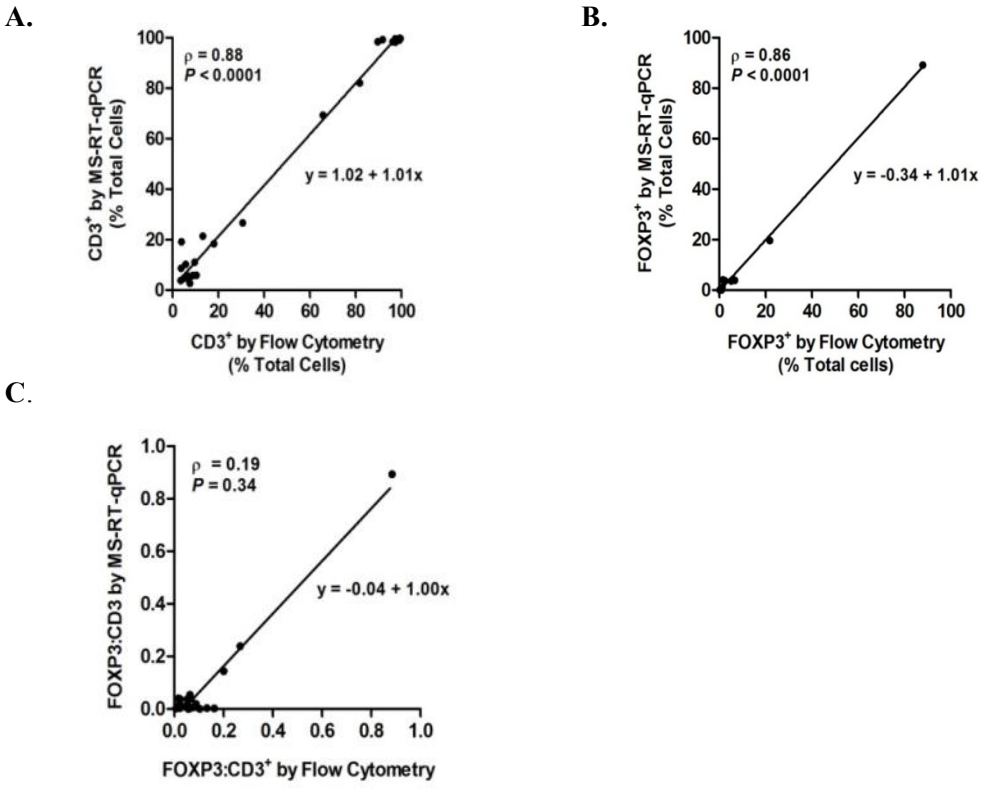


Figure 3.31: Comparison between the MSRT-qPCR and Flow Cytometry

$CD3^+$, $CD3^+CD4^+$, $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{high}CD127^{-/dim}$, $CD3^+CD8^+$, $CD3^-$, $CD20^+$, $CD3^-CD56^+$, $CD14^+$ and $CD15^+$ cells were isolated from healthy donor PBMC (from leucocyte cones) using antibody-coated magnetic MicroBeads (Miltenyi Biotech). The sorted cell populations (*N*=25) were analysed for $CD3^+$ and $FOXP3^+$ cells using both the MSRT-qPCR assay and flow cytometry. The figure shows **(A)** $CD3^+$ cells (% total cells); **(B)** $FOXP3^+$ cells (% total cells); **(C)** $FOXP3^+$ to $CD3^+$ ratio for each method. For each figure, the data from the MSRT-qPCR and flow cytometry assays were compared using the Spearman correlation (ρ).

3.7 CONCLUSIONS

Accurate and reliable methods to quantify Tregs and lymphocyte populations are essential when performing prospective clinical studies of Tregs post-alloHSCT. The work in this chapter described the design, development, optimisation and testing of two multi-colour flow cytometry assays to measure putative Tregs (absolute counts and %CD4 T cells) and lymphocyte populations (T, $CD4^+$ T, $CD8^+$ T, B and NK cells) from lymphopenic post-transplant blood samples and HSC grafts. By careful selection of the antigens, fluorochromes, titration of the antibodies and use of appropriate controls, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells can be detected with good specificity and sensitivity. In addition, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell assay was reproducible, reliable and had acceptable variability. $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell quantification was also dependent on the sample anti-coagulant and storage time, highlighting the need for a standardised method when performing the clinical study. Despite potential errors in the lymphocyte assay, modifying the data analysis produced a method that showed very strong correlation with a commercial assay.

This work also developed and tested a quantitative MSRT-qPCR assay to detect the unmethylated *FOXP3* enhancer (TSDR) specific to $CD4^+CD25^+FOXP3^+$ nTregs (Sehouli *et al.,* 2011). Use of BSMS primers and probes allowed differential detection of the methylated and unmethylated *FOXP3*, *CD3* and *GAPDH* target regions and quantification of putative Tregs and T cells. In keeping with the findings of Baron and colleagues (2007) and Sehouli and colleagues (2011), unmethylated *FOXP3* TSDR and *CD3* were only detected in high numbers from isolated cell populations containing high proportions of $CD4^+CD25^{high}CD127^{-/dim}$ T cells and T cells respectively. Conversely, unmethylated *FOXP3* TSDR was not detected in other haemopoietic cell lines ($CD8^+$ T cells, B cells, NK cells, monocytes and granulocytes). Quantification of putative Tregs and T cells by the MSRT-qPCR and by flow cytometry showed strong correlation, indicating that the MSRT-qPCR Treg assay may provide an alternative method to measure putative Tregs and T-cell numbers.

Finally, the functional experiments demonstrated that the putative Tregs being measured in this project do have *in vitro* suppressive function, being able to suppress the proliferation of anti-CD3/CD28 activated autologous $CD4^{+}CD25^{-}$ T cells. Although putative Tregs cannot be sorted on the basis of FOXP3, flow cytometry analysis confirmed that a high proportion of the isolated cells were $CD25^{+}FOXP3^{+}CD127^{dim}$.

Chapter 4

Regulatory T Cells in the Haematopoietic Stem Cell Graft

4. REGULATORY T CELLS IN THE HAEMATOPOIETIC STEM CELL GRAFT

4.1 INTRODUCTION

The cellular composition of the HSC graft is an important factor influencing the success of human alloHSCT. High $CD34^+$ cell doses in the graft are associated with faster neutrophil engraftment and lymphocyte recovery and lower rates of disease relapse (Nakamura *et al.,* 2008). However, high $CD34^+$ counts are also associated with increased cGvHD (Sohn *et al.,* 2003). Higher doses of $CD56^+$ NK cells and $CD8^+$ cytotoxic T cells in the grafts have been associated with faster neutrophil engraftment, improved lymphocyte reconstitution, reduced non-relapse mortality (NRM) and improved overall survival (OS) (Kim *et al.,* 2006). The concentration of $CD20^+$ cells in PBSCH may also predict the incidence of aGvHD and transplant related mortality (TRM) (Iori *et al.,* 2008).

The impact of Treg numbers in the HSC graft remains less clear. In mouse GvHD models, depletion of $CD25^+$ cells from the grafts significantly increased GvHD (Johnson *et al.,* 2002). Conversely, co-transfer of $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ conventional T cells was able to prevent GvHD but did not inhibit the GvT response (Edinger *et al.,* 2003). In human studies, low $CD4^+FOXP3^+$ cell counts in PBSCH have been associated with increased rates of aGvHD (Pabst *et al.,* 2007; Wolf *et al.,* 2007). However, only one study showed $CD4^+FOXP3^+$ cell numbers were significant in multivariate analysis (Wolf *et al.,* 2007). Similarly, the $CD3^+$ to $CD4^+CD25^{high}FOXP3^+$ ratio has also been associated with the incidence of aGvHD (Pastore *et al.,* 2012). However, these findings were only observed in the setting of myeloablative conditioning. The impact of graft Tregs on engraftment, cGvHD, CMV reactivation, relapse, NRM and OS remains uncertain.

To determine the influence of Tregs in the graft on these transplant outcomes, a prospective observational clinical study of alloHSCT was established in Oxford. The work presented in this

chapter describes this study and in particular tests the hypotheses that low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell doses in the graft are independently associated with increased rates of acute and chronic GvHD, higher NRM and inferior survival. Since Tregs may also be important for T-cell reconstitution and protecting the stem cell niche, this study will also test the hypotheses that low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell doses in the graft are associated with increased rates of CMV reactivation and delayed engraftment (Fujisaki *et al.,* 2011; Gaidot *et al.,* 2011). However, in keeping with the studies in mice performed by Edinger and colleagues (2003), it will examine the hypothesis that human $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the grafts do not affect disease relapse. Importantly, this study will explore the hypothesis that it is the balance between $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and conventional T cells that is more important than the absolute $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell dose. It will also ascertain whether any potential effect of $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the graft remains significant when assessed by multivariate analysis including other significant transplant co-variables.

4.2 SPECIFIC METHODS

4.2.1 CLINICAL STUDY

The clinical study and data collection were performed as per 2.1.

4.2.2 FLOW CYTOMETRY

Aliquots of the HSC graft (1ml in EDTA) were obtained from the Stem Cell Services Laboratory (NHSBT, Oxford) on the day of transplantation. HSC grafts were diluted with PBS (Lonza) to obtain a total nucleated cell count between 10-20x10^9/l. Due to the higher number of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the HSC graft compared to the post-transplant blood samples, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell and lymphocyte flow cytometry assays were performed by directly staining 50µl of diluted graft. Flow cytometry was run as per Chapter 3.

4.2.3 SUPPRESSIVE FUNCTION

Aliquots of the HSC graft (4ml in acid-citrate dextrose (ACD)) were obtained from the Stem Cell Services Laboratory (NHSBT, Oxford) on the day of transplantation. Sterile PBMC were isolated as per Methods 2.3.1. *In vitro* proliferation assays were set-up and analysed as per Methods 2.3.6.

4.2.4 STATISTICS AND GRAPHS

Statistics were analysed as per 2.5. Univariate analysis for the incidence of engraftment, GvHD, CMV activation, relapse and NRM were analysed using cumulative incidence with competing risks. Multivariate analysis was performed using the Fine and Gray method. Overall survival and relapse-free survival were analysed using Kaplan-Meier estimation. Multivariate analysis was tested by Cox Proportional Hazards. The variables used in multivariate analysis were the main variables being studied ($CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ T cells (%$CD4^+$ T cells) in the graft) and any co-variable with $P<0.1$ in univariate analysis.

4.3 RESULTS

In this chapter, the results will review the alloHSCT enrolled in this study and will describe the cellular contents of the HSC grafts, including $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells, $CD34^+$ cells, T cells ($CD3^+$), B cells ($CD20^+$) and NK cells ($CD3^-CD56^+$). It will then examine transplant related outcomes (GvHD, relapse, NRM, OS, engraftment, infection and CMV) and determine whether the incidence of these events is significantly associated with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) and/or the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ T cells (%$CD4^+$ T cells) in the HSC graft. By univariate analysis, it will also ascertain which other transplant related variables are significantly associated with these events and will test whether the effect of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells remains significant in multivariate analysis. All incidence values quoted are at one year post-transplant unless stated otherwise.

4.3.1 PATIENT AND TRANSPLANT CHARACTERISTICS

From 1st December 2008 to 31st August 2011, 107 patients received an alloHSCT at the Oxford University Hospitals NHS Trust. 106 patients were recruited into this study receiving a total of 110 transplants. One patient declined to take part. Four patients required a second transplant, three due to primary graft failure and one due to relapsed disease. HSC graft samples were unavailable from nine transplants (four frozen UCB; five missed). HSC grafts and clinical data were, therefore, available for analysis from 101 alloHSCT. Median follow up was 256 days (10-958 days).

The recipient, donor and transplant characteristics are summarised in Tables 4.1 & 4.2. The median age of the recipients was 48 years (18-74 years) with more transplants performed in male patients (62.4%). The diagnoses transplanted are AML (*N*=35), MDS (*N*=11), ALL (*N*=13), CML (*N*=3), NHL (*N*=15), HL (*N*=4), CLL (*N*=7), Myeloma (*N*=3), Myeloproliferative disease (*N*=9) and Aplastic anaemia (*N*=1). When diagnoses were grouped as per Methods 2.1.5, 58.4% transplants were for leukaemia, 28.7% for lymphoproliferative disorders and 12.9% for other haematological

disorders. The majority of alloHSCT used a RIC regimen (84%) and PBSCH were used as the source of HSC in most transplants (96%). More transplants used an unrelated donor (60%), while *in vivo* T-cell depletion with Alemtuzumab was used in 63 transplants (62%). The indications for using Alemtuzumab were unrelated donor (*N*=61), HLA-mismatch (*N*=1) and Hodgkin's lymphoma (*N*=1).

Recipient		***N***	**%**
Age	**Median** (years/range)	**48** (18-74)	**-**
Gender	**Male**	**63**	**62.4**
CMV Serology	**IgG Positive**	**48**	**47.5**
Diagnosis	**Leukaemia**	**59**	**58.4**
	AML	35	34.7
	MDS	11	10.9
	ALL	13	12.9
	Lymphoproliferative	**29**	**28.7**
	NHL	15	14.9
	CLL	7	6.9
	HL	4	4.0
	Myeloma	3	3.0
	Other	**13**	**12.9**
	MP	9	8.9
	CML	3	3.0
	AA	1	1.0
Status at alloHSCT	**CR**	**53**	**52.5**
	CR1	32	31.7
	CR2+	21	20.8
	PR	**19**	**18.8**
	Active	**27**	**26.7**

Table 4.1: Baseline Characteristics of the Transplant Recipients

Number (*N*) and percentage (%) of all 101 alloHSCTs.

Donor		**No.**	**%**
Age	**Median** (years/range)	**40** (19-72)	**-**
Gender	**Male**	**69**	**68.3**
CMV Serology	**IgG positive**	**32**	**31.7**
Transplant		**N**	**%**
Donor	**Sibling**	**40**	**39.6**
	Unrelated	**61**	**60.4**
Source	**PBSCH**	**97**	**96.0**
	BM	**4**	**4.0**
Conditioning	**RIC**	**85**	**84.2**
	Flu/Mel	83	82.2
	Fly/Cy	2	2.0
	MA	**16**	**15.8**
	Cy/TBI	13	12.9
	Cy/Bu	2	2.0
	Cy/ALG	1	1.0
Alemtuzumab	**Yes**	**63**	**62.4**
HLA mismatch	**Yes**	**18**	**17.8**
Gender mismatch	**Female-to-Male**	**18**	**17.8**
	Other	**83**	**82.2**
ABO mismatch	**Matched/minor**	**61**	**60.4**
	Major/Bidirectional	**40**	**39.6**

Table 4.2: Baseline Characteristics of the Donors and Transplants

Number (*N*) and percentage (%) of all 101 alloHSCTs.

4.3.2 ANALYSIS OF THE HSC GRAFTS

For the 101 HSC grafts analysed in this study, 97 (96%) were derived from GCSF-stimulated PBSCH and four (4%) were obtained from BM harvests. This section describes the cellular content of the HSC grafts as analysed by the flow cytometry assays in Chapter 3.3-3.4.

A) $CD34^+$ CELLS

In this study, the median $CD34^+$ dose transplanted was $6.10x10^6$/kg (0.00-19.8) (Figure 4.1A-B) and is consistent with previous reported values in GCSF-stimulated PBSCH (Pabst *et al.,* 2007; Wolf *et al.,* 2007). Seventeen (16.8%) transplants received less than 4 $x10^6$/kg $CD34^+$ cells. This was the minimum target $CD34^+$ count used for HSC collection at this centre based upon the previous published studies (Ilhan *et al.,* 1999). However, four of these grafts were BM harvests which are known to contain lower $CD34^+$ and total nucleated cell counts (Hassan *et al.,* 1996; Singhal *et al.,* 2000). In this study, the median $CD34^+$ dose in the PBSC and BM harvests were $6.30x10^6$/kg ($1.10\text{-}19.8x10^6$/kg) and $1.10x10^6$/kg ($0.00\text{-}1.70x10^6$/kg) respectively (P=0.0009) (Figure 4.1C).

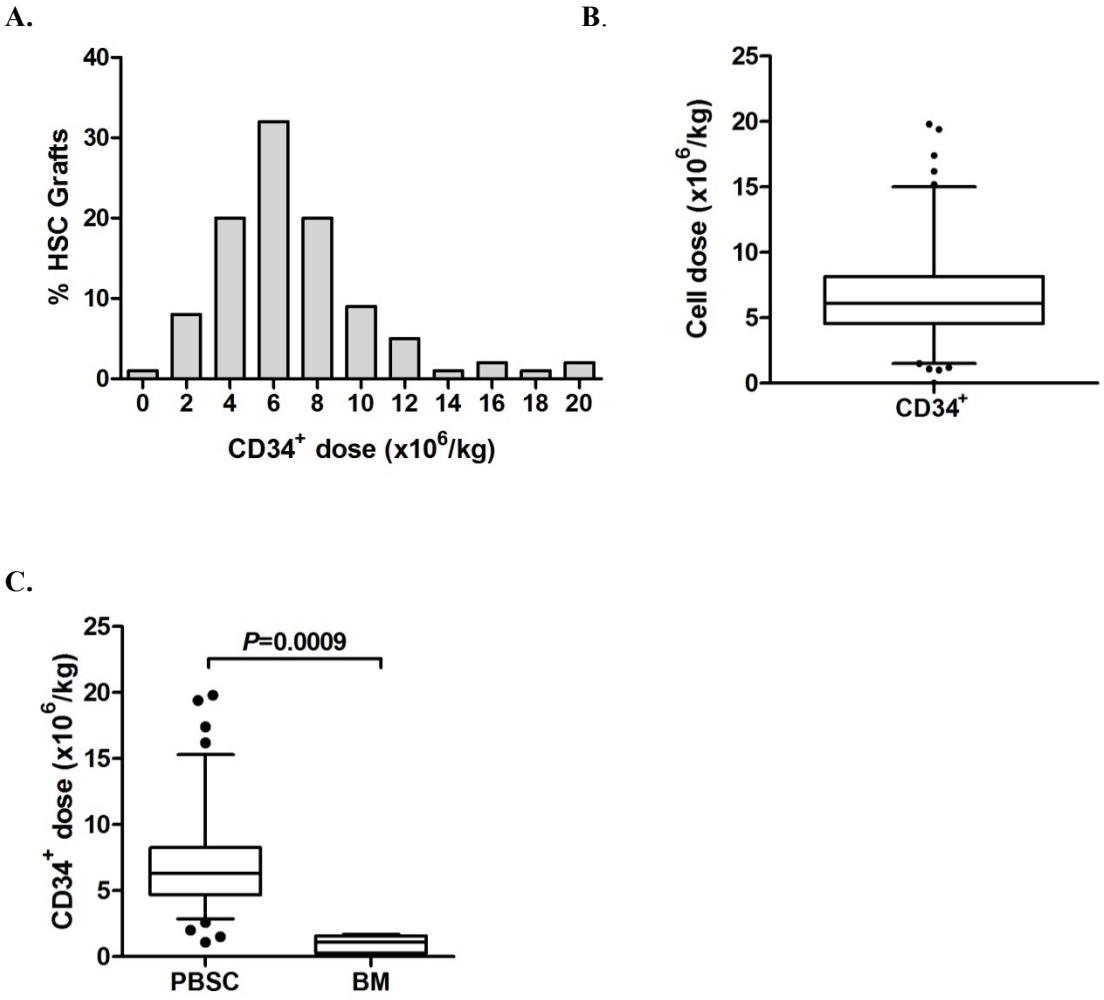


Figure 4.1: CD34+ Cell Dose within the HSC Grafts

The $CD34^+$ cell doses ($x10^6$/kg) in 101 allogeneic HSC grafts were measured using flow cytometry *(data supplied by Stem Cell Services Laboratory (National Health Service Blood and Transplant (NHSBT), Oxford, UK)*. Figure shows **(A)** Frequency distribution; **(B)** Box-and-whiskers plot of the $CD34^+$ cell dose in all 101 HSC grafts; **(C)** Comparison of the $CD34^+$ dose from PBSC (N=97) and BM harvests (N=4). Data were analysed using Mann-Whitney test.

B) CD3+ CELLS

The donor T-cell content in the HSC grafts is an important factor for sustained engraftment and the development of GvHD (Urbano-Ispizua *et al.,* 2001; Urbano-Ispizua *et al.,* 2002). In this study, the median T-cell ($CD3^+$), $CD4^+$ T-cell ($CD3^+CD4^+$) and $CD8^+$ T-cell ($CD3^+CD8^+$) dose transplanted was $274x10^6$/kg (21.0-$801x10^6$/kg), $170x10^6$/kg (12.0-$694x10^6$/kg), $81.0x10^6$/kg (6.00-$251x10^6$/kg) respectively (Figure 4.2A-B). The absolute T-cell dose was significantly higher in PBSCH compared to BM harvests ($282x10^6$/kg (87.0-$801x10^6$/kg) v $22.5x10^6$/kg (21.0-$43.0x10^6$/kg); *P*=0.0008) (Figure 4.2C). The median CD4:CD8 T-cell ratio was 2.1 (0.8-9.0).

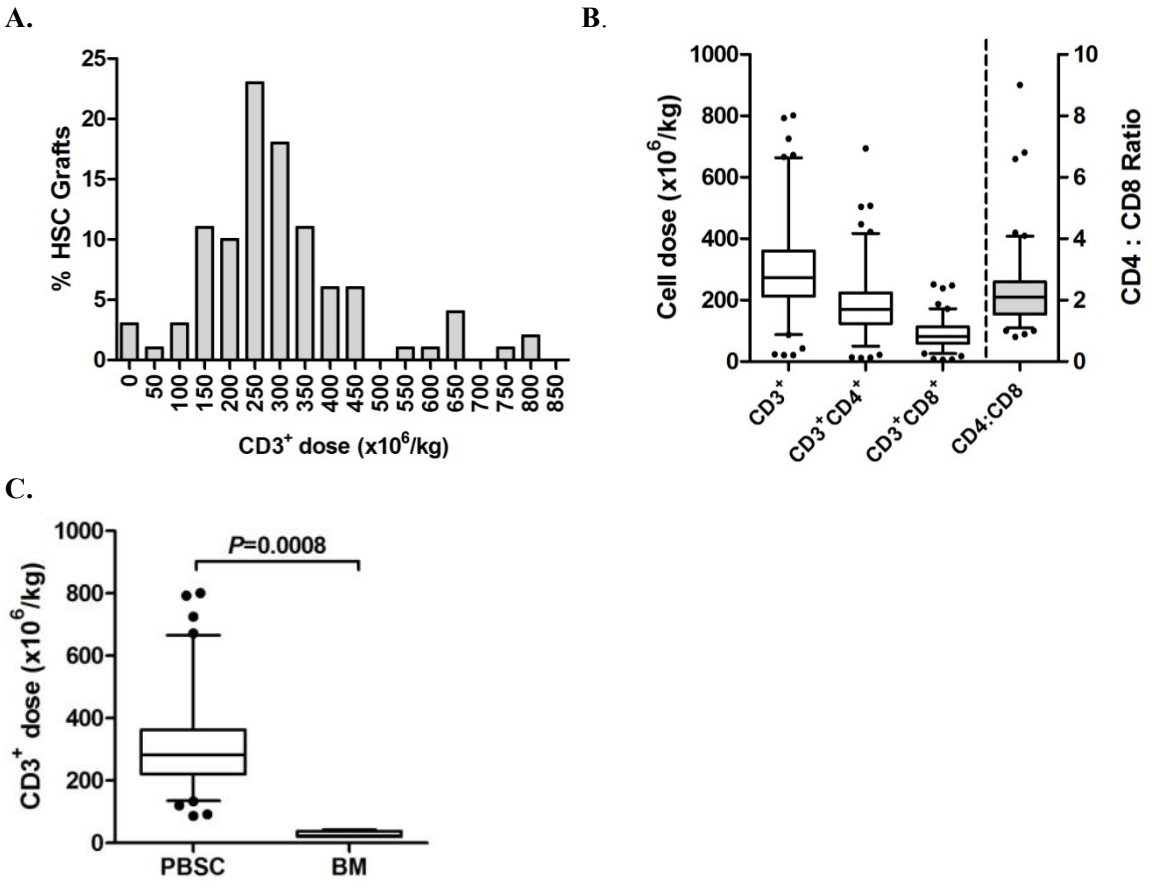


Figure 4.2: CD3+ Cell Dose within the HSC Grafts

The $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cell doses ($x10^6$/kg) in 101 allogeneic HSC grafts were analysed by flow cytometry (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD19 PE, CD16 PerCp-Cy5.5, CD56 APC). Figure shows **(A)** Frequency distribution; **(B)** Box-and-whiskers plot of the $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cell dose in all 101 HSC grafts; **(C)** Comparison of the $CD3^+$ dose from PBSC (N=97) and BM harvests (N=4). Data were analysed using Mann-Whitney test.

C) CD19+ (B CELLS) AND CD3-CD56+ (NK CELLS)

The HSC graft content of donor $CD19^+$ cells and $CD3^-CD56^+$ cells may also affect engraftment, GvHD and NRM (Kim *et al.,* 2006; Iori *et al.,* 2008). In this study the median $CD19^+$ and $CD3^-CD56^+$ dose transplanted was $60.0x10^6$/kg ($6.00\text{-}245x10^6$/kg) and $31.0x10^6$/kg ($2.00\text{-}101x10^6$/kg) respectively (Figure 4.3A-C). Absolute $CD19^+$ and $CD3^-CD56^+$ doses were significantly higher in PBSCH than BM harvests ($61.0x10^6$/kg ($13.0\text{-}245x10^6$/kg) v $7.00x10^6$/kg ($6.00\text{-}8.00x10^6$/kg), *P*=0.0008; $34.0x10^6$/kg ($8.00\text{-}101x10^6$/kg) v $2.00x10^6$/kg ($2.00\text{-}4.0010^6$/kg), *P*=0.0008) (Figure 4.3D).

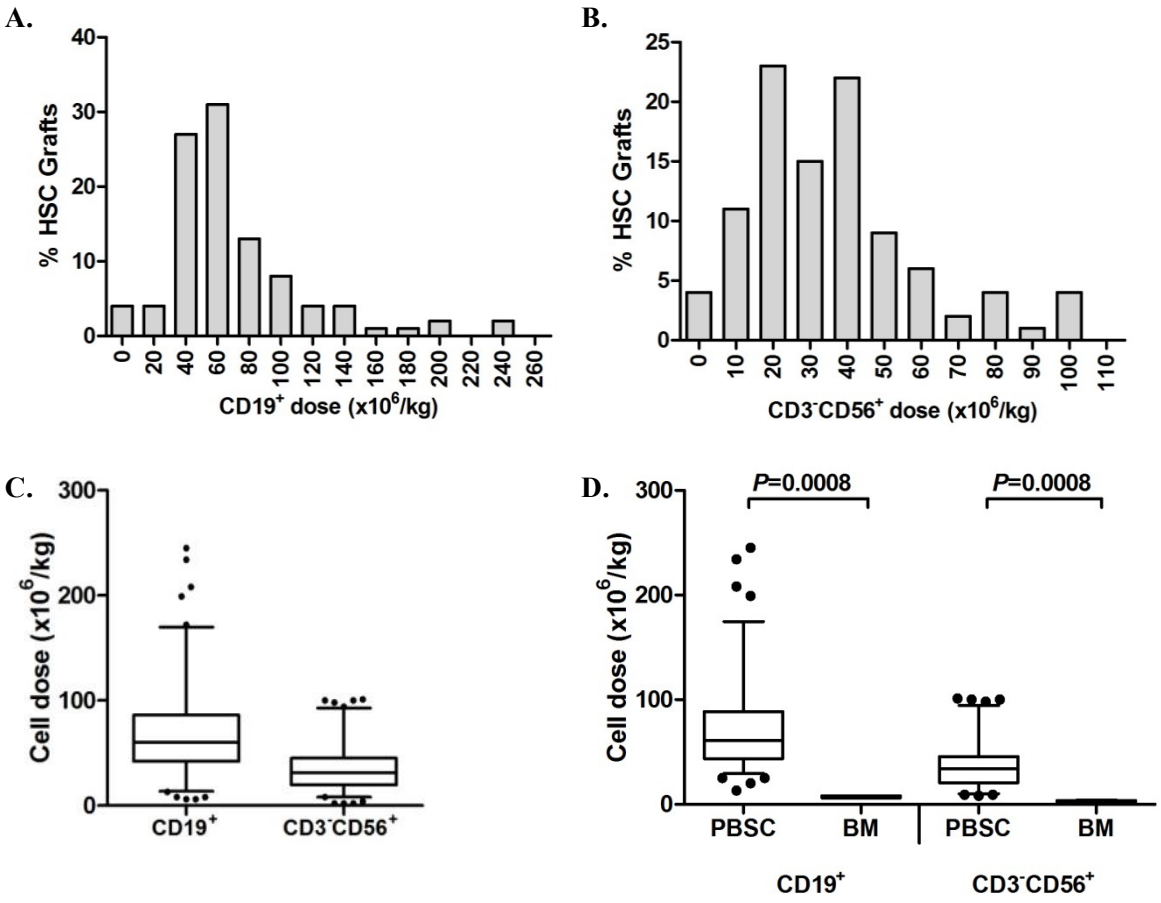


Figure 4.3: $CD19^+$ and $CD3^-CD56^+$ Cell Dose in the HSC Grafts

The $CD19^+$ and $CD3^-CD56^+$ cell doses ($x10^6$/kg) in 101 allogeneic HSC grafts were analysed by flow cytometry (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD19 PE, CD16 PerCp-Cy5.5 and CD56 APC). Frequency distributions of **(A)** $CD19^+$ dose; **(B)** $CD3^-CD56^+$ dose in the HSC grafts. **(C)** Box-and-whiskers plots of the $CD19^+$ and $CD3^-CD56^+$ cells in all 101 HSC grafts. **(D)** Comparison of the $CD19^+$ and $CD3^-CD56^+$ cells dose from PBSC (N=97) and BM harvests (N=4). Data were analysed using Mann-Whitney test.

D) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T CELLS

In contrast with the cellular populations described above, the Treg content in human HSC grafts has not been extensively studied. To address this issue, the absolute numbers and relative proportions of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the HSC grafts were determined by flow cytometry. It is acknowledged that whilst this may be an imperfect phenotypic definition of Tregs, it is considered a valid approach for this analysis. The median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose transplanted was $4.60x10^6$/kg ($0.20\text{-}20.6x10^6$/kg). This represented 2.94% (0.81-8.56%) of the $CD4^+$ T cells and 1.80% (0.47-5.24%) of the total T cells (Figure 4.4). As above, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose was higher in PBSCH than in BM harvests ($4.73x10^6$/kg ($0.83\text{-}20.6x10^6$/kg) v $0.34x10^6$/kg ($0.19\text{-}1.14x10^6$/kg), P=0.0008) (Figure 4.5A). However, there was no significant difference when $CD25^+FOXP3^+CD127^{-/dim}$ cells were expressed as $\%CD4^+$ T cells (2.94% (0.81-8.56%) v 2.71% (1.31-5.26%), P=0.75) (Figure 4.5B).

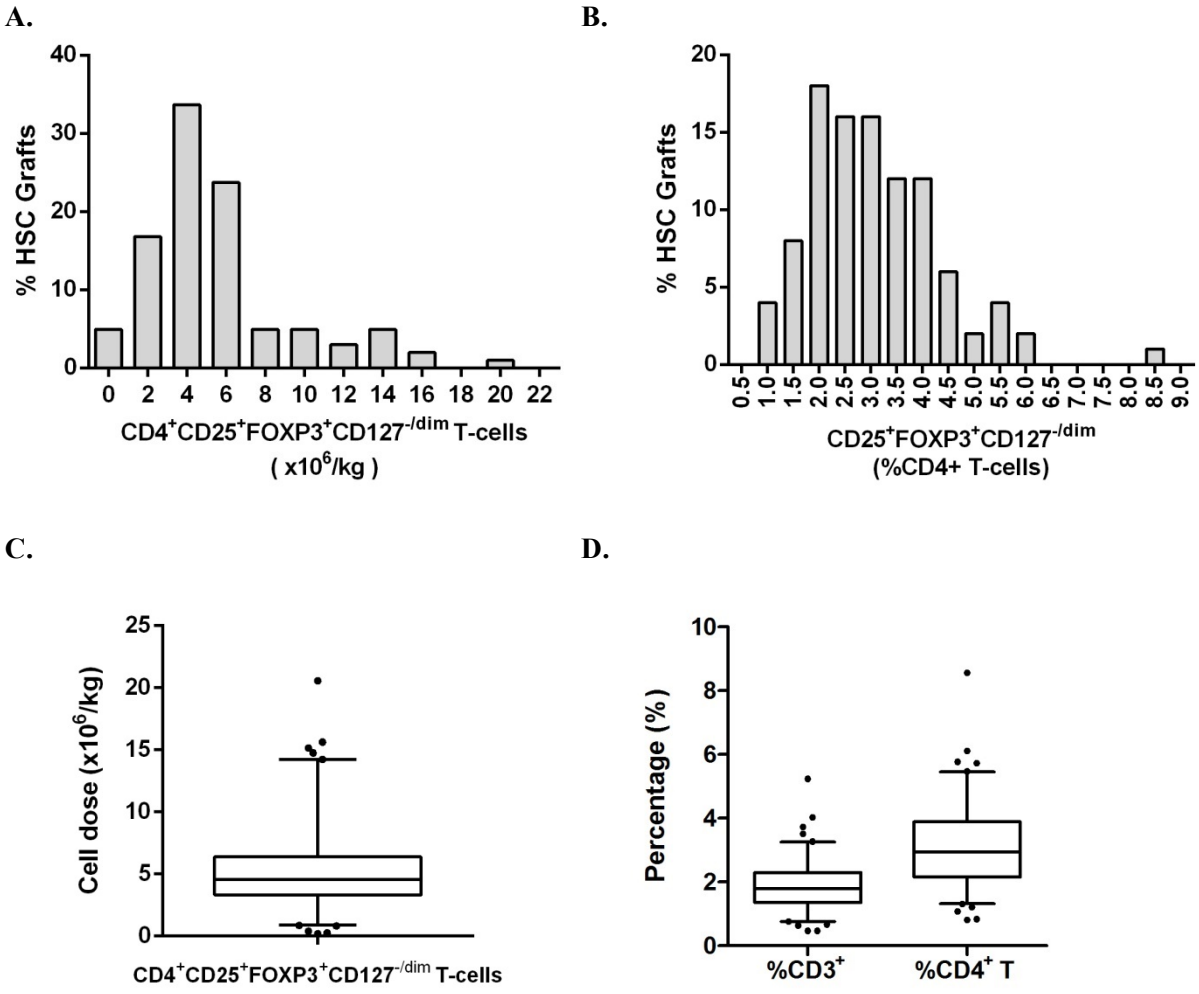


Figure 4.4: CD4⁺CD25⁺FOXP3⁺CD127⁻/ᵈⁱᵐ T Cells in the HSC Grafts

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in 101 allogeneic HSC grafts were analysed using flow cytometry (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD25PE, FOXP3 APC, CD127 PerCp-Cy5.5). Frequency distributions of **(A)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg); **(B)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). Box-and-whiskers plots of **(C)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg); **(D)** $CD25^+FOXP3^+CD127^{-/dim}$ cells expressed as %T cells and %$CD4^+$ T cells.

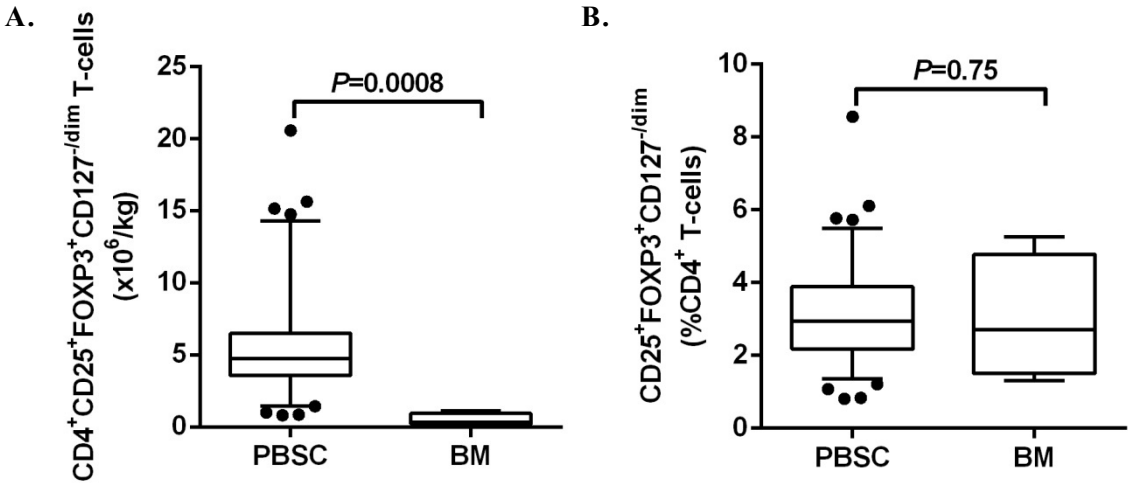


Figure 4.5 $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Dose in PBSC and BM Harvests

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in 101 allogeneic HSC grafts were analysed using flow cytometry (anti-CD3 PE-Cy7, CD4 FITC, CD8 APC-AF570, CD25PE, FOXP3 APC, CD127 PerCp-Cy5.5). Comparison of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in PBSC and BM harvests expressed as **(A)** Absolute counts ($x10^6$/kg); **(B)** %$CD4^+$ T cells. Data were analysed using Mann-Whitney test.

E) CORRELATION OF THE HSC COUNTS

Within the HSC grafts, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose showed a moderate correlation with $CD3^+$ cell dose (Spearman correlation coefficient ρ=0.69; *P*<0.0001), $CD19^+$ cell dose (ρ=0.59; *P*<0.0001) and $CD3^-CD56^+$ cell dose (ρ=0.56; *P*<0.0001). There was only a weak correlation with $CD34^+$ cell dose (ρ=0.23; *P*=0.02) (Figure 4.6A-B). There was no significant correlation, however, between the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), and the $CD34^+$ dose (ρ=-0.01; *P*=0.99), $CD3^+$ cell dose (ρ=-0.07; *P*=0.19), $CD19^+$ cell dose (ρ=0.13; *P*=0.19) or $CD3^-CD56^+$ cell dose (ρ=0.15; *P*=0.14) (Figure 4.6C-D).

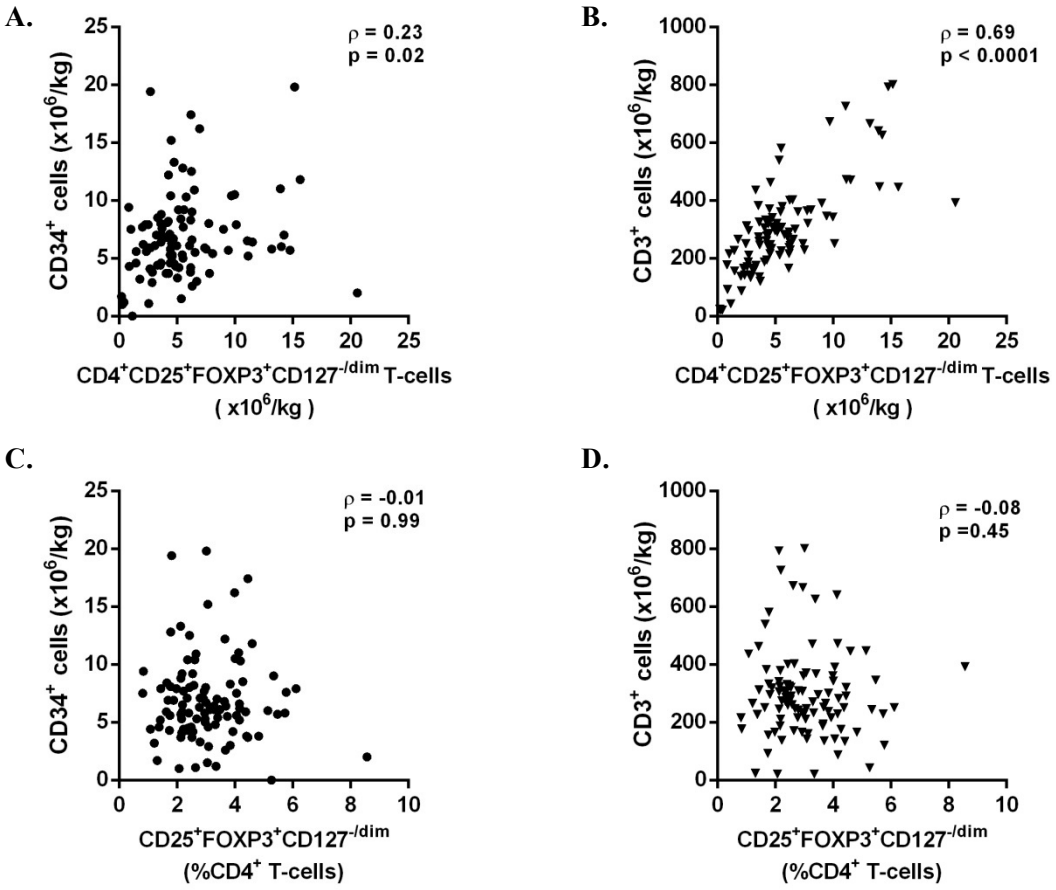


Figure 4.6: Correlation between $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells and Other Cells in the Graft

$CD34^+$ cells, $CD3^+$ cells and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute dose and $\%CD4^+$ T cells) in 101 allogeneic HSC grafts were analysed using flow cytometry. Correlation between the HSC graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ($x10^6$/kg) and **(A)** $CD34^+$ dose ($x10^6$/kg); **(B)** $CD3^+$ dose ($x10^6$/kg). Correlation between the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) in the HSC graft and **(C)** $CD34^+$ dose ($x10^6$/kg); **(D)** $CD3^+$ dose ($x10^6$/kg). Data were analysed using Spearman Correlation.

Taken together, these data demonstrate that there is wide variability of the cellular contents of HSC grafts used in allogeneic HSCT. As well as variation in the total cell numbers, there is also marked variation in the relative proportions of the different cell types, including $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. To improve the success of allogeneic HSCT, it will be necessary to understand what affect the different cells types and/or cell numbers (absolute and relative proportions) within the HSC grafts have on transplant-related outcomes.

4.3.3 FUNCTION OF HSC GRAFT $CD4^+CD25^+CD127^{-/DIM}$ T CELLS

As well as measuring $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell numbers within the HSC grafts, it was important to determine whether these putative Tregs actually had suppressive function. Unfortunately, sufficient cell numbers were not available from the majority of graft samples to set up *in vitro* suppression assays. Therefore, in order to test the general hypothesis that putative Tregs in HSC grafts do have suppressive function, *in vitro* suppression assays were set up for the largest graft samples available (*N*=3 separate grafts). Using the *in vitro* suppression assays described in Methods 2.3.6, isolated $CD4^+CD25^+CD127^{-/dim}$ T cells suppressed anti-CD3/CD28 driven proliferation of autologous $CD4^+CD25^-$ T cells by a mean of 22.9 ± 8.44%, 6.00 ± 3.12% and 0.60 ± 0.60% at $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:1, 1:2 and 1:4 respectively (*N*=3 separate experiments, each from a different graft). No suppression was observed at $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:8 to 1:32. Only the suppression observed with a $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratio of 1:1 reached statistical significance compared to the control well ($CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell 0:2) (22.9 ± 8.44% v 1.6 ± 0.96, $P<0.01$) (Figure 4.7).

In summary, these data show that graft $CD4^+CD25^+CD127^{-/dim}$ T cells (putative Tregs) are able to suppress anti-CD3/CD28 driven proliferation of autologous $CD4^+CD25^-$ T cells.

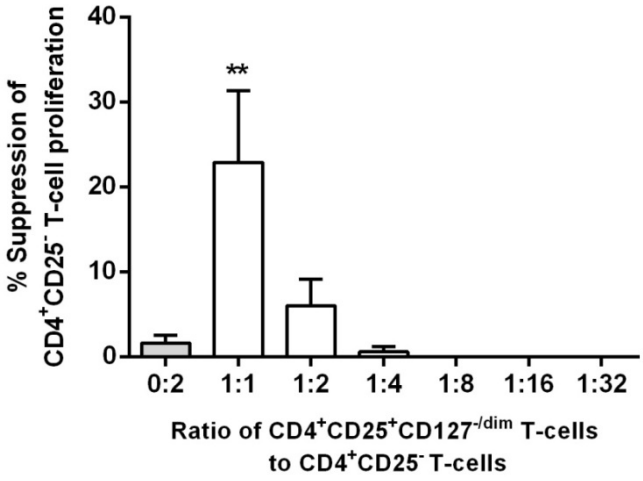


Figure 4.7: *In vitro* Suppressive Function of $CD4^+CD25^+CD127^{-/dim}$ T Cells in the HSC Grafts

$5x10^4$ CFSE-labelled (0.5µM) $CD4^+CD25^-$ T cells were activated for five days in 96-well U-bottomed plates with plate bound anti-CD3 (5µg/ml) and soluble anti-CD28 (1µg/ml) in the presence or absence of autologous $CD4^+CD25^+CD127^{-/dim}$ T cells ($CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios of 1:1 to 1:32). Figure shows the mean %suppression (i.e. suppression of anti-CD3/C28 driven proliferation of $CD4^+CD25^-$ T cells) from three separate experiments (each experiment was performed using cells isolated from different HSC grafts). Data were analysed using repeated-measures ANOVA with post-hoc Bonferroni tests with all $CD4^+CD25^+CD127^{-/dim}$ T-cell to $CD4^+CD25^-$ T-cell ratios compared to control group i.e. 0:2.

4.3.4 SEPARATING THE HSCT BY HSC GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

To test the hypotheses that low putative Tregs in the HSC graft are associated with increased risk of GvHD, delayed engraftment, CMV reactivation, NRM and inferior survival, the alloHSCT were divided into two groups, using the graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell 25th centile (absolute and relative counts (%$CD4^+$ T cells)) as a cut-off. This cut off was chosen as it was hypothesised that only those transplants with the lowest $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the grafts would have inferior outcomes. This hypothesis is consistent with Oxford's historical data relating to $CD34^+$ cell dose in the grafts. As part of this project, the Oxford BMT database was established, containing records of all alloHSCT previously performed in Oxford (*N*=243). On review of these data, patients transplanted with grafts containing $CD34^+$ cell doses in the bottom quartile (<3.8x10^6/kg) had significantly worse five year overall survival (47.6% (31.5-57.7% v 61.0% (52.0-71.6%), *P*=0.006). In this same cohort, however, no difference was observed when the transplants were separated according to the median HSC $CD34^+$ cell dose (5.6x10^6/kg) (55.3% (46.0-66.6%) v 55.4 (44.3-69.2%), *P*=0.46) (Figure 4.8).

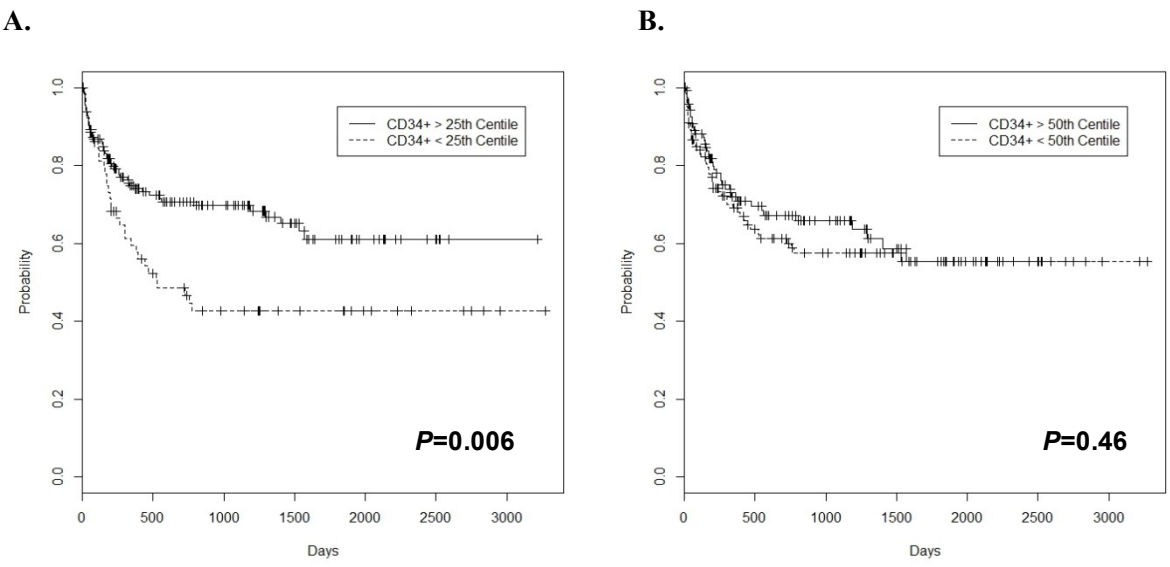


Figure 4.8: Overall Survival According to the $CD34^+$ Dose in the HSC Graft

The effect of the HSC $CD34^+$ cell dose on overall survival in 243 historical alloHSCT performed in Oxford. The transplants were divided by **(A)** HSC $CD34^+$ dose 25th centile (3.8x10^6/kg); **(B)** Median HSC $CD34^+$ dose (5.6x10^6/kg).

The 101 HSCT in this study were therefore divided into two groups according to the 25th centile for the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose in the HSC graft (</> $3.32x10^6$/kg). The baseline characteristics of these two groups are shown in Table 4.3. The low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell grafts were collected from younger donors (34 years (21-39) v 41 years (19-72), *P*=0.02) and contained more BM harvests (4/25 v 0/76, *P*=0.003).

Recipient		**Low Tregs** *N* = 25	**High Tregs** *N* = 76	***P***
Age	Median (years/range)	47 (18-66)	49 (18-74)	0.86
Gender	Male	19	44	0.24
CMV Serology	IgG Positive	13	35	0.65
Diagnosis	Leukaemia	16	43	
	Lymphoproliferative	6	23	0.80
	Other	3	10	
Status at alloHSCT	CR	14	40	
	PR	4	15	0.92
	Active disease	7	21	
Donor				
Age	Median (years/range)	34 (21-39)	41 (19-72)	**0.02**
Gender	Male	17	52	1.00
CMV	IgG positive	11	21	0.14
Transplant				
Donor	Sibling	6	34	0.10
Stem Cells	PBSCH	21	76	**0.003**
Conditioning	RIC	20	65	0.54
Alemtuzumab	Yes	19	44	0.15
HLA mismatch	Yes	3	15	0.55
F to M mismatch	Yes	6	12	0.37
ABO mismatch	Major/Bidirectional	3	17	0.39

Table 4.3: Baseline Characteristics of the Low and High $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell Groups

The 101 alloHSCT were grouped using the 25th centile of the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose in the HSC grafts (</> $3.32x10^6$/kg). Continuous variables were analysed using Mann-Whitney Test. Categorical variables were analysed using the Fisher's Test or Chi-Square Test (more than two levels). 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells

The study transplants were also divided into two groups according to the 25th centile for the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the HSC graft (</> 2.16%). The baseline characteristics of these two groups are shown in Table 4.4. The high $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cell (%$CD4^{+}$ T cells) group had a greater proportion of recipients with a PR at transplantation (19/76 v 0/25) but lower proportions with CR or AD (15/25 v39/75 and 10/25 v 18/76 respectively, *P*=0.02). The high group also had a non-significant trend towards a higher proportion of grafts from male donors (55/76 v 13/25, *P*=0.08).

Recipient		**Low %Tregs** *N* = 25	**High %Tregs** *N* = 76	***P***
Age	Median (years/range)	48 (18-65)	48 (18-74)	0.56
Gender	Male	18	45	0.34
CMV Serology	IgG Positive	13	35	0.50
Diagnosis	Leukaemia	16	43	
	Lymphoproliferative	5	24	0.54
	Other	4	9	
Status at alloHSCT	CR	15	39	
	PR	0	19	**0.02**
	Active	10	18	
Donor				
Age	Median (years/range)	39 (21-39)	40 (19-72)	0.49
Gender	Male	13	55	0.08
CMV	IgG positive	9	25	0.65
Transplant				
Donor	Sibling	8	32	0.48
Stem Cells	PBSCH	23	74	0.26
Conditioning	RIC	20	65	0.53
Alemtuzumab	Yes	17	46	0.64
HLA mismatch	Yes	2	16	0.23
F to M mismatch	Yes	7	11	0.14
ABO mismatch	Major/Bidirectional	2	12	0.09

Table 4.4: Baseline Characteristics of the Low and High $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cell (%$CD4^{+}$ T cells) Groups

The 101 alloHSCTs were grouped using the 25th centile of the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the HSC grafts (</> 2.16%). Continuous variables were analysed using Mann-Whitney Test. Categorical variables were analysed using the Fisher's Test or Chi-Square Test (more than two levels). '%Tregs' = $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells).

Having stratified the HSC transplants into 'low' and 'high' groups based upon the absolute or relative proportions of $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells within the HSC grafts, the following work in this chapter will now describe the incidence of acute GvHD (4.3.5), chronic GvHD (4.3.6), disease relapse/non-relapse mortality (4.3.7), overall survival (4.3.8), neutrophil and platelet engraftment (4.3.9), mortality due to infection (4.3.10) and CMV reactivation (4.3.11) according to these groups. For each transplant outcome, the data will first be analysed for the entire study population (*N*=101 HSCT) and then separately for the 'low' and 'high' absolute and relative $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell groups described above. The study outcomes will also be analysed according to other important transplant related variables (defined within each section), e.g. intensity of the conditioning (RIC vs MAC). Finally, within each section, multivariate analysis will be used to determine whether absolute and/or relative $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell dose within the HSC graft are independently associated with each transplant outcome assessed.

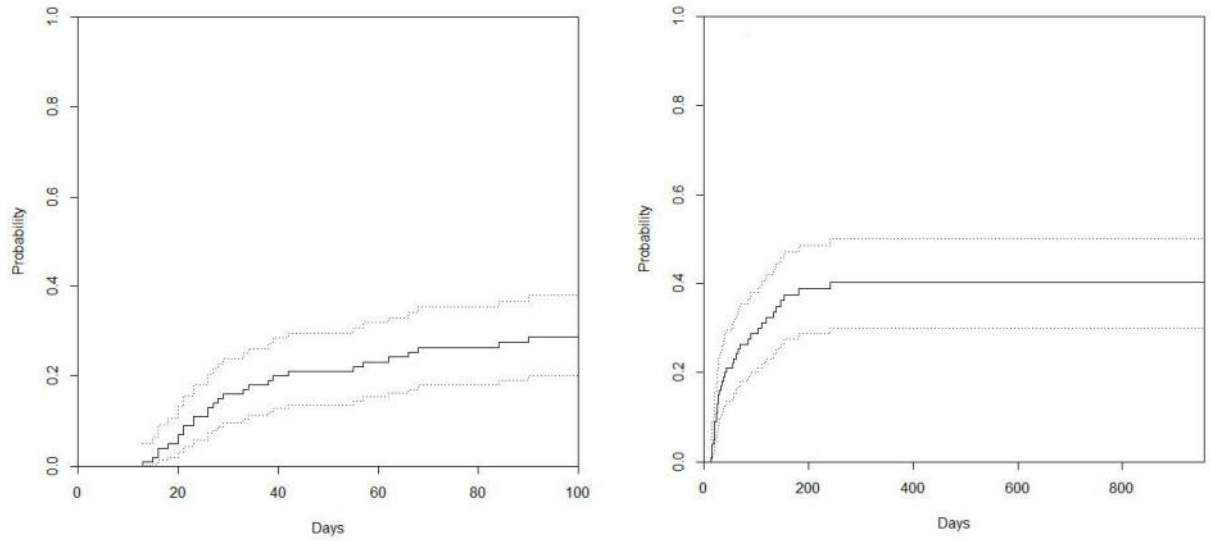
4.3.5 ACUTE GVHD

The incidence and severity of acute GvHD (aGvHD) in this study was assessed using the criteria established in the Consensus Conference Classification of aGvHD and the NIH Consensus Classification of GvHD (Przepiorka *et al.,* 1995; Filipovich *et al.,* 2005) (Table 1.1).

Using the Consensus Conference Classification of aGvHD, the incidence of aGvHD was 48.9% (38.6-58.5%) at day 100, with an incidence of grade II-IV aGvHD of 28.7% (20.0-37.9%) and grade III-IV aGvHD of 15.3% (5.1-17.0%) (Figure 4.9A). Grade I aGvHD was most frequent (41.7%) but severe aGvHD (grades III-IV) accounted for almost one third of cases (31.2%). Single organ aGvHD was most common (72.9%) but all three sites (skin, gut and liver) were affected in 8.3% of cases. Skin was the most frequently affected organ, involved in 77.1% of cases (Table 4.5).

In the NIH Consensus Criteria of GvHD, aGvHD is dependent upon the presence of diagnostic symptoms/signs and rather than time. Using these criteria, 62 transplants developed aGvHD, producing an incidence of 67.4% (56.1-76.3%) at one year, with an incidence of grade II-IV aGvHD of 40.2% (29.9-50.3%) (Figure 4.9B). 48 cases of aGvHD started before day 100 (77.4%) with an additional fourteen cases (22.6%) commencing after 100 days. 31 cases (50%) were defined as classical aGvHD and there were 10 cases (16.1%) of persistent aGvHD, seven cases (11.3%) of recurrent aGvHD and 14 cases (22.6%) of late-onset aGvHD. The majority of the late-onset aGvHD occurred before day 150 (71.4%) but the latest time of onset was day 241. Single organ aGvHD remained the most common pattern (71.0%) and skin was still the most frequently affected organ (79.0%). The incidence and patterns of aGvHD are summarised in Figure 4.9 and Table 4.5.

A. **B.**



Grade	Consensus Conference (Day 100)		NIH Consensus Criteria (Day 365)	
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI
I-IV	48.9	38.6 - 58.5	67.4	56.1 - 76.3
II-IV	28.7	20.0 - 37.9	40.2	29.9 - 50.3
III-IV	15.3	5.10 - 17.0	21.7	13.8 - 30.8

Figure 4.9: Acute GvHD

Cumulative incidence of acute GvHD in 101 alloHSCT assessed according to **(A)** Consensus Conference Classification (Przepiorka *et al.,* 1995); **(B)** NIH Consensus Criteria of aGvHD (Filipovich *et al.,* 2005). Incidence curves shown are for grade II-IV aGvHD. Dashed lines show the 95% confidence intervals (%).

	Consensus Conference		NIH Consensus Criteria	
	N	**%**	**N**	**%**
Grade				
All	48	100	62	100
I	20	41.7	25	40.3
II	13	27.1	17	27.4
III	10	20.8	14	22.6
IV	5	10.4	6	9.7
Site				
Skin	37	77.1	49	79.0
Gut	21	43.8	24	38.7
Liver	7	14.6	12	19.4
No. Sites				
1	35	72.9	44	71.0
2	9	18.8	13	21.0
3	4	8.3	5	8.1
NIH Type				
Classic	n/a	-	31	50.0
Persistent	n/a	-	10	16.1
Recurrent	n/a	-	7	11.3
Delayed	n/a	-	14	22.6

Table 4.5: Characteristics of Acute GvHD

Grade, site and type of acute GvHD in 101 alloHSCT assessed according to the Consensus Conference Classification (Przepiorka *et al.,* 1995) and the NIH Consensus Criteria of GvHD (Filipovich *et al.,* 2005). The number and percentage of all GvHD cases for the two criteria are shown.

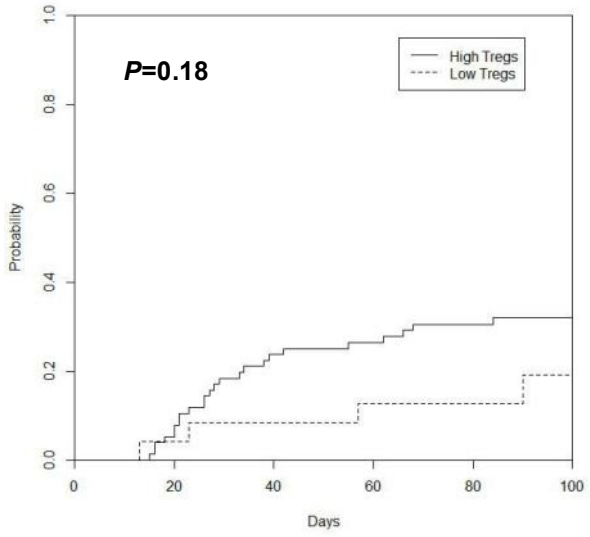
A) GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

Low doses of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) within the HSC grafts were not associated with any significant difference in the incidence of grades I-IV, II-IV or III-IV aGvHD at 100 days post-transplant as classified by the Consensus Conference Classification of aGvHD (Figures 4.10A & 4.11A). Neither was there any significant difference in the incidence of grades I-IV, II-IV or III-IV aGvHD at 365 days post-transplant as classified by the NIH Consensus Criteria of GvHD (Figures 4.10B & 4.11B).

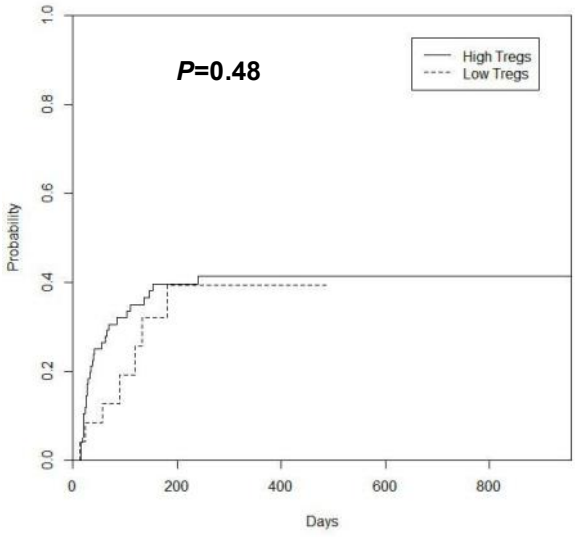
B) OTHER TRANSPLANT VARIABLES

The other transplant variables considered in univariate analysis of aGvHD are shown in Tables 4.6 & 4.7. In this study, only younger recipient age (below median age of 48 years) was significantly associated with an increased incidence of grade II-IV aGvHD. Using the Consensus Conference Classification of aGvHD, the cumulative incidence of grade II-IV aGvHD by day 100 was 39.6% (25.7-53.3%) and 18.5% (8.98-30.7%) in the younger and older recipient groups respectively (P=0.02) (Table 4.6). Similarly, using the NIH Consensus Criteria, the cumulative incidence of grade II-IV aGvHD by day 365 was 50.9% (34.7-65.13%) and 30.3% (17.6-44.0%) in the younger and older groups (P=0.03) (Table 4.7). None of the variables were significantly associated with the incidence of either grade I-IV aGvHD or grade III-IV aGvHD using either classification.

A.



B.



A. Consensus Conference Classification of aGvHD – Day 100

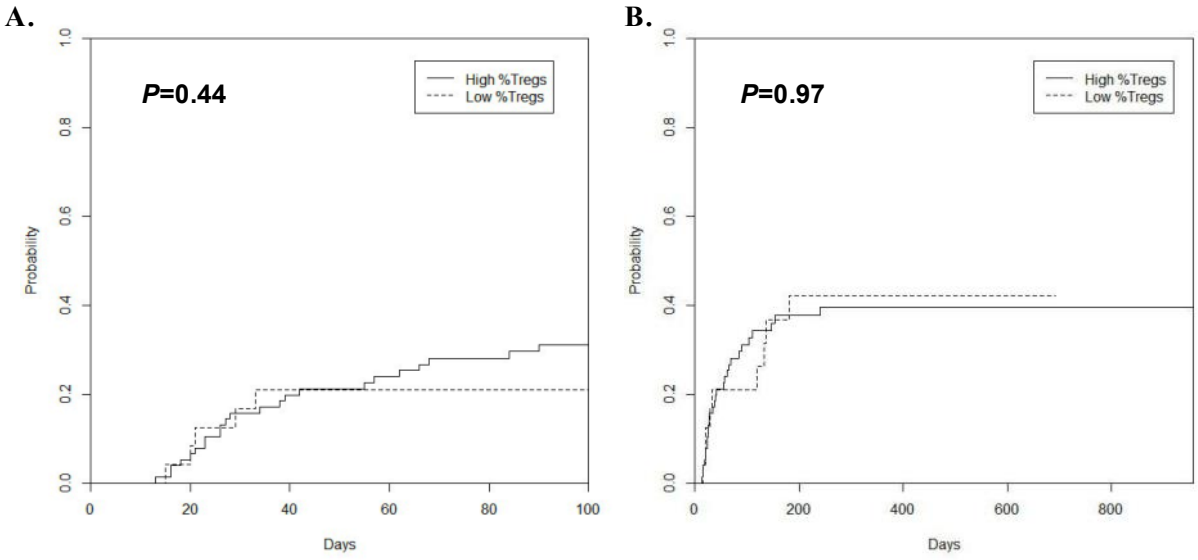
Grade	Low Tregs (N=25)		High Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
I-IV	*44.0*	*22.4 - 63.8*	*50.6*	*38.7 - 61.4*	*0.46*
II-IV	19.2	5.40 - 39.2	32.0	21.7 - 42.7	0.18
III-IV	15.0	3.30 - 37.7	15.9	8.70 - 25.1	0.71

B. NIH Consensus Criteria of aGvHD – Day 365

Grade	Low Tregs (N=25)		High Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
I-IV	75.0	42.8 - 90.7	65.7	52.8 - 75.9	0.98
II-IV	39.5	15.8 - 62.6	41.3	29.7 - 52.5	0.48
III-IV	21.4	5.94 - 43.0	22.3	13.3 - 32.6	0.78

Figure 4.10: Acute GvHD According to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell Dose ($x10^6$/kg) in the Graft

Cumulative incidence of aGvHD according to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose in the graft ($x10^6$/kg) using **(A)** Consensus Conference Classification (Przepiorka *et al.,* 1995); **(B)** NIH Consensus Criteria (Filipovich *et al.,* 2005). 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Low Tregs <$3.32x10^6$/kg, High Tregs >$3.32x10^6$/kg. Incidence curves show grade II-IV aGvHD.



A. Consensus Conference Classification of aGvHD – Day 100

Grade	Low %Tregs (N=25)		High %Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
I-IV	33.3	15.5 - 52.4	53.7	41.5 - 64.4	0.19
II-IV	20.9	7.36 - 39.0	31.2	20.9 - 42.0	0.44
III-IV	8.35	1.37 - 23.8	17.6	9.86 - 27.1	0.31

B. NIH Consensus Criteria of aGvHD – Day 365

Grade	Low %Tregs (N=25)		High %Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
I-IV	62.1	35.9 - 80.1	69.1	56.1 - 79.0	0.43
II-IV	42.1	20.3 - 62.6	39.6	27.9 - 51.0	0.97
III-IV	18.5	5.41 - 37.6	22.7	13.6 - 33.3	0.61

Figure 4.11: Acute GvHD According to $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the Graft

Cumulative incidence of aGvHD according to the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft using **(A)** Consensus Conference Classification of aGvHD (Przepiorka *et al.,* 1995); **(B)** NIH Consensus Criteria of GvHD (Filipovich *et al.,* 2005). '%Tregs' = $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells). Low %Tregs <2.16%; High %Tregs > 2.16%. Incidence curves show grade II-IV aGvHD.

Variable	Group	II-IV aGvHD (%)	95% CI (%)	*P*
Recipient Age	< Median	39.6	25.7 - 53.3	**0.02**
	> Median	18.5	8.98 - 30.7	
Donor Age	< Median	31.0	20.3 - 42.3	0.41
	> Median	23.5	10.1 - 40.2	
Conditioning	RIC	27.0	17.8 - 37.0	0.35
	MAC	37.5	14.5 - 60.7	
Donor	Sibling	25.3	12.9 - 39.6	0.57
	Unrelated	31.1	19.6 - 43.4	
HSC Source	PBSCH	27.7	19.0 - 37.1	0.14
	BM	50.0	12.7 - 89.6	
Alemtuzumab	Yes	30.1	18.9 - 42.0	0.76
	No	26.6	13.6 - 41.5	
D to R Gender	F to M	16.7	3.87 - 37.3	0.25
	Other	31.3	21.4 - 41.7	
HLA match	Matched	29.9	20.2 - 40.2	0.48
	Mismatch	23.5	6.82 - 45.8	
Graft $CD34^+$ dose	Low	24.3	9.55 - 42.5	0.76
	High	30.0	19.9 - 40.8	
Graft $CD3^+$ dose	Low	33.2	13.2 - 54.9	0.82
	High	27.9	18.3 - 38.4	
Graft $CD19^+$ dose	Low	25.0	9.89 - 43.6	0.95
	High	29.6	19.6 - 40.2	
Graft $CD3^-CD56^+$ dose	Low	25.0	9.86 - 43.6	0.92
	High	29.7	19.7 - 40.4	

Table 4.6: Univariate Analysis of Acute GvHD (A)

Cumulative incidence of grade II-IV aGvHD according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at 100 days. Acute GvHD was assessed using the Consensus Conference Classification of aGvHD (Przepiorka *et al.,* 1995).

Variable	Group	II-IV aGvHD (%)	95% CI (%)	*P*
Recipient Age	< Median	50.9	34.7 - 65.1	**0.03**
	> Median	30.3	17.6 - 44.0	
Donor Age	< Median	40.6	28.1 - 52.7	0.75
	> Median	39.2	21.0 - 57.1	
Conditioning	RIC	37.1	26.6 - 47.9	0.19
	MAC	64.8	18.2 - 89.6	
Donor	Sibling	46.7	29.4 - 62.3	0.56
	Unrelated	35.3	22.9 - 47.9	
HSC Source	PBSCH	39.6	29.1 - 49.8	0.14
	BM	50.0 (day 63)	1.27 - 89.6	
Alemtuzumab	Yes	34.0	22.1 - 46.4	0.35
	No	49.6	31.3 - 65.5	
D to R Gender	F to M	41.7	30.3 - 52.7	0.43
	Other	34.7	10.6 - 60.7	
HLA match	Matched	42.1	30.6 - 53.1	0.41
	Mismatch	31.0	10.3 - 54.6	
Graft $CD34^+$ dose	Low	36.6	16.0 - 57.3	0.76
	High	41.3	29.5 - 52.8	
Graft $CD3^+$ dose	Low	33.2	13.2 - 54.9	0.72
	High	41.6	29.9 - 52.9	
Graft $CD19^+$ dose	Low	31.2	13.0 - 51.5	0.66
	High	42.6	30.6 - 54.0	
Graft $CD3^-CD56^+$ dose	Low	30.4	12.9 - 50.0	0.58
	High	43.0	30.9 - 54.5	

Table 4.7: Univariate Analysis of Acute GvHD (B)

Cumulative incidence of grade II-IV acute GvHD according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at one year unless stated otherwise. Acute GvHD was assessed using the NIH Consensus Criteria of GvHD (Filipovich *et al.,* 2005).

C) MULTIVARIATE ANALYSIS

The variables considered in multivariate analysis of aGvHD were the HSC graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose, proportion of $CD25^+FOXP3^+CD127^{-/dim}$ (%$CD4^+$ T cells) and the recipient's age. The $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cell dose and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the HSC graft were not associated with aGvHD using ether classification of aGvHD. Only younger recipient age was significantly associated with increased risk of grade II-IV aGvHD (Consensus Conference Classification: HR 2.56 (1.16-2.56), *P*=0.02; NIH Consensus Criteria: HR 2.08 (1.08-3.98), *P*=0.03) (Table 4.8). There were no independent factors associated with grades I-IV or grades III-IV using ether classification of aGvHD.

Taken together, these data indicate that the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell content (absolute counts or relative proportions of total $CD4^+$ T cells) of the HSC graft do not predict the development of acute GvHD.

A. Consensus Conference Classification of aGvHD

Variable	Group	HR	95% CI	*P*
Recipient Age	< Median	2.56	1.16 - 2.56	**0.02**
	> Median	1	-	-

B. NIH Consensus Criteria of aGvHD

Variable	Group	HR	95% CI	*P*
Recipient Age	< Median	2.08	1.08 - 3.98	**0.03**
	> Median	1	-	-

Table 4.8: Multivariate Analysis of Acute GvHD

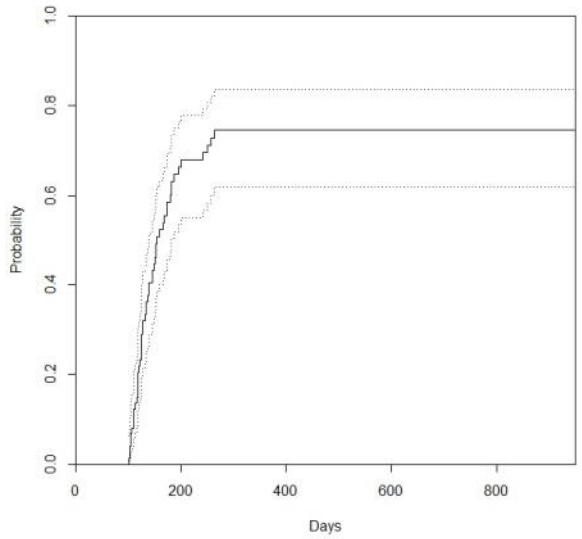
Multivariate analysis of grade II-IV aGvHD. aGvHD was assessed using **(A)** Consensus Conference Classification of aGvHD (Przepiorka *et al.,* 1995); **(B)** NIH Consensus Criteria of GvHD (Filipovich *et al.,* 2005). Hazard ratios (HR), 95% confidence interval (CI) and *P* values were calculated using the Fine and Gray regression model.

4.3.6 CHRONIC GVHD

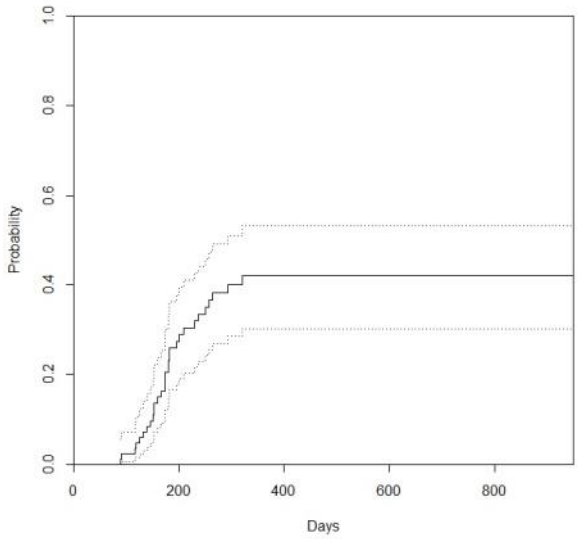
In this study, 75 transplants were evaluable for the development of cGvHD beyond day 100 using the Shulman classification of cGvHD (Shulman *et al.,* 1980). In all cases, cGvHD started within the first year post-transplant, with an incidence of 74.7% (61.9-83.8%) at one year. The incidence of extensive cGvHD was 55.6% (41.5-67.6%) at one year (Figure 4.12A). Extensive cGvHD (70.6% of cases) was more frequent than limited cGvHD, with 66.7% of cases involving two or more sites and 21.6% of cases involving four or more sites. The skin was the major organ involved (86.3% of cases) but other commonly affected sites were the eyes (37.3%), GI tract (mouth 25.5%, other 33.3%) and liver (25.5%). Muscles, joints, lung and hair were involved in a minority of cases only (<10%) (Table 4.9).

According to the NIH Consensus Criteria (2005), cGvHD can occur any time post-transplant and therefore all 101 transplants were evaluable for the onset of cGvHD. In this setting, the overall incidence was 42.0% (30.1-53.5%) at one year, with an incidence of moderate and severe cGvHD of 28.3% (18.1-39.3%) (Figure 4.12B). All cases of cGvHD started within the first year post-transplant. 50.0% of the cases were defined as "Classic cGvHD" and the other 50.0% were "Overlap cGvHD". The skin, eyes, mouth and GI tract remained the most commonly affected organs (93.3%, 63.3%, 46.7% and 26.7% of cases respectively). 86.7% of cases involved two or more organs with 40.0% of cases involving four or more sites (Table 4.9). There were only two cases of cGvHD commencing before day 100 (days 89 and 90) and both were overlap cGvHD. 32 cases of cGvHD using the Shulman criteria (62.7%) were not classified as cGvHD by the NIH Consensus Criteria. 31 of these episodes were re-defined as late aGvHD (fourteen delayed, ten persistent and seven recurrent) and one failed to meet the diagnostic or distinctive criteria used in the NIH Classification. Although eleven transplants with late aGvHD subsequently developed cGvHD (four cases of Classic cGvHD and seven cases of Overlap cGvHD), the remaining 20 cases showed no diagnostic/distinctive symptoms or signs of cGvHD.

A.



B.



Shulman Classification (N=75)			**NIH Consensus Criteria (N=101)**		
Grade	Incidence (%)	95% CI (%)	**Grade**	Incidence (%)	95% CI (%)
All	74.7	61.9 - 83.8	All	42.0	30.1 - 53.3
Ext	55.6	41.5 - 67.6	Mod&Sev	28.3	18.1 - 39.3

Figure 4.12: Chronic GvHD

(A) Cumulative incidence of cGvHD in 75 alloHSCT (survival beyond 100 days post-transplant) as assessed according to Shulman Classification of cGvHD (Shulman *et al.,* 1980); **(B)** Cumulative incidence of cGvHD in 101 alloHSCTs assessed according to the NIH Consensus Classification (Filipovich *et al.,* 2005). Incidence curves shown are all grades of cGvHD. Dashed lines show the 95% confidence intervals.

	Shulman Classification (N=75)		NIH Consensus Criteria (N=101)	
	N	%	N	%
Grade				
All	51	100	30	100
Limited	15	29.4	n/a	-
Extensive	36	70.6	n/a	-
Mild	n/a	-	10	33.3
Mod	n/a	-	14	46.7
Severe	n/a	-	6	20.0
Site				
All	51	100	30	100
Skin	44	86.3	28	93.3
Eyes	19	37.3	19	63.3
GI	17	33.3	8	26.7
Mouth	13	25.5	14	46.7
Liver	13	25.5	7	23.3
Muscles	4	7.8	4	13.3
Joints	4	7.8	4	13.0
Lung	3	5.9	3	10.0
Hair	2	3.9	2	6.7
GU	0	0.0	0	0.0
No. Sites				
1	17	33.3	4	13.3
2	16	31.4	10	33.3
3	7	13.7	4	13.3
4+	11	21.6	12	40.0
NIH Type				
Classic	n/a	-	15	50.0
Overlap	n/a	-	15	50.0

Table 4.9: Characteristics of Chronic GvHD

Grade, site and type of cGvHD, assessed according to the Shulman Classification (Shulman *et al.,* 1980) and the NIH Consensus Classification of GvHD (Filipovich *et al.,* 2005). Table shows the number and percentage of all GvHD.

A) GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

As with aGvHD, low doses of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%CD4 T cells) within the HSC grafts were not associated with any significant difference in the incidence of cGvHD (all grades) or extensive cGvHD as defined by the Shulman Classification (Figures 4.13A & 4.14A). Neither were they associated with any difference in the incidence of all cases of cGvHD nor moderate/severe cGvHD according to the NIH Consensus Classification of cGvHD (Figures 4.13B & 4.14B).

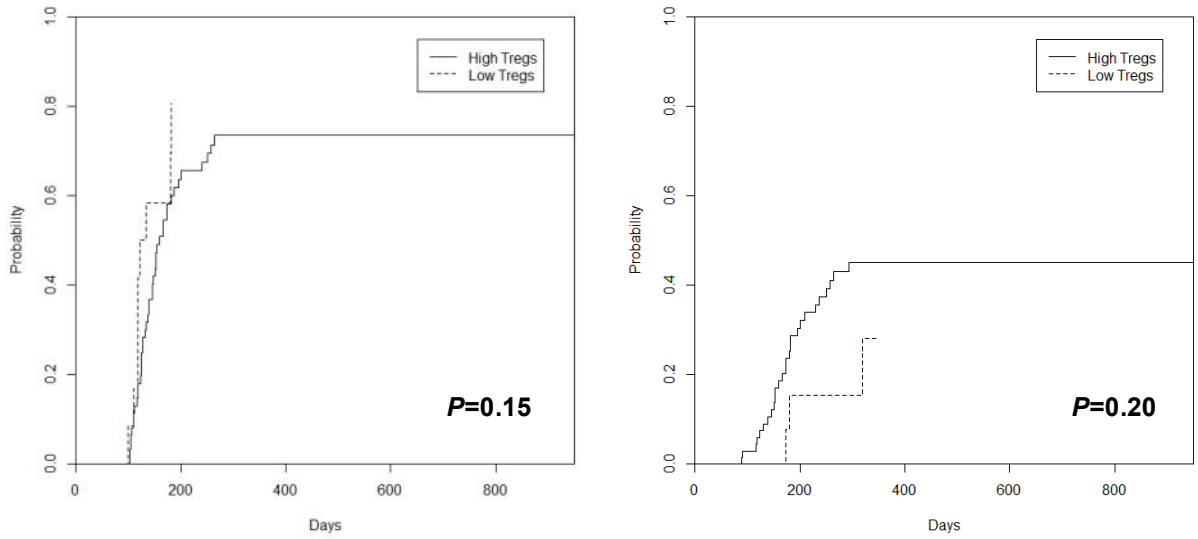
B) OTHER TRANSPLANT VARIABLES

The other transplant variables considered in univariate analysis of chronic GvHD (all grades) are shown in Tables 4.10 & 4.11. Stem cell source was not assessed using the Shulman Classification as there were no transplants using a BM harvest with follow-up beyond 107 days.

Using the Shulman Classification of cGvHD, younger recipient age (87.6% (68.0-95.6) v 62.1% (42.4-76.7); *P*=0.01) and the occurrence of previous grade II-IV aGvHD by day 100 (88.5% (53.4-97.7%) v 68.9% (day 341) (52.9-80.5%), *P*=0.03) were both significantly associated with an increased incidence of cGvHD at one year (Table 4.10).

Using the NIH Consensus Classification of cGvHD, younger recipient age (53.3% (34.0-69.4) v 32.2% (17.7-47.6); *P*=0.07), sibling donors (55.4% (35.4-71.5) v 31.2% (17.5-45.9); *P*=0.06), and not using Alemtuzumab (55.3% (35.0-71.7) v 32.0% (18.4-46.5); *P*=0.09) all showed a non-significant trends towards increased incidence of cGvHD by one year post-transplant. Of note, in this study, donor type (sibling vs unrelated) and Alemtuzumab were very strongly confounded since it is the donor type that is the main determinant of whether the alloHSCT uses Alemtuzumab conditioning (Table 4.11).

A. **B.**



A. Shulman Classification of cGvHD (*N*=75)

Grade	Low Tregs (N=12)		High Tregs (N=63)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
All	69.4 (day 180)	25.8 - 90.7	73.5	59.2 - 83.5	0.15
Extensive	46.0	13.7 - 73.8	56.0	40.7 - 68.7	0.37

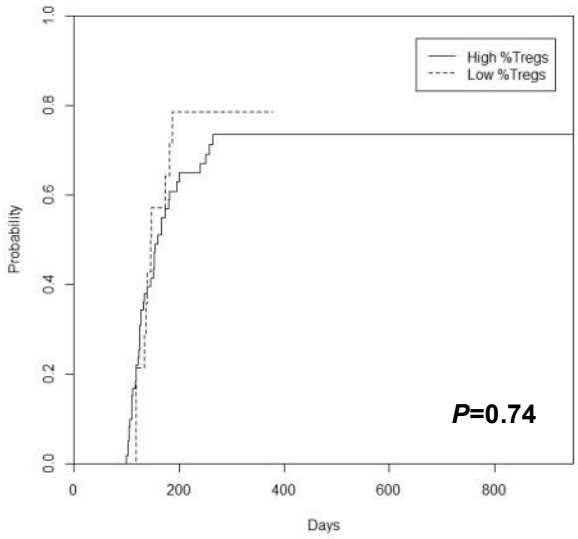
B. NIH Consensus Criteria of cGvHD (*N*=101)

Grade	Low Tregs (N=25)		High Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
All	28.1 (day 346)	3.89 - 60.9	45.1	31.9 - 57.4	0.20
Mod&Sev	28.1 (day 346)	3.89 - 60.9	28.6	17.6 - 40.7	0.83

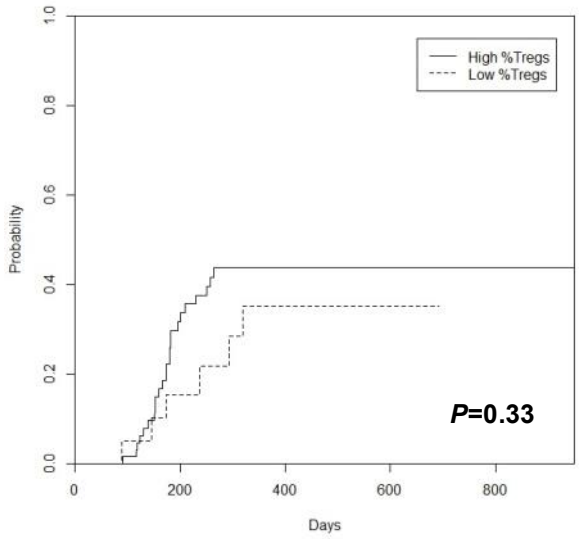
Figure 4.13: Chronic GvHD According to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Dose ($x10^6$/kg) in the Graft

Cumulative incidence of cGvHD according to $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) in the graft. **(A)** Shulman Classification of cGvHD (Shulman *et al.,* 1980), N=75; **(B)** NIH Consensus Criteria of cGvHD (Filipovich *et al.,* 2005), N=101. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Low Tregs <$3.32x10^6$/kg, High Tregs >$3.32x10^6$/kg. Incidence curves shown are all grades of cGvHD. Values quoted are at one year post-transplant unless stated otherwise.

A.



B.



A. Shulman Classification of cGvHD (*N*=75)

cGvHD	**Low %Tregs (N=14)**		**High %Tregs (N=61)**		***P***
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
All	78.6	40.7 - 93.7	73.5	58.6 - 83.8	0.74
Extensive	61.9	24.4 - 85.0	54.2	38.2 - 67.7	0.35

B. NIH Consensus Criteria of cGvHD (*N*=101)

cGvHD	**Low %Tregs (N=25)**		**High %Tregs (N=76)**		***P***
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
All	35.2	9.43 - 51.3	43.7	30.1 - 56.5	0.33
Mod&Sev	28.5	9.43 - 51.3	28.2	16.6 - 40.9	0.97

Figure 4.14: Chronic GvHD According to $CD25^{+}FOXP3^{+}CD127^{-/dim}$ Cells (%$CD4^{+}$ T Cells) in the Graft

Cumulative incidence of cGvHD according to the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells) in the graft. **(A)** Shulman Classification of cGvHD (Shulman *et al.,* 1980); **(B)** NIH Consensus Criteria of cGvHD (Filipovich *et al.,* 2005). '%Tregs' = $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells). Low %Tregs <2.16%, High %Tregs >2.16%. Incidence curves shown are all grades of cGvHD. Values quoted are at one year post-transplant.

Variable	Group	cGvHD (%)	95% CI (%)	*P*
Recipient Age	< Median	87.6	68.0 - 95.6	**0.01**
	> Median	62.1	42.4 - 76.7	
Donor Age	< Median	78.0	62.3 - 87.7	0.35
	> Median	68.5	39.8 - 85.7	
Conditioning	RIC	73.3	59.4 - 83.1	0.96
	MAC	86.7 (day 341)	15.4 - 98.9	
Donor	Sibling	77.6	54.9 - 89.8	0.68
	Unrelated	71.3	53.8 - 83.2	
Alemtuzumab	Yes	72.7	55.7 - 84.0	0.45
	No	75.8	52.0 - 88.9	
D to R Gender	F to M	77.8 (day 257)	14.8 - 96.8	0.98
	Other	74.1	60.1 - 83.9	
HLA match	Matched	72.7	58.9 - 82.5	0.41
	Mismatch	100 (day 195)	100 - 100	
II-IV aGvHD	No	68.9	52.9 - 80.5	**0.03**
	Yes	88.5 (day 341)	53.4 - 97.7	
Graft $CD34^+$ dose	Low	65.8 (day 341)	29.1 - 86.7	0.24
	High	76.8	62.4 - 86.2	
Graft $CD3^+$ dose	Low	100 (day 257)	100 - 100	0.11
	High	71.1	57.0 - 81.2	
Graft $CD19^+$ dose	Low	71.3	36.2 - 89.3	0.61
	High	75.6	60.7 - 85.5	
Graft $CD3^-CD56^+$ dose	Low	76.0	39.0 - 92.3	0.40
	High	74.3	59.4 - 84.4	

Table 4.10: Univariate Analysis of Chronic GvHD (A)

Cumulative incidence of cGvHD according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at one year unless stated otherwise. Chronic GvHD was assessed using the Shulman Classification of cGvHD (Shulman *et al.,* 1980).

Variable	Group	cGvHD (%)	95% CI (%)	*P*
Recipient Age	< Median	53.3	34.0 - 69.4	**0.07**
	> Median	32.2	17.7 - 47.6	
Donor Age	< Median	42.3	25.8 - 55.4	0.59
	> Median	40.7	18.4 - 62.0	
Conditioning	RIC	41.0	28.3 - 53.3	0.73
	MAC	49.1	14.6 - 76.9	
Donor	Sibling	55.4	35.4 - 71.5	**0.06**
	Unrelated	31.2	17.5 - 45.9	
Alemtuzumab	Yes	32.0	18.4 - 46.5	**0.09**
	No	55.3	35.0 - 71.7	
Stem cell source	PBSC	43.3	31.1 - 54.9	0.83
	BM	0.00 (day 107)	-	
D to R Gender	F to M	51.3 (day 293)	15.9 - 78.5	0.50
	Other	39.7	27.1 - 52.2	
HLA	Matched	42.8	30.2 - 54.8	0.54
	Mismatch	37.9	5.50 - 72.3	
II-IV aGvHD	No	40.6	26.8 - 54.0	0.69
	Yes	43.9	21.0 - 64.8	
Graft $CD34^+$ dose	Low	42.1	17.4 - 65.2	0.84
	High	41.1	27.8 - 54.0	
Graft $CD3^+$ dose	Low	60.8 (day 257)	15.4 - 87.6	0.43
	High	40.6	27.9 - 52.9	
Graft $CD19^+$ dose	Low	41.7	17.7 - 64.3	0.84
	High	41.6	27.8 - 54.5	
Graft $CD3^-CD56^+$ dose	Low	27.8	9.25 - 50.2	0.18
	High	46.3	32.1 - 59.3	

Table 4.11: Univariate Analysis of Chronic GvHD (B)

Cumulative incidence of cGvHD according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at one year unless stated otherwise. Chronic GvHD was assessed using the NIH Consensus Classification of cGvHD (Filipovich *et al.,* 2005).

C) MULTIVARIATE ANALYSIS

The variables explored in multivariate analysis of cGvHD (all grades) according to the Shulman classification were graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), recipient age and previous acute GvHD (grade II-IV). In multivariate analysis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the graft (absolute and %$CD4^+$ T cells) were not associated with cGvHD. However, younger recipient age was significantly associated with increased cGvHD (HR 2.08 (1.18-3.66), *P*=0.01) (Table 4.12A). When using the NIH Classification, the variables used were graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), recipient age and Alemtuzumab conditioning. Donor type was not included since Alemtuzumab and donor type were strongly confounded. In contrast to the hypothesis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) in the graft were significantly associated with a lower incidence of cGvHD (HR 0.33 (0.11-0.96), *P*=0.04) when taking the other variables into consideration. Younger recipient age was also significantly associated with increased cGvHD (HR 2.22 (1.08-4.57), *P*=0.03) (Table 4.12B).

A. Shulman Classification of cGvHD

Variable	**Group**	**HR**	**95% CI**	***P***
Recipient Age	< Median	2.08	1.18 - 3.66	**0.01**
	> Median	1	-	-

B. NIH Consensus Criteria of GvHD

Variable	**Group**	**HR**	**95% CI**	***P***
Recipient Age	< Median	2.22	1.08 - 4.57	**0.03**
	> Median	1	-	-
HSC Treg count	High	1	-	-
($x10^{6/}$kg)	Low	0.33	0.11 - 0.96	**0.04**

Table 4.12: Multivariate Analysis of Chronic GvHD

Multivariate analysis of cGvHD (all grades). cGvHD was assessed using **(A)** Shulman Classification (Shulman *et al.,* 1980); **(B)** NIH Consensus Classification of cGvHD (Filipovich *et al.,* 2005). Hazard ratios (HR), 95% confidence interval (CI) and *P*-values were calculated using the Fine and Gray regression model. 'Treg' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells.

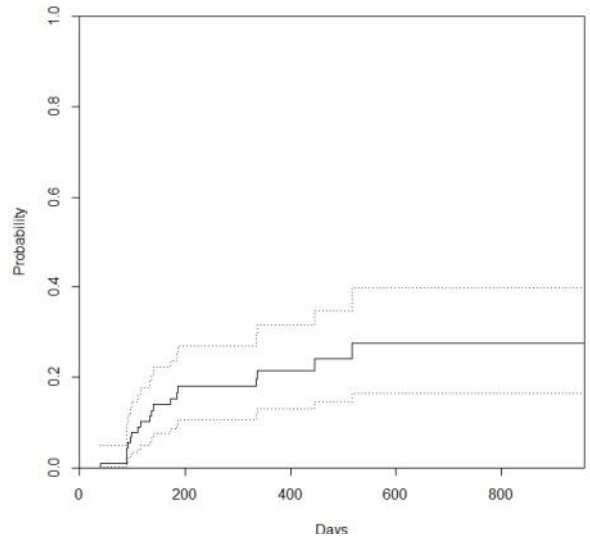
4.3.7 RELAPSE AND NON-RELAPSE MORTALITY

Nineteen patients' disease relapsed (AML 14, ALL 1, MDS 2, MP 1, Myeloma 1). The probability of relapse was 21.6% (13.1-31.5%) and 27.6% (6.10-47.6%) at one and two years respectively. NRM was 23.5% (14.9-33.3%) at one year which had sustained a plateau. The probability of RFS was 56.1% (45.8-68.6%) and 50.0% (38.5-64.7%) at one and two years respectively (Figure 4.15). As disease relapse had not reached a plateau by one year, the cumulative incidence of relapse was assessed at one and two years post-transplant.

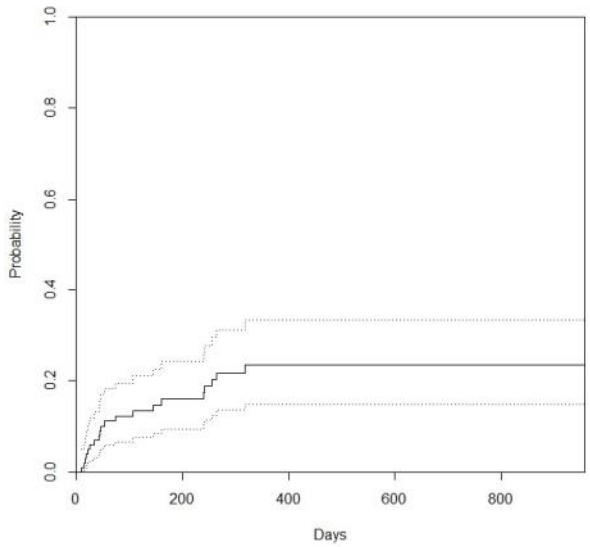
A) GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

Low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were not associated with an increased risk of disease relapse. However, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses were associated with significantly higher NRM at one year (43.3% (16.1-68.1) v 18.5% (9.94-29.0%) *P*=0.01) (Figure 4.16). Similarly, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were also associated with significantly higher NRM at one year (38.6% (16.6-60.3) v 19.2% (10.3-30.2%), *P*=0.047) (Figure 4.17).

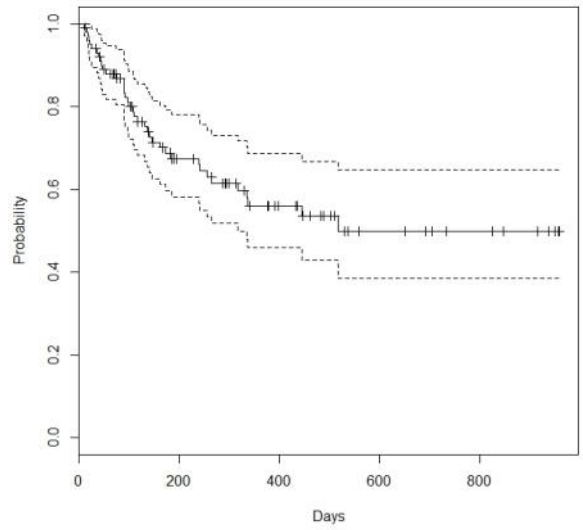
A.



B.



C.

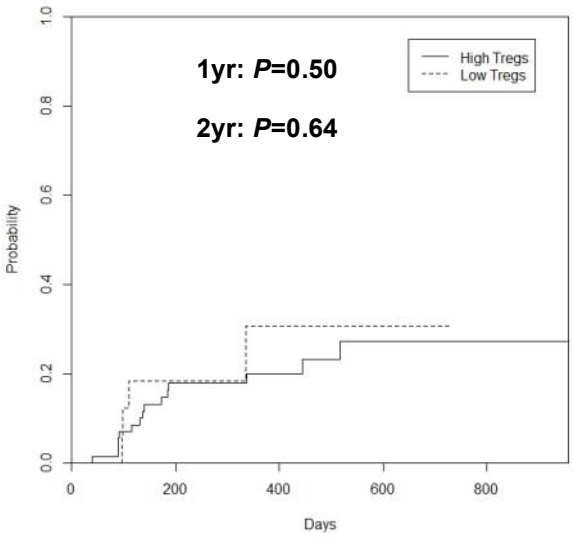


Outcome	**One Year**		**Two Years**	
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)
Relapse	21.6	13.1 - 31.5	27.6	6.10 - 47.6
NRM	23.5	14.9 - 33.3	23.5	14.9 - 33.3
RFS	56.1	45.8 - 68.6	50.0	38.5 - 64.7

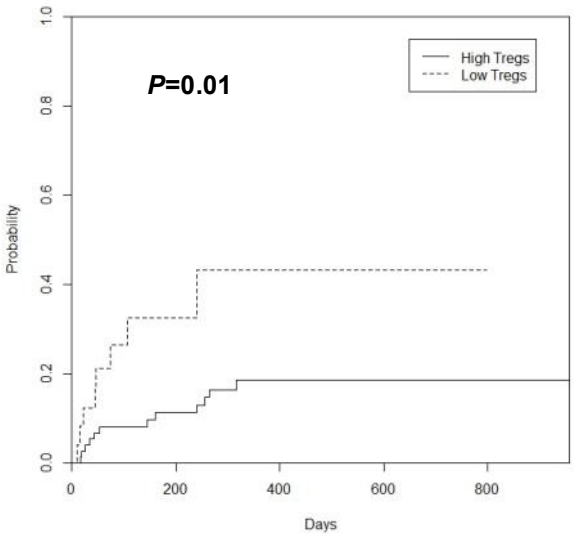
Figure 4.15: Relapse, Non-Relapse Mortality and Relapse-Free Survival

Cumulative incidence of **(A)** Relapse; **(B)** Non-Relapse Mortality for 101 alloHSCT. (**C**) Kaplan-Meier estimate of Relapse-Free Survival in 101 alloHSCT. Dashed lines show the 95% confidence intervals (%).

A.



B.

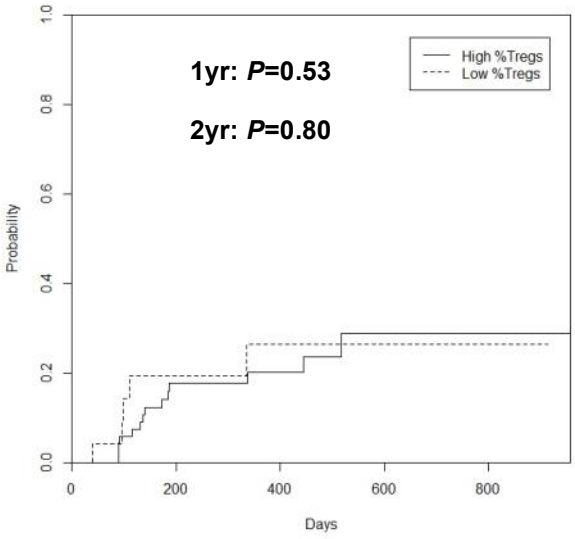


Outcome	Low Tregs (N=25)		High Tregs (N=75)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
Relapse (1yr)	30.7	6.44 - 60.1	20.0	11.1 - 30.8	0.50
Relapse (2yr)	30.7	6.44 - 60.1	27.3	15.0 - 41.2	0.64
NRM	43.3	16.1 - 68.1	18.5	9.94 - 29.0	**0.01**

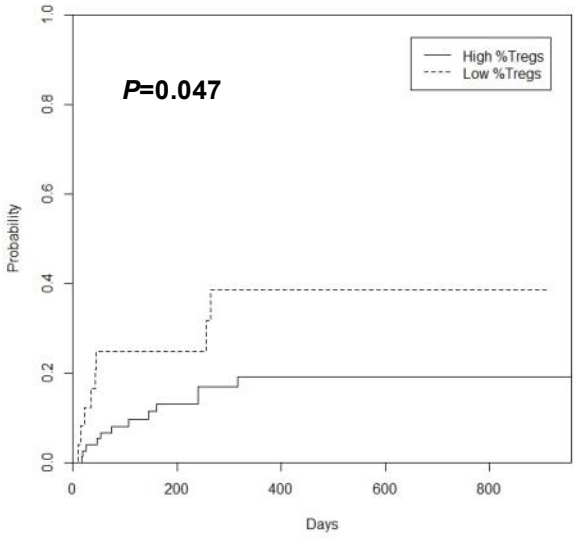
Figure 4.16: Relapse and Non-Relapse Mortality According to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Dose ($x10^6$/kg) in the Graft

Cumulative incidence of **(A)** Relapse; **(B)** Non-Relapse Mortality according to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) in the graft. 'Treg' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Low Tregs <$3.32x10^6$/kg; High Tregs >$3.32x10^6$/kg. All values quoted are at one year post-transplant unless stated otherwise.

A.



B.



Outcome	Low %Tregs (N=25)		High %Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
Relapse (1yr)	26.4	8.60 - 48.4	20.2	10.9 - 31.5	0.53
Relapse (2yr)	26.4	8.60 - 48.4	28.8	149 - 44.3	0.80
NRM	38.6	16.6 - 60.3	19.2	10.3 - 30.2	**0.047**

Figure 4.17: Relapse and Non-Relapse Mortality According to $CD25^+FOXP3^+CD127^{-/dim}$ Cells (%$CD4^+$ T Cells) in the Graft

Cumulative incidence of **(A)** Relapse; **(B)** Non-Relapse Mortality according to the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft. '%Tregs' = $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). Low %Tregs <2.16%; High %Tregs >2.16%. All values quoted are at one year post-transplant unless stated otherwise.

B) OTHER TRANSPLANT VARIABLES

The other transplant variables considered in univariate analysis of disease relapse are shown in Tables 4.13 & 4.14. At one year post-transplant, only the patient's diagnosis (leukaemia 32.7% (19.7-46.4%), lymphoproliferative 0.00%, other diagnoses 14.8% (0.37-52.1%), *P*=0.005) was significantly associated with the incidence of relapse. Status at transplant (CR 28.9% (16.2-42.8%), PR 0.00% and AD 21.1% (5.63-43.1%); P=0.06) and HLA matching (Matched (18.3% (9.85-28.7) v Mismatched 41.0% (12.4-68.4), *P*=0.099) showed a non-significant association with relapse (Table 4.13). At two years post-transplant, diagnosis (leukaemia 37.5% (22.1-52.3%), lymphoproliferative 9.36% (0.42-35.7%), other diagnoses 14.8% (day 692) (0.37-52.1%), *P*=0.007) and status at transplant (CR 36.6% (18.1-55.3%), PR 0.00% and AD 30.1% (9.00-55.0%); *P*=0.049) were both significantly associated with the probability of relapse (Table 4.14).

The transplant variables considered in univariate analysis of NRM are shown in Table 4.15. Those variables significantly associated with higher NRM by one year were recipient CMV IgG positive (31.8% (17.7-46.9%) v 16.6 (6.93-29.8%), *P*=0.04), BM harvests (100% (day 107) v 21.1% (12.7-30.9%), *P*=0.0001) and low $CD3^{-}CD56^{+}$ cell doses in the graft (42.2% (20.1-62.9%) v 17.6 (9.09-28.3%), *P*=0.01) (Table 4.15). Low $CD34^{+}$ and $CD3^{+}$ cell doses in the graft showed non-significant trends towards higher non-relapse mortality (38.6% (17.1-59.9%) v 18.9 (10.1-29.7%), *P*=0.06 and 41.2% (13.5-67.5%) v 19.4 (10.8-30.0%), *P*=0.05).

Variable	Group	Relapse (%)	95% CI (%)	*P*	*P**
Recipient Age	< Median	19.6	8.19 - 34.6	0.41	-
	> Median	23.5	12.0 - 37.2		
Donor Age	< Median	18.4	9.27 - 30.0	0.18	-
	> Median	28.2	11.9 - 47.1		
Diagnosis	Leukaemia#	32.7	19.7 - 46.4	**0.005**	-
	Lympho'	0.00	-		**0.003**
	Other	14.8	0.37 - 52.1		0.13
Disease Status	CR#	28.9	16.2 - 42.8	**0.06**	-
	PR	0.00	-		**0.03**
	AD	21.1	5.63 - 43.1		0.32
Conditioning	RIC	22.9	13.3 - 33.9	0.66	-
	MAC	14.6	2.00 - 38.8		
Donor	Sibling	20.6	8.84 - 35.7	0.96	-
	Unrelated	23.1	11.3 - 37.3		
Stem cell source	PBSC	22.3	13.5 - 32.5	0.66	-
	BM	0.00 (day 107)	-		
Alemtuzumab	Yes	24.2	12.4 - 38.1	0.83	-
	No	18.5	7.30 - 33.7		
D to R Gender	F to M	29.2	7.84 - 55.1	0.25	-
	Other	20.3	11.2 - 31.3		
HLA match	Matched	18.3	9.85 - 28.7	**0.099**	-
	Mismatch	41.0	12.4 - 68.4		
Graft $CD34^+$ dose	Low	15.7	3.61 - 35.7	0.56	-
	High	23.4	13.3 - 35.2		
Graft $CD3^+$ dose	Low	15.7	2.04 - 41.4	0.55	-
	High	23.2	13.5 - 34.4		
Graft $CD19^+$ dose	Low	11.1	1.71 - 30.4	0.28	-
	High	24.8	14.4 - 36.7		
Graft $CD3^-CD56^+$ dose	Low	15.3	3.55 - 34.9	0.58	-
	High	23.6	13.4 - 35.3		

Table 4.13: Univariate Analysis of Disease Relapse (One Year)

Cumulative incidence of disease relapse in 101 alloHSCT according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at one year unless stated otherwise. * Compared to reference level (#).

Variable	Group	Relapse (%)	95% CI (%)	*P*	*P* *
Recipient Age	< Median	25.7	10.7 - 43.7	0.39	-
	> Median	29.0	14.3 - 45.5		
Donor Age	< Median	26.5	13.4 - 41.6	0.30	-
	> Median	28.2	11.9 - 47.1		
Diagnosis	Leukaemia#	37.5	22.1 - 52.3	**0.007**	-
	Lympho'	9.36	0.42 - 35.7		**0.005**
	Other	14.8 (day 692)	0.37 - 52.1		0.11
Disease Status	CR#	36.6	18.1 - 55.3	**0.049**	-
	PR	0.00	-		**0.02**
	AD	30.1	9.00 - 55.0		0.41
Conditioning	RIC	29.5	17.2 - 43.0	0.56	-
	MAC	14.6	2.00 - 38.8		
Donor	Sibling	20.6	8.84 - 35.7	0.65	-
	Unrelated	36.1	16.4 - 56.3		
Stem cell source	PBSC	28.5	17.0 - 41.1	0.66	-
	BM	0.00 (day 107)	-		
Alemtuzumab	Yes	37.2	17.4 - 57.1	0.47	-
	No	18.5	7.30 - 33.7		
D to R Gender	F to M	29.2	7.84 - 55.1	0.43	-
	Other	28.1	15.1 - 42.5		
HLA match	Matched	24.7	13.4 - 37.7	0.11	-
	Mismatch	41.0 (day 461)	12.4 - 68.4		
Graft $CD34^+$ dose	Low	15.7	3.61 - 35.7	0.39	-
	High	31.6	17.5 - 46.7		
Graft $CD3^+$ dose	Low	15.7 (day 537)	2.04 - 41.4	0.43	-
	High	30.4	17.5 - 44.4		
Graft $CD19^+$ dose	Low	11.1 (day 650)	1.71 - 30.4	0.18	-
	High	32.6	18.7 - 47.2		
Graft $CD3^-CD56^+$	Low	15.3	3.55 - 34.9	0.44	-
dose	High	31.1	3.55 - 34.9		

Table 4.14: Univariate Analysis of Disease Relapse (Two Years)

Cumulative incidence of disease relapse in 101 alloHSCT according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at two years unless stated otherwise. * Compared to reference level (#).

Variable	Group	Relapse (%)	95% CI (%)	*P*
Recipient Age	< Median	26.5	13.2 - 41.8	0.99
	> Median	21.1	10.6 - 33.9	
Donor Age	< Median	27.1	16.2 - 39.3	0.28
	> Median	14.7	4.28 - 31.1	
Recipient CMV	Neg	16.6	6.93 - 29.8	**0.04**
IgG	Pos	31.8	17.7 - 46.9	
Donor CMV	Neg	21.4	11.9 - 32.7	0.77
IgG	Pos	27.8	11.4 - 47.0	
Conditioning	RIC	24.1	14.6 - 34.9	0.99
	MAC	18.8	4.29 - 41.1	
Donor	Sibling	22.9	10.4 - 38.4	0.85
	Unrelated	23.4	12.6 - 36.2	
Stem cell source	PBSC	21.1	12.7 - 30.9	**0.0001**
	BM	100 (day 107)	100 - 100	
Alemtuzumab	Yes	22.5	12.1 - 34.9	0.96
	No	24.4	11.0 - 40.6	
D to R Gender	F to M	34.6	10.6 - 60.6	0.34
	Other	21.2	12.3 - 31.8	
HLA match	Matched	24.0	14.6 - 34.8	0.98
	Mismatch	16.7	3.89 - 37.2	
Graft $CD34^+$ dose	Low	38.6	17.1 - 59.9	**0.06**
	High	18.9	10.1 - 29.7	
Graft $CD3^+$ dose	Low	41.2	13.5 - 67.5	**0.05**
	High	19.4	10.8 - 30.0	
Graft $CD19^+$ dose	Low	35.6	14.5 - 57.6	0.16
	High	20.1	11.1 - 31.1	
Graft $CD3^-CD56^+$ dose	Low	42.2	20.1 - 62.9	**0.01**
	High	17.6	9.09 - 28.3	

Table 4.15: Univariate Analysis of Non-Relapse Mortality

Cumulative incidence of non-relapse mortality in 101 alloHSCT according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at one year unless stated otherwise.

C) MULTIVARIATE ANALYSIS

The variables used in multivariate analysis of disease relapse were the graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), diagnosis, disease status at transplant and HLA matching. Unfortunately, multivariate analysis of these data was not possible due to failure of convergence of the competing risks regression model when including the variables diagnosis or disease status.

The variables included in multivariate analysis of NRM were the graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell, $CD34^+$, $CD3^+$ and $CD3^-CD56^+$ cell dose, the graft proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), HSC source and the recipient's CMV IgG status. Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft remained independently associated with increased NRM (HR 2.57 (1.05-6.29), *P*=0.04). Other graft variables significantly associated with higher NRM were low $CD34^+$ dose (HR 2.55 (1.02-6.36), *P*=0.045) and low $CD3^-CD56^+$ cell dose (HR 3.78 (1.52-9.40), *P*=0.004). Positive CMV IgG status (HR 2.66 (1.09-6.48), *P*=0.03) was also significantly associated with higher NRM (Table 4.16).

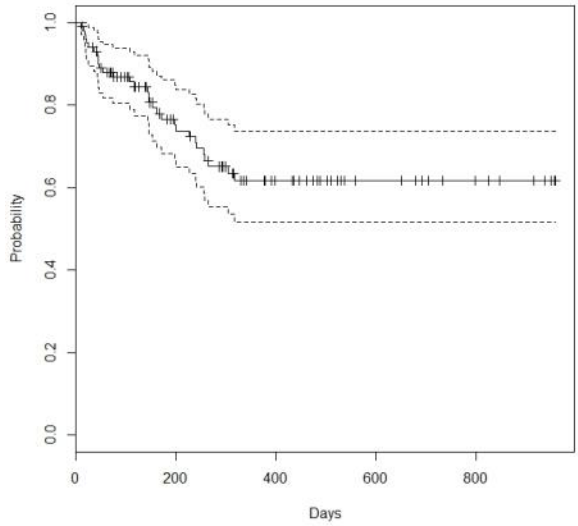
Variable	Group	HR	95% CI	*P*
Graft %Tregs	High	1		
	Low	2.57	1.05 - 6.29	**0.04**
Graft $CD34^+$ dose	High	1		
	Low	2.55	1.02 - 6.36	**0.045**
Graft $CD3^-CD56^+$ dose	High	1		
	Low	3.78	1.52 - 9.40	**0.004**
Recipient CMV IgG	Negative	1		
	Positive	2.66	1.09 - 6.48	**0.03**

Table 4.16: Multivariate Analysis of Non-Relapse Mortality

Multivariate analysis of NRM. Hazard ratios (HR), 95% confidence interval (CI) and *P* values using the Fine and Gray regression model. '%Tregs' = $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells).

4.3.8 OVERALL SURVIVAL

In this series, the probability of overall survival (OS) had reached a plateau by one year at 61.6% (51.6-73.7%) (Figure 4.18). 31 patients died with the most common cause of death being relapse of the underlying haematological disease (*N*=11). Other causes of death were GvHD (*N*=8), infection (*N*=7), intra-cranial haemorrhage (*N*=1), veno-occlusive disease (*N*=1), superior vena cava obstruction (*N*=1), pneumonitis (*N*=1) and progressive neuropathy (*N*=1) (Table 4.17).



Outcome	Six Months		One Year	
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)
OS	76.6	68.2 - 86.0	61.6	51.6 - 73.7

Figure 4.18: Overall Survival

Kaplan-Meier estimate of overall survival in 101 alloHSCT. Dashed lines show the 95% confidence intervals (%)

Table 4.17: Cause of Death

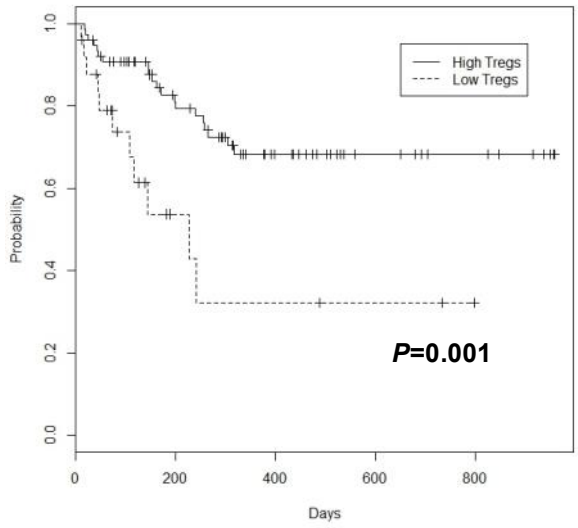
Cause	N	%
All	31	100
Disease relapse	11	35.5
GvHD	8	25.8
Infection	7	22.6
Intra-cranial haemorrhage	1	3.22
Veno-occlusive disease	1	3.22
Superior vena cava obstruction	1	3.22
Pneumonitis	1	3.22
Progressive neuropathy	1	3.22

A) GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

In keeping with the NRM data, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses in the graft were significantly associated with inferior overall survival (32.2% (13.9-74.7) v 68.2% (57.4-81.1); *P*=0.001) (Figure 4.19). In univariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were also significantly associated with inferior overall survival (41.1% (23.4-71.9%) v 67.8% (56.5-81.3%), *P*=0.007) (Figure 4.20).

B) OTHER TRANSPLANT VARIABLES

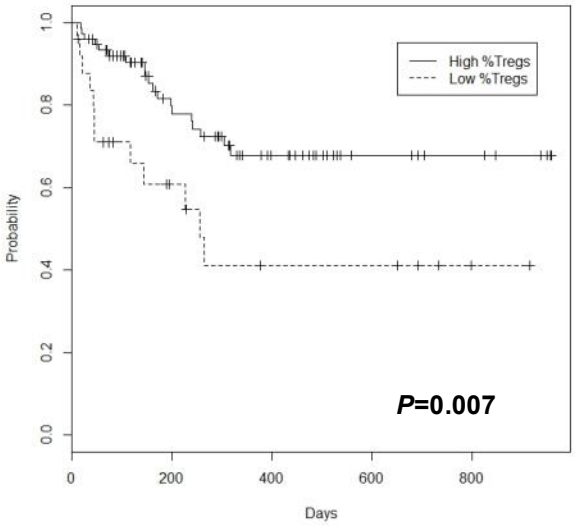
The other transplant variables considered in univariate analysis of overall survival are shown in Table 4.18. In univariate analysis, variables significantly associated with inferior overall survival were use of BM harvests (0.00% (day 107) v 63.6% (53.4-75.8%), *P*=0.0001), female-to-male donation (34.2 % (15.7-74.2%) v 67.2% (56.5-79.9%), *P*=0.02) and positive recipient CMV serology (49.2 % (34.9-69.2%) v 71.3% (58.4-87.1%), *P*=0.02). In relation to the graft, only low $CD3^-CD56^+$ cell doses were also significantly associated with inferior overall survival (40.8 % (23.6-70.5%) v 68.4% (57.2-81.7%), *P*=0.01) (Table 4.18).



Outcome	Low Tregs (N=25)		High Tregs (N=76)		*P*
	Probability (%)	95% CI (%)	Probability (%)	95% CI (%)	
OS	32.2	13.9 - 74.7	68.2	57.4 - 81.1	**0.001**

Figure 4.19: Overall Survival According to the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-Cell Dose ($x10^{6}$/kg) in the Graft

Kaplan-Meier estimate of overall survival according to the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell dose ($x10^{6}$/kg) in the graft. 'Tregs' = $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells. Low Tregs $<3.32x10^{6}$/kg, High Tregs $>3.32x10^{6}$/kg. Values quoted are at one year post-transplant.



Outcome	Low %Tregs (N=25)		High %Tregs (N=76)		*P*
	Probability (%)	95% CI (%)	Probability (%)	95% CI (%)	
OS	41.1	23.4 - 71.9	67.8	56.5 - 81.3	**0.007**

Figure 4.20: Overall Survival According to $CD25^{+}FOXP3^{+}CD127^{-/dim}$ Cells (%$CD4^{+}$ T Cells) in the Graft

Kaplan-Meier estimate of overall survival according to the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft. '%Tregs' = $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells). Low %Tregs <2.16%, High %Tregs >2.16%. Values quoted are at one year post-transplant.

On next page

Table 4.18: Univariate Analysis of Overall Survival

Probability of overall survival according to transplant related variables in 101 alloHSCT. Overall survival (%) and 95% confidence intervals (%) at one year unless stated otherwise.

Variable	Group	OS (%)	95% CI (%)	*P*
Recipient Age	< Median	68.1	54.3 - 85.4	0.16
	> Median	56.0	42.6 - 73.7	
Donor Age	< Median	61.5	49.7 - 76.0	0.83
	> Median	61.9	44.3 - 86.5	
Recipient CMV IgG	Pos	49.2	34.9 - 69.2	**0.02**
	Neg	71.3	58.4 - 87.1	
Donor CMV	Pos	46.7	29.9 - 72.8	0.16
IgG	Neg	68.5	57.1 - 82.1	
D to R Gender	F to M	34.2	15.7 - 74.2	**0.02**
	Other	67.2	56.5 - 79.9	
Diagnosis	Leukaemia[#]	55.9	43.0 - 72.7	0.45
	Lympho'	74.8	59.0 - 95.0	
	Other	59.2	33.8 - 100	
Disease Status	CR[#]	59.1	46.0 - 76.0	0.64
	PR	72.0	51.7 - 100	
	AD	59.5	40.6 - 87.3	
HSC source	PBSC	63.6	53.4 - 75.8	**0.0001**
	BM	0.00 (day 107)	-	
Donor	Sibling	58.5	43.7 - 78.3	0.72
	Unrelated	64.9	52.4 - 80.4	
Conditioning	RIC	59.9	48.9 - 73.3	0.78
	MAC	75.0	56.5 - 99.5	
Alemtuzumab	Yes	66.4	54.2 - 81.3	0.48
	No	55.7	40.4 - 76.8	
HLA match	Matched	60.5	49.7 - 73.6	0.82
	Mismatch	69.4	45.9 - 100	
Graft $CD34^+$ dose	Low	49.9	31.4 - 79.2	0.16
	High	65.5	54.3 - 79.1	
Graft $CD3^+$ dose	Low	48.7	26.6 - 89.0	0.12
	High	64.6	53.7 - 77.7	
Graft $CD19^+$ dose	Low	52.0	32.9 - 82.0	0.28
	High	64.2	52.9 - 78.0	
Graft $CD3^-CD56^+$ dose	Low	40.8	23.6 - 70.5	**0.01**
	High	68.4	57.2 - 81.7	

C) MULTIVARIATE ANALYSIS

The variables used in multivariate analysis of OS were the graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell and $CD3^-CD56^+$ cell dose, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft, HSC source, the recipient's CMV IgG status and donor-to-recipient gender. In multivariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) continued to be significantly associated with inferior OS (HR 2.58 (1.24-5.35); *P*=0.01). Other factors associated with worse OS were low $CD3^-CD56^+$ cell doses in the graft (HR 3.08 (1.47-6.46); *P*=0.003) and a positive recipient CMV serology (HR 2.54 (1.21-5.32); *P*=0.01) (Table 4.19).

Variable	Group	HR	95% CI	*P*
Graft %Tregs	High	1	-	-
	Low	2.58	1.24 - 5.34	**0.01**
Graft $CD3^-CD56^+$	High	1	-	-
	Low	3.08	1.47 - 6.46	**0.003**
Recipient CMV	Negative	1	-	-
IgG serology	Positive	2.54	1.21 - 5.32	**0.01**

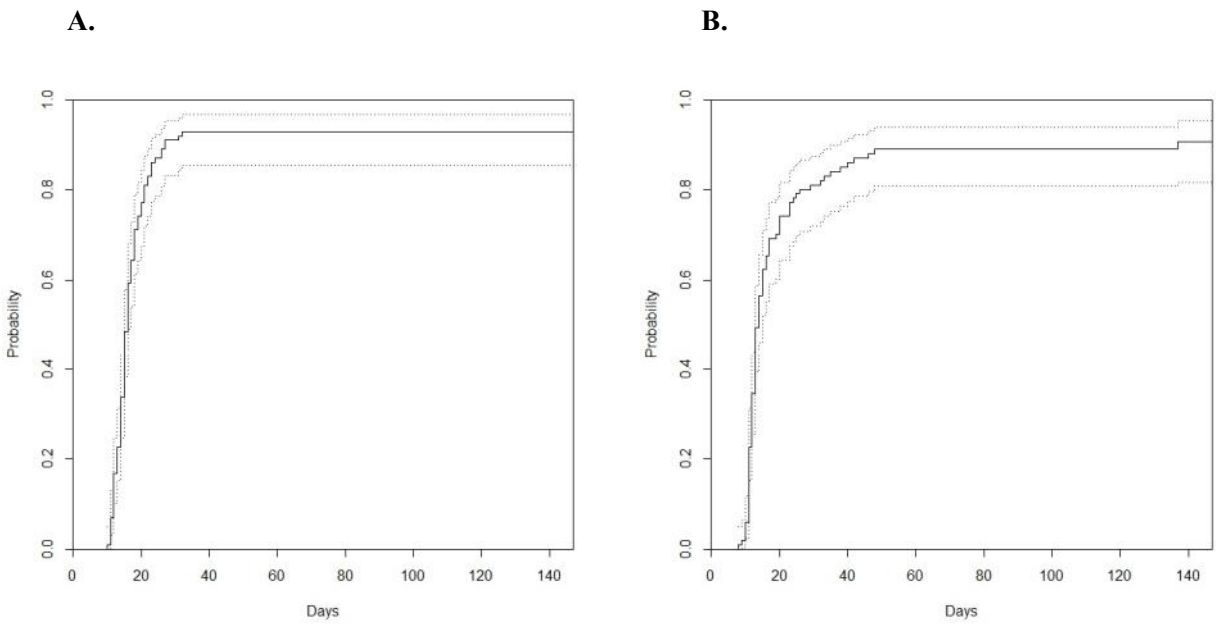
Table 4.19: Multivariate Analysis of Overall Survival

Multivariate analysis of OS in the 101 alloHSCTs. Hazard ratios (HR), 95% confidence interval (CI) and *P* values were calculated using Cox Proportional Hazards. '%Tregs' = $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells).

In keeping with the original hypotheses, these findings indicate that low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft is an independent predictor of higher NRM and inferior OS. However, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses in the graft (absolute counts or %$CD4^+$ T cells) do not predict the occurrence of disease relapse. Since, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were not associated with increased GvHD (Chapter 4.3.5-4.3.6), it was hypothesised that they may be associated with other causes of NRM e.g. poor engraftment, infection, CMV reactivation. Therefore, these other outcomes will now be analysed.

4.3.9 ENGRAFTMENT

In view of the significant association observed between the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft and NRM, this study then investigated the effect of graft $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells on other causes of NRM. In this series, primary graft failure occurred following three alloHSCT, although all three patients subsequently received a second transplant. Four patients died prior to attaining neutrophil engraftment and seven died prior to platelet engraftment. The median time to neutrophil and platelet engraftment was 15 days (10-32) and 13 days (8-137) respectively (Figure 4.21).



Engraftment	Day 30		Day 100	
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)
Neutrophil (>0.5x10^{9}/l)	91.1	83.3 - 95.4	93.1	85.6 - 96.7
Platelet (>50x10^{9}/l)	81.2	72.0 - 87.6	89.1	80.9 - 93.9

Figure 4.21: Neutrophil and Platelet Engraftment

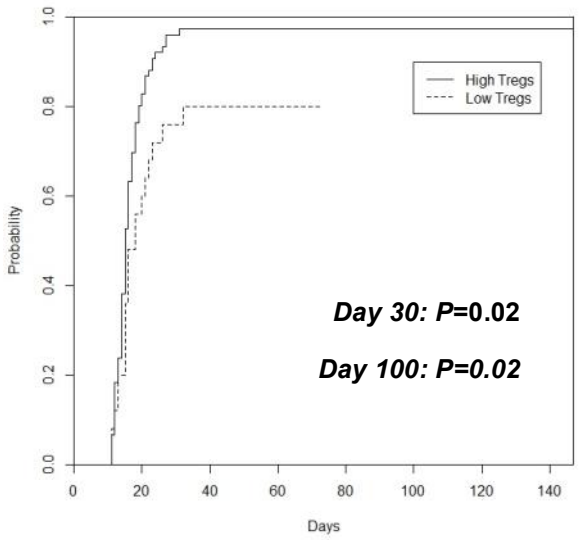
Cumulative incidence of **(A)** Neutrophil engraftment; **(B)** Platelet engraftment in 101 alloHSCT. Dashed lines show the 95% confidence intervals (%).

A) GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

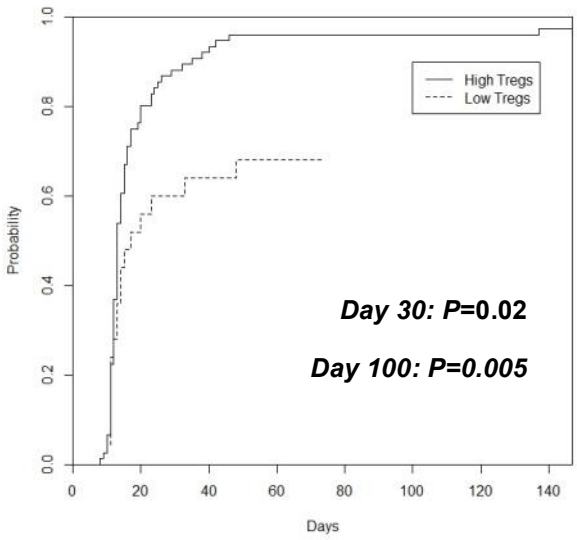
In univariate analysis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the graft were significantly associated with a lower probability of both neutrophil and platelet engraftment (>$0.5x10^9$/l and >$50x10^9$/l respectively) at day 30 (76.0% (52.8-88.9%) v 96.1% (87.0-98.8), *P*=0.02 and 60.0% (37.6-76.6%) v 88.2% (78.1-93.8), *P*=0.02 respectively). The median time to neutrophil engraftment was 16 days and 15 days in the low and high $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell groups respectively. The median time to platelet engraftment was 13 days in both the low and high $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell groups (Figure 4.22).

In univariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were also associated with a significantly lower probability of neutrophil engraftment by day 30 (76.0 % (52.8-88.9%) v 96.1% (87.0-98.8%), *P*=0.03). Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were associated with non-significant trend towards a lower probability of day 30 platelet engraftment (68.0 % (45.1-83.0%) v 85.8% (75.1-91.8%), *P*=0.052. The median time to neutrophil engraftment was 15 days in both the low and high $CD25^+FOXP3^+CD127^{-/dim}$ (%$CD4^+$ T cells) groups. The median time to platelet engraftment was 15 and 13 days in the low and high $CD25^+FOXP3^+CD127^{-/dim}$ (%$CD4^+$ T cells) groups respectively (Figure 4.23).

A.



B.

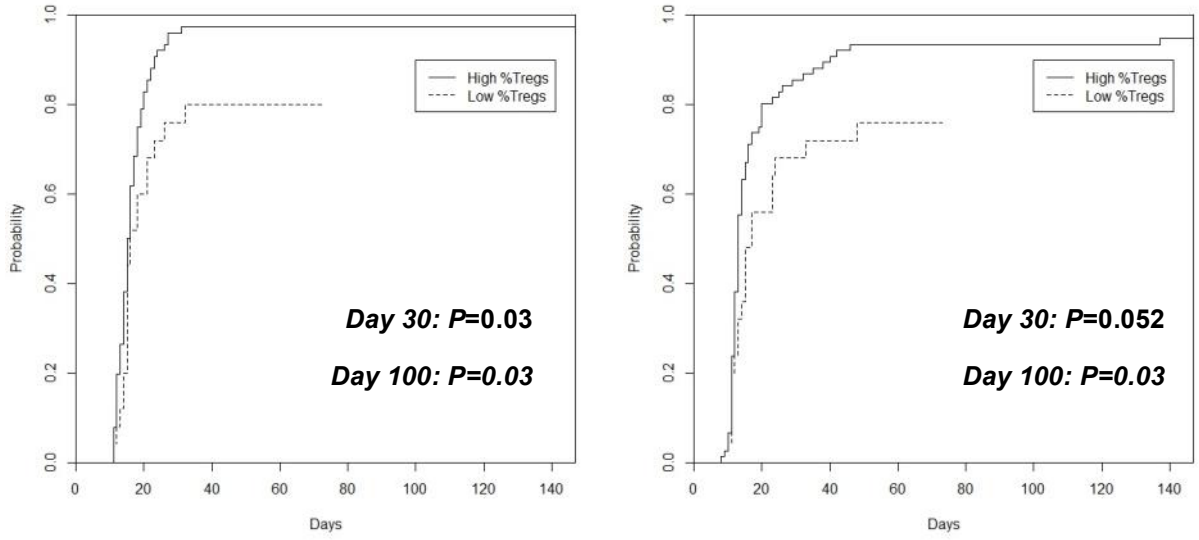


Engraftment	Low Tregs (N=25)			High Tregs (N=76)			*P*
	Median (Days)	Incidence (%)	95% CI (%)	Median (Days)	Incidence (%)	95% CI (%)	
Neutrophil (>0.5x10^9/l)	16	76.0	52.8 - 88.9	15	96.1	87.0 - 98.8	**0.02**
Platelet (>50x10^9/l)	13	60.0	37.6 - 76.6	13	88.2	78.1 - 93.8	**0.02**

Figure 4.22: Engraftment According to the CD4$^+$CD25$^+$FOXP3$^+$CD127$^{-/dim}$ T-Cell Dose (x10^6/kg) in the Graft

Cumulative incidence of **(A)** Neutrophil; **(B)** Platelet engraftment according to the CD4$^+$CD25$^+$FOXP3$^+$CD127$^{-/dim}$ T-cell dose (x10^6/kg) in the graft. 'Tregs' = CD4$^+$CD25$^+$FOXP3$^+$CD127$^{-/dim}$ T cells. Low Tregs <3.32x10^6/kg, High Tregs >3.32x10^6/kg. Values quoted are at day 30.

A. **B.**



Engraftment	Low %Tregs (N=25)			High %Tregs (N=76)			
	Median (Days)	Incidence (%)	95% CI (%)	Median (Days)	Incidence (%)	95% CI (%)	*P*
Neutrophil (>0.5x10^9/l)	15	76.0	52.8 - 88.9	15	96.1	87.0 - 98.8	**0.03**
Platelet (>50x10^9/l)	15	68.0	45.1 - 83.0	13	85.5	75.1 - 91.8	**0.052**

Figure 4.23: Engraftment According to CD25$^+$FOXP3$^+$CD127$^{-/dim}$ cells (%CD4$^+$ T Cells) in the Graft

Cumulative incidence of **(A)** Neutrophil; **(B)** Platelet engraftment according to the proportion of CD25$^+$FOXP3$^+$CD127$^{-/dim}$ cells (%CD4$^+$ T cells) in the graft. '%Tregs' = CD25$^+$FOXP3$^+$CD127$^{-/dim}$ (%CD4$^+$ T cells). Low %Treg <2.16%, High %Treg >2.16%. Values quoted are at day 30.

B) OTHER TRANSPLANT VARIABLES

The other transplant variables considered in univariate analysis of engraftment are shown in Tables 4.20 & 4.21. The probability of successful neutrophil engraftment by day 30 was significantly lower when using low $CD34^+$ cell doses (76.0% (52.7-88.9%) v 96.1% (87.0-98.8%), *P*=0.0007), with a median time to engraftment of 19 and 15 days in the low and high groups respectively. The probability of engraftment by day 30 was also lower when transplanting 'other' diagnoses (94.9% (83.4-98.59%), 96.6% (65.0-99.7%) and 61.5% (28.9-82.7%) for the leukaemia, lymphoproliferative and other groups respectively, *P*=0.006). The median time to engraftment for these three groups was 16, 15 and 15 days. Use of BM harvest showed a non-significant trend towards a lower probability of neutrophil engraftment by day 30 (50.0% (2.26-88.1%) v 92.8% (85.1-99.6%), *P*=0.09) (Table 4.20).

Similarly, the probability of platelet engraftment by day 30 was significantly lower when using low graft $CD34^+$ cell doses (56.0% (34.1-73.2%) v 89.5% (79.6-94.7%), *P*=0.0001). The median time to engraftment was 19 and 13 days for low and high $CD34^+$ groups respectively. Using BM harvest was also associated with a significantly lower probability of platelet engraftment by day 30 (25.0% (0.33-71.4%) v 83.5% (74.3-89.6%), *P*=0.03). The median time to engraftment with PBSC and BM harvests was 13 and 20 days respectively. The probability of day 30 platelet engraftment was also significantly lower when transplanting 'other' diagnoses (84.8% (72.3-91.9%), 93.1% (70.6-98.5%) and 38.5% (13.0-63.9%) for the leukaemia, lymphoproliferative and other groups respectively, *P*=0.0006). The median time to platelet engraftment for these groups was 13, 13 and 20 days respectively (Table 4.21).

Variable	Group	Median (Days)	Engraftment (%)	95% CI (%)	*P*	*P* *
Recipient Age	< Median	15	93.9	80.6 - 98.2	0.74	
	> Median	15	88.5	75.2 - 94.9		
Donor Age	< Median	15	90.0	79.7 - 95.2	0.87	
	> Median	16	93.5	70.3 - 98.7		
Diagnosis	Leukaemia[#]	16	94.9	83.4 - 98.5		-
	Lympho'	15	96.6	65.0 - 99.7	**0.006**	0.14
	Other	15	61.5	28.9 - 82.7		**0.01**
HSC source	PBSC	15	92.8	85.1 - 96.6	**0.09**	
	BM	18	50.0	2.26 - 88.1		
Donor	Sibling	17	92.5	76.2 - 97.8	0.20	
	Unrelated	15	90.2	78.8 - 95.6		
Conditioning	RIC	15	90.6	81.7 - 95.3	0.13	
	MAC	20	93.8 (day 27)	27.0 - 99.7		
Alemtuzumab	Yes	15	90.5	79.4 - 95.7	0.21	
	No	17	92.1	75.1 - 97.7		
D to R Gender	F to M	18	94.4 (day 27)	33.9 - 99.7	0.20	
	Other	15	90.4	81.3 - 95.2		
HLA match	Matched	15	91.6	82.7 - 96.0	0.72	
	Mismatch	15	88.9	57.0 - 97.6		
Graft $CD34^+$ dose	Low	19	76.0	52.7 - 88.9	**0.0007**	
	High	15	96.1	87.0 - 98.8		
Graft $CD3^+$ dose	Low	16	84.0	60.7 - 94.1	0.15	
	High	15	93.4	84.2 - 97.4		
Graft $CD19^+$ dose	Low	15	88.0	64.1 - 96.4	0.71	
	High	16	92.1	82.7 - 96.5		
Graft $CD3^-CD56^+$	Low	15	88.0	64.4 - 96.4	0.76	
dose	High	16	92.1	82.7 - 96.5		

Table 4.20: Univariate Analysis of Neutrophil Engraftment

Probability of neutrophil engraftment by day 30 in the 101 alloHSCTs according to transplant related variables. Median time to engraftment (days), probability of engraftment at 30 days (unless otherwise stated) and the 95% confidence intervals. * Compared to reference level (#).

Variable	Group	Median (Days)	Engraftment (%)	95% CI (%)	*P*	*P* *
Recipient Age	< Median	14	83.7	69.3 - 91.7	0.93	
	> Median	13	78.9	64.6 - 97.9		
Donor Age	< Median	12	78.6	66.7 - 86.6	0.65	
	> Median	14	87.1	67.4 - 95.3		
Diagnosis	Leukaemia[#]	13	84.8	72.3 - 91.9	**0.0006**	-
	Lympho'	13	93.1	70.6 - 98.5		**0.09**
	Other	20	38.5	13.0 - 63.9		**0.002**
HSC source	PBSC	13	83.5	74.3 - 89.6	**0.03**	
	BM	20	25.0	0.33 - 71.4		
Donor	Sibling	15	82.5	65.9 - 91.5	0.34	
	Unrelated	12	80.3	67.6 - 88.5		
Conditioning	RIC	13	81.2	71.0 - 88.1	0.14	
	MAC	21	81.3	47.6 - 94.4		
Alemtuzumab	Yes	12	81.0	68.6 - 88.8	0.18	
	No	15	81.6	64.3 - 91.1		
D to R Gender	F to M	16	77.8	48.0 - 91.8	0.21	
	Other	13	81.9	71.6 - 88.8		
HLA match	Matched	13	80.7	70.3 - 87.8	0.93	
	Mismatch	13	83.3	53.4 - 94.8		
Graft $CD34^+$ dose	Low	19	56.0	34.1 - 73.2	**0.0001**	
	High	13	89.5	79.6 - 94.7		
Graft $CD3^+$ dose	Low	13	68.0	45.0 - 83.0	0.14	
	High	13	85.5	75.1 - 91.8		
Graft $CD19^+$ dose	Low	13	72.0	48.8 - 86.1	0.53	
	High	13	84.2	73.6 - 90.8		
Graft $CD3^-$ $CD56^+$ dose	Low	13	80.0	56.8 - 91.6	0.66	
	High	13	81.6	70.6 - 88.8		

Table 4.21: Univariate Analysis of Platelet Engraftment

Probability of platelet engraftment by day 30 in the 101 alloHSCTs according to the transplant related variables. Median time to engraftment (days), probability of engraftment at 30 days and the 95% confidence intervals. * Compared to reference level (#).

C) MULTIVARIATE ANALYSIS

The variables examined in multivariate analysis of engraftment were the graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells, proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) and $CD34^+$ cell dose, the HSC source and patient's diagnosis.

In multivariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were associated with a lower probability of neutrophil engraftment by day 30 (HR 0.55 (0.33-0.94), *P*=0.03), although the absolute graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were no longer significant. Other significant variables were low graft $CD34^+$ cell doses (HR 0.41 (0.28-0.61), $P=8.7x10^{-6}$) and 'other' diagnoses (HR 0.41 (0.18-0.91), *P*=0.03 (compared to leukaemia)) (Table 4.22A).

For platelets, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft also remained significantly associated with poor platelet engraftment by day 30 (HR 0.55 (0.31-0.96), *P*=0.04). Low graft $CD34^+$ doses (HR 0.32 (0.19-0.50), $P=5.8x10^{-6}$) and 'other' diagnoses (HR 0.26 (0.11-0.59), *P*=0.002 (compared to leukaemia)) were also independent factors of poor platelet engraftment (Table 4.22B).

A. Neutrophil Engraftment

Variable	Group	HR	95% CI	*P*
Graft %Tregs	High	1	-	-
	Low	0.55	0.33 - 0.94	**0.03**
Graft $CD34^+$	High	1	-	-
	Low	0.41	0.28 - 0.61	**$8.7x10^{-6}$**
Diagnosis	Leukaemia	1	-	-
	Lympho'	1.23	0.78 - 1.93	0.37
	Other	0.41	0.18 - 0.91	**0.03**

B. Platelet Engraftment

Variable	Group	HR	95% CI	*P*
Graft %Tregs	High	1	-	-
	Low	0.55	0.31 - 0.96	**0.04**
Graft $CD34^+$	High	1	-	-
	Low	0.32	0.19 - 0.52	**$5.8x10^{-6}$**
Diagnosis	Leukaemia[#]	1	-	-
	Lympho'	1.37	0.88 - 2.14	0.16
	Other	0.26	0.11 - 0.59	**0.002**

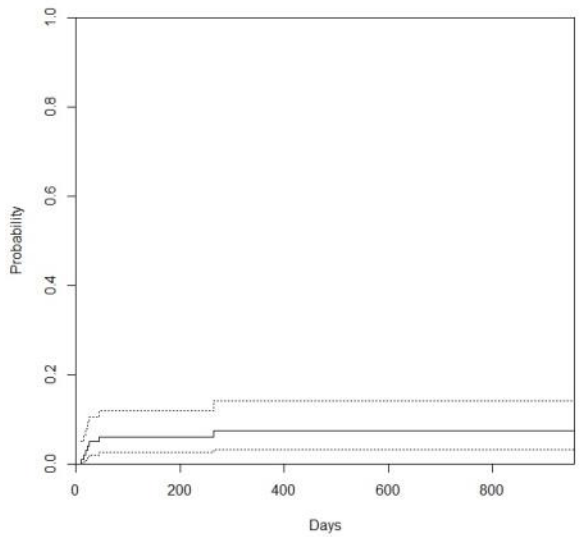
Table 4.22: Multivariate Analysis of Engraftment

Multivariate analysis of **(A)** Neutrophil engraftment; **(B)** Platelet engraftment in the 101 allogeneic HSCT. Hazard ratio (HR), 95% confidence interval (CI) and *P* values using the Fine and Gray regression model. '%Tregs' = $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells)

4.3.10 INFECTION

Delayed neutrophil engraftment after alloHSCT is associated with increased risk of infection, particularly fungal infections such as invasive aspergillosis (Mikulska *et al.,* 2009). In view of the findings above that low proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft are significantly associated with inferior neutrophil engraftment, it was hypothesised that low proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) may also be associated with increased risk of infection. Although in this project, the cumulative incidence of infective episodes, fever or antibiotic/antifungal usage was not specifically measured, the incidence of mortality from infection (all causes) was available. Therefore, the association between $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the graft and mortality from infection was investigated.

The overall incidence of mortality due to infection was 7.46% (3.22-14.1%) by one year post-transplant (Figure 4.24).



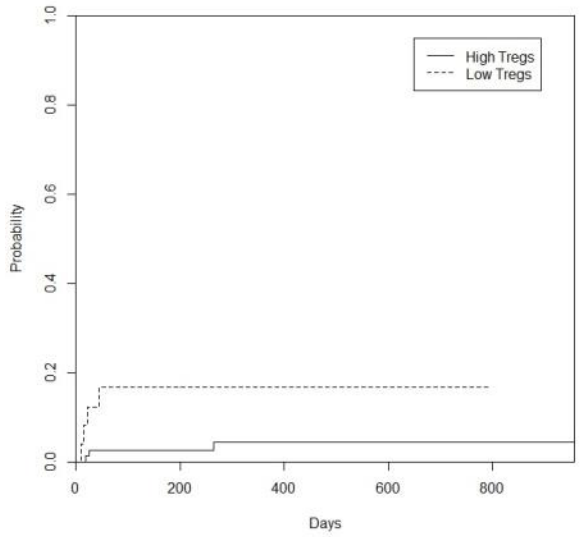
Outcome	Six Months		One Year	
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)
Death from infection	6.02	2.45 - 11.9	7.46	3.22 - 14.1

Figure 4.24: Mortality directly due to Infection (All Causes)

Cumulative incidence of mortality due to infection (all causes) in the 101 alloHSCTs. Dashed lines show the 95% confidence intervals (%).

A) GRAFT $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS

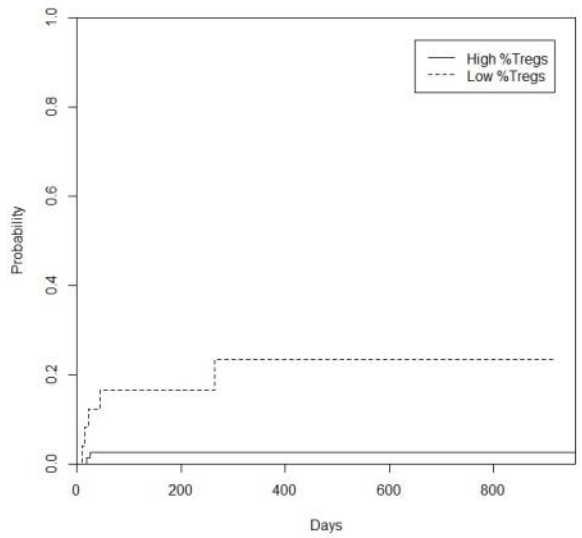
In univariate analysis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses in the graft were significantly associated with higher rates of mortality from infection at one year post-transplant (16.7% (5.05-34.3%) v 4.36% (1.12-11.2%, *P*=0.02) (Figure 4.25). Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were also significantly associated with a higher probability of death due to infection at one year post-transplant (23.4 % (7.53-44.2%) v 2.63% (0.49-8.28), *P*=0.002) (Figure 4.26).



Outcome	Low Tregs (N=25)		High Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
Death from infection	16.7	5.05 - 34.3	4.36	1.12 - 11.2	**0.02**

Figure 4.25: Mortality directly due to Infection According to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Dose (x10^6/kg) in the Graft

Cumulative incidence of mortality due to infection according to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose (x10^6/kg) in the graft. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Low Tregs <3.32x10^6/kg, High Tregs >3.32x10^6/kg. Values quoted are at one year post-transplant



Outcome	Low %Tregs (N=25)		High %Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
Septic death	23.4	7.53 - 44.2	2.63	0.49 - 8.28	**0.002**

Figure 4.26: Mortality directly due to Infection According to $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T Cells) in the Graft

Cumulative incidence of mortality due to infection according to the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft. '%Tregs' = $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells). Low %Tregs <2.16%, High %Tregs >2.16%. Values quoted are at one year post-transplant.

B) OTHER TRANSPLANT VARIABLES

Other transplant variables considered in univariate analysis of mortality from infection are shown below in Table 4.23. Of those variables analysed, only low $CD3^{-}CD56^{+}$ cell dose in the graft were also associated with significantly increased risk of mortality due to infection (22.7% (7.63-42.6%) v 2.63% (0.05-8.28%), *P*=0.003) (Table 4.23).

Variable	Group	Mortality (%)	95% CI (%)	*P*
Recipient Age	< Median	5.16	0.85 - 15.8	0.27
	> Median	9.81	3.56 - 19.9	
Donor Age	< Median	7.25	2.65 - 15.0	0.87
	> Median	8.12	1.24 - 23.7	
Stem cell source	PBSC	5.22	1.93 - 11.0	0.11
	BM	25.0 (day 107)	0.33 - 71.4	
Donor	Sibling	5.00	0.88 - 15.0	0.51
	Unrelated	9.27	3.26 - 19.2	
Conditioning	RIC	7.65	3.05 - 15.0	0.91
	MAC	6.25	0.36 - 25.5	
Alemtuzumab	Yes	8.92	3.15 - 18.5	0.57
	No	5.26	0.92 - 15.7	
D to R Gender	F to M	5.56	0.34 - 23.1	0.80
	Other	7.86	3.13 - 15.4	
HLA match	Matched	6.50	2.36 - 13.6	0.39
	Mismatch	11.1	1.73 - 30.4	
Graft $CD34^+$ dose	Low	12.0	2.91 - 28.0	0.25
	High	5.89	1.84 - 13.4	
Graft $CD3^+$ dose	Low	12.7	3.06 - 29.5	0.18
	High	5.62	1.78 - 12.8	
Graft $CD19^+$ dose	Low	12.7	3.06 - 29.5	0.22
	High	5.81	1.82 - 13.3	
Graft $CD3^-CD56^+$ dose	Low	22.7	7.63 - 42.6	**0.003**
	High	2.63	0.05 - 8.28	

Table 4.23: Univariate Analysis of Mortality due to Infection

Cumulative incidence of mortality due to infection in the 101 allogeneic HSCT according to transplant related variables. Probability of death (%) and 95% confidence intervals (%) are at one year unless otherwise stated.

C) MULTIVARIATE ANALYSIS

The transplant variables used in multivariate analysis of mortality due to infection were the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose, $CD3^-CD56^+$ cell dose and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft. Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) (HR 9.08 (1.89-43.8), *P*=0.006) and low $CD3^-CD56^+$ cell dose (HR 8.85 (1.73-45.2), *P*=0.009) in the graft were both independently associated with increased infective mortality within the first year (Table 4.24)

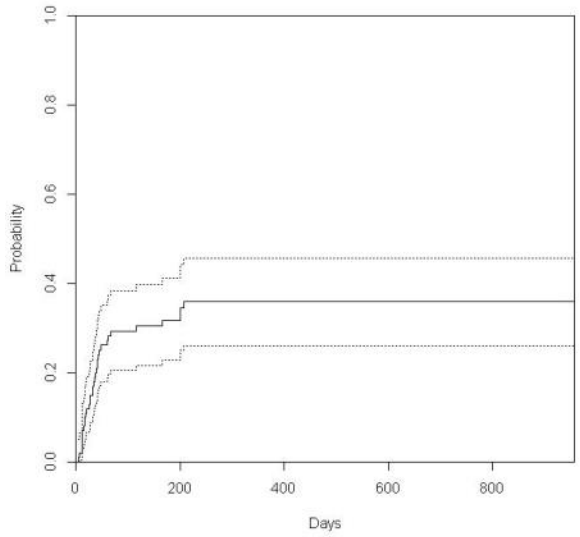
Variable	Group	HR	95% CI	*P*
HSC %Tregs	High	1		
	Low	9.08	1.89 - 43.8	**0.006**
HSC $CD3^-CD56^+$	High	1		
	Low	8.85	1.73 - 45.2	**0.009**

Table 4.24: Multivariate Analysis of Mortality due to Infection

Multivariate analysis of mortality due to infection in the 101 allogeneic HSCT. Hazard ratios (HR), 95% confidence interval (CI) and *P* values were calculated using the Fine and Gray regression model. '%Tregs' = $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells)

4.3.11 CMV

Finally, the effect of graft $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells on CMV reactivation was assessed. CMV DNA in the peripheral blood (>1000 copies/ml) was detected in 34 transplants with a median time to positive CMV PCR being 34 days (4-208). The probability of CMV activation for all transplants was 35.8% (26.1-45.7%) at one year (Figure 4.27).



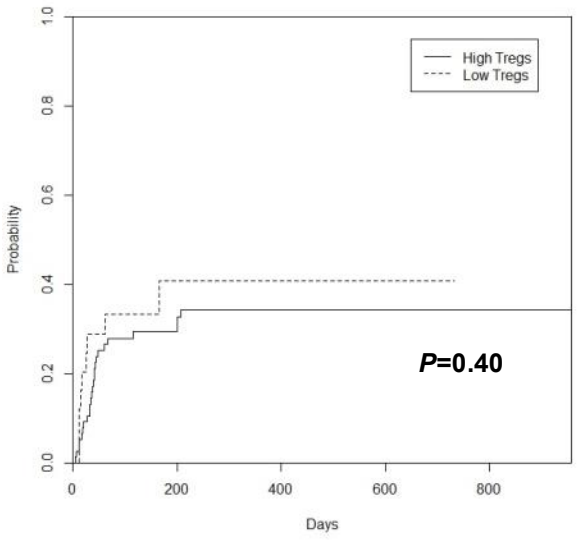
Outcome	Six Months		One Year	
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)
CMV	31.8	22.7 - 41.2	35.8	26.1 - 45.7

Figure 4.27: CMV Reactivation

Cumulative incidence of CMV reactivation in the 101 allogeneic HSCT. Dashed lines show the 95% confidence intervals (%).

A) GRAFT CD4+CD25+FOXP3+CD127-/DIM T CELLS

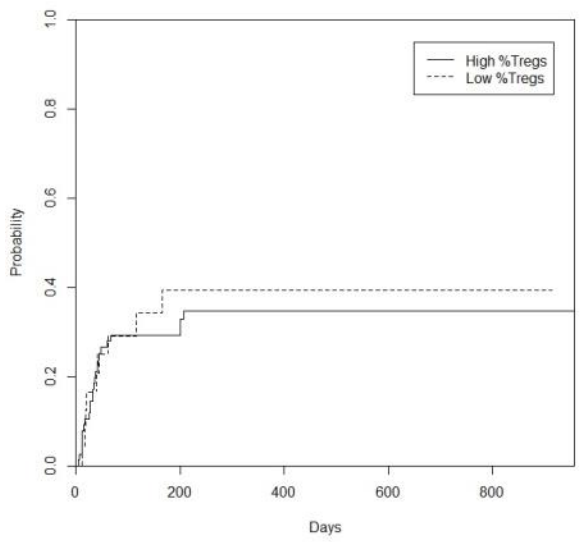
In univariate analysis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft were not associated with a significant difference in the incidence of CMV reactivation (Figures 4.28 & 4.29).



Outcome	Low Tregs (N=25)		High Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
CMV	40.9	18.6 - 62.1	34.2	23.4 - 45.3	0.40

Figure 4.28: CMV Reactivation According to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Dose ($x10^6$/kg) in the Graft

Cumulative incidence of CMV reactivation according to the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose ($x10^6$/kg) in the graft. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Low Tregs <3.32x10^6/kg, High Tregs >3.32x10^6/kg. Values quoted are at one year post-transplant.



Outcome	Low %Tregs (N=25)		High %Tregs (N=76)		*P*
	Incidence (%)	95% CI (%)	Incidence (%)	95% CI (%)	
CMV	39.3	19.0 - 59.2	34.6	23.7 - 45.9	0.74

Figure 4.29: CMV Reactivation According to $CD25^{+}FOXP3^{+}CD127^{-/dim}$ Cells (%$CD4^{+}$ T Cells) in the Graft

Cumulative incidence curve of CMV activation according to the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft. '%Tregs' = $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells). Low %Tregs <2.16%, High %Tregs >2.16%. Values quoted are at one year post-transplant.

B) OTHER TRANSPLANT VARIABLES

Transplant variables considered in univariate analysis of CMV reactivation are shown in Table 4.25. As expected, previous recipient and donor exposure to CMV were significantly associated with higher rates of CMV reactivation (72.7% (54.7-84.5) v 5.66% (1.4-14.2), $P=5.4x10^{-11}$ and 57.2% (36.2-73.6%) v 25.9% (15.9-37.2%), $P=0.003$ respectively). Low $CD3^{-}CD56^{-}$ cell dose in the graft also showed a non-significant trend towards a lower incidence of CMV reactivation (17.7% (5.1-36.4) v 41.3% (29.6-52.6), $P=0.06$) (Table 4.25).

Variable	Group	CMV (%)	95% CI (%)	*P*
Recipient Age	< Median	35.2	21.2 - 49.4	0.84
	> Median	36.7	23.2 - 50.3	
Donor Age	< Median	35.0	23.4 - 46.8	0.87
	> Median	37.7	20.1 - 55.2	
Recipient CMV IgG	Neg	5.66	1.40 - 14.2	**$5.4x10^{-11}$**
	Pos	72.7	54.7 - 84.5	
Donor CMV IgG	Neg	25.9	15.9 - 37.2	**0.003**
	Pos	57.2	36.2 - 73.6	
HSC source	PBSC	26.2	17.8 - 35.3	0.96
	BM	25.0 (day 63)	0.33 - 71.4	
Donor	Sibling	30.9	16.3 - 46.7	0.14
	Unrelated	38.4	26.1 - 50.6	
Conditioning	RIC	36.5	25.8 - 47.3	0.89
	MAC	31.3	10.7 - 54.6	
Alemtuzumab	Yes	38.8	26.6 - 50.8	0.10
	No	30.0	15.3 - 46.3	
D to R Gender	F to M	55.6	23.2 - 79.0	0.41
	Other	32.4	22.4 - 42.9	
HLA match	Matched	34.8	24.2 - 45.7	0.57
	Mismatch	38.9	16.6 - 60.8	
Graft $CD34^+$ dose	Low	48.4	25.4 - 68.1	0.21
	High	31.8	21.3 - 42.9	
Graft $CD3^+$ dose	Low	41.3	21.5 - 60.2	0.12
	High	33.0	22.3 - 44.0	
Graft $CD19^+$ dose	Low	39.3	18.7 - 59.4	0.60
	High	34.6	23.7 - 45.8	
Graft $CD3^-CD56^+$ dose	Low	17.7	5.10 - 36.4	**0.06**
	High	41.3	29.6 - 52.6	

Table 4.25: Univariate Analysis of CMV Reactivation

Cumulative incidence of CMV reactivation in the 101 alloHSCT according to transplant related variables. Cumulative incidence (%) and 95% confidence intervals (%) at one year unless otherwise stated.

C) MULTIVARIATE ANALYSIS

The variables used in multivariate analysis of CMV activation were the graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), $CD3^-CD56^+$ cell dose and the recipient and donor CMV IgG serology. Neither the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose nor the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) within the grafts were significantly associated with CMV disease. Only previous recipient exposure to CMV (positive CMV IgG serology) was independently associated with significantly higher rates of CMV reactivation (HR 19.1 (5.76-63.5), P=1.4x10^{-6}) (Table 4.26).

Variable	Group	HR	95% CI	*P*
Recipient CMV	Negative	1	-	-
IgG Serology	Positive	19.1	5.76 - 63.5	**1.4x10^{-6}**

Table 4.26: Multivariate Analysis of CMV Reactivation

Multivariate analysis of CMV reactivation in 101 alloHSCT. Hazard ratios (HR), 95% confidence interval (CI) and *P* values were calculated using the Fine and Gray regression model.

Taken together, these data suggest that the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft is an independent predictor of neutrophil engraftment and mortality due to infection. Furthermore, this observation was independent of the $CD34^+$ dose in the graft which is already known to be an independent predictor of neutrophil engraftment (Kamel *et al.,* 2005; Specchia *et al.,* 2006; Pastore *et al.,* 2008). However, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the graft do not predict the occurrence of CMV reactivation. In light of these findings, it may be hypothesised that $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells within the graft are directly or indirectly involved with HSC engraftment. In keeping with this hypothesis, mouse $FOXP3^+$ Tregs have been shown to co-localise with HSC on the endosteal surface of bone, possibly forming an immune privileged site for HSC engraftment (Fujisaki *et al.,* 2011).

4.4 CONCLUSIONS

In this project, a prospective, observational clinical study was performed to examine the cellular composition of HSC grafts and investigate whether low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cells in the grafts ($x10^6$/kg and $\%CD4^+$ T cells) were associated with higher rates of GvHD and other transplant related events following alloHSCT. The main conclusions from this chapter are:

- The median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose in the HSC grafts was $4.60x10^6$/kg ($0.20\text{-}20.6x10^6$/kg), representing 2.94% (0.81-8.56%) of the $CD4^+$ T cells.
- Absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell, $CD34^+$, $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cell doses were significantly lower in BM harvests than PBSCH. However, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) was not significantly different between the two sources.
- $CD4^+CD25^+CD127^{-/dim}$ T cells within the HSC grafts were functional as demonstrated by *in vitro* suppression assays.

- The incidence of acute GvHD (grade II-IV) was 28.7% at day 100 and 40.2% at one year post-transplant using the Consensus Conference and NIH Consensus classifications of GvHD (Przepiorka *et al.,* 1995; Filipovich *et al.,* 2005).
- Single organ aGvHD was most common with skin being the most frequently affected site.
- In contrast to the original hypothesis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) in the HSC grafts were not associated with increased aGvHD.

- The incidence of chronic GvHD was 74.7% and 42.0% at one year using the Shulman criteria and NIH Consensus classification of cGvHD (Przepiorka *et al.,* 1995; Filipovich *et al.,* 2005).
- All cases of cGvHD commenced within the first year post-transplant.
- Multiple organ cGvHD was most common with skin being the most frequently affected site.

- As with aGvHD, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were not associated with increased cGvHD.

- The incidence of relapse, NRM and OS at one year was 21.6%, 23.5% and 61.6% respectively.
- In multivariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were significantly associated with higher NRM (HR 2.57 (1.05-6.29), *P*=0.04) and inferior OS (HR 2.58 (1.24-5.34), *P*=0.01) at one year. This association was independent of low $CD34^+$ doses in the graft, which were also associated with increased NRM (HR 3.78 (1.52-9.40), *P*=0.004). It was also independent of low $CD3^-CD56^+$ cell doses which were associated with significantly higher NRM (HR 3.78 (1.52-9.40), *P*=0.004) and worse OS (HR 3.08 (1.47-6.46), *P*=0.003).
- In keeping with previous mouse and human studies, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were not associated with increased relapse (Edinger *et al.,* 2003; Wolf *et al.,* 2007).

- The probability of neutrophil and platelet engraftment by day 30 was 91.1% and 91.2%.
- In multivariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were significantly associated with lower neutrophil and platelet engraftment by day 30 (HR 0.55 (0.32-0.94), *P*=0.03 and 0.55 (0.31-0.96), *P*=0.04 respectively). This association was independent of low $CD34^+$ dose in the graft, which was also associated with inferior neutrophil and platelet engraftment (HR 0.41 (0.28-0.61), $P=8.7\times10^{-6}$ and 0.32 (0.19-0.52), $P=5.8\times10^{-6}$).
- Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were significantly associated with increased mortality due to infection (HR 9.08 (1.89-43.8), *P*=0.006). This was independent of the $CD3^-CD56^+$ cell dose in the graft, which was also associated with higher mortality from infection (HR 8.85 (1.73-45.2), *P*=0.009).

- Low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were not associated with an increased incidence of CMV infection.

Although no effect was seen with GvHD, these novel findings do support the hypothesis that $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells within the graft are significantly associated with alloHSCT outcomes, particularly engraftment, infection and mortality. Increasing the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at the time of transplantation may be a promising strategy to improve transplant outcomes.

Chapter 5

Regulatory T-cell Reconstitution post Allogeneic Haematopoietic Stem Cell Transplantation

5. REGULATORY T-CELL RECONSTITUTION POST ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

5.1 INTRODUCTION

Reconstitution of the new donor-derived immune system post-alloHSCT is essential to protect the recipient from life-threatening infections. Failed engraftment and delayed neutrophil recovery are associated with increased bacterial and fungal infections (Mikulska *et al.,* 2009). Similarly, low T cells, CMV-specific $CD8^+$ T cells and $CD56^+$ NK cells post-transplant are all associated with increased risk of CMV reactivation (Pastore *et al.,* 2011; Torelli *et al.,* 2011).

The dynamics of immune reconstitution are also critical for the development of GvHD and the GvT response. Donor-derived $CD4^+$ and $CD8^+$ T cells have been implicated in the pathogenesis of GvHD and it has been hypothesised that a deficiency in $CD4^+CD25^+FOXP3^+$ Tregs during immune reconstitution may cause GvHD (Matthews *et al.,* 2009; Pastore *et al.,* 2012). In mouse transplant models, depletion of $CD4^+CD25^+$ T cells produced life-threating GvHD (Hoffmann *et al.,* 2002; Taylor *et al.,* 2002). Likewise, in humans, $FOXP3^+$ cells in the peripheral blood (PB) and tissues are possibly reduced in GvHD (Rezvani *et al.,* 2006, Rieger *et al.,* 2006; Magenau *et al.,* 2010).

In this chapter, cellular immune reconstitution will be studied by analysing PB counts for twelve months post-transplant in the 101 alloHSCT recruited into the clinical study in Chapter 4. Using the assays developed in Chapter 3, leucocyte (neutrophil, lymphocyte, monocyte, eosinophil and basophil), lymphocyte (T-cell, $CD4^+$ T-cell, $CD8^+$ T-cell, $CD19^+$ cell and $CD3^-CD56^+$ cell) and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell (putative Treg) counts (absolute counts ($x10^6$/l) and $\%CD4^+$ T cells) will be measured post-alloHSCT. This chapter will describe the pattern and kinetics of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell reconstitution and in particular, will test the hypotheses that low and/or falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the PB in the first few months post-

alloHSCT are associated with increased risk of GvHD. It will also test the hypothesis that $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the PB are lower at the time of active GvHD symptoms.

Understanding which transplant factors influence Treg reconstitution post-alloHSCT may be important when designing future conditioning regimens and transplant protocols. Therefore, this chapter will perform exploratory univariate and multivariate analysis of which pre-, peri- and post-transplant variables are significantly associated with low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells post-transplant. In view of the previous findings that low proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft are associated with higher NRM and inferior OS (Chapter 4), this chapter will also test the hypothesis that low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the PB are associated with these events. It will also examine the relationship between PB $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and the incidence of CMV infection and disease relapse.

Finally, this work will test whether it is possible to measure putative Tregs post-alloHSCT using the MSRT-qPCR assay developed in Chapter 3.6. It will assess the correlation between the results obtained from the flow cytometry and MSRT-qPCR assays. Since FOXP3 may be transiently expressed in activated T cells, it will also test the hypothesis that detecting FOXP3 expression in human $CD4^{+}$ T cells post-alloHSCT may over-estimate putative Treg numbers, particularly when GvHD is present.

5.2 SPECIFIC METHODS

5.2.1 CLINICAL STUDY

The clinical study, transplant outcomes, sample and data collection were performed as per Methods 2.1.

5.2.2 MEASURING LEUCOCYTE COUNTS AND LYMPHOCYTE POPULATIONS

Blood samples (10ml in EDTA) were collected from the study participants at 1, 2, 3, 4, 5, 6, 9 and 12 months post-transplant. A FBC and leucocyte differential were obtained from an automated analyser (Sysmex XE2100). $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and lymphocyte populations were analysed using the flow cytometry assays described in Chapter 3.3-3.4. $CD3^{+}$ cells and $FOXP3^{+}$ cells were also measured using the MSRT-qPCR assay described in Chapter 3.6.

5.2.3 STATISTICS

Statistics were analysed using the principles described in Methods 2.5. For immune reconstitution, each cell subtype is presented as the median count, the range and coefficient of variation. Box plots show the median, interquartile range and 5^{th} and 95^{th} centiles. Multivariate linear regression of post-transplant counts were analysed as per Methods 2.5.1. Univariate analysis for GvHD, CMV reactivation, relapse and NRM were analysed using cumulative incidence with competing risks. Multivariate analysis was performed using the Fine and Gray method. Univariate analysis of OS was analysed using Kaplan-Meier estimation. Multivariate analysis was tested by Cox Proportional Hazards.

5.3.1 IMMUNE RECONSTITUTION POST-TRANSPLANT

A) SAMPLE COLLECTION AND PROCESSING

During the course of this study (1st December 2008 until 31st August 2011), 589 post-alloHSCT blood samples were collected and processed as described in Methods 2.2. The data from 61 blood samples was excluded from analysis as the samples were not collected from any of the 101 transplants with graft data. An additional 60 samples were excluded from analysis as they were collected beyond one year post-transplant (N=23), were duplicated samples (N=22) or involved laboratory processing errors (N=15). From the 101 alloHSCT being followed, 468 of a potential 515 samples (90.9%) were collected and analysed (37 missed samples, 10 processing errors) (Table 5.1).

Month	Samples Collected			Insufficient follow-up	Death / 2nd HSCT	Potential Samples	Missed /Error
	N	Median (day)	Range (day)	N	N	N	N
1	91	34	24-48	1	7	93	1 / 1
2	82	61	54-76	4	12	84	2 / 1
3	67	89	82-110	10	15	76	7 / 2
4	55	124	117-138	16	16	69	11 / 3
5	52	153	145-167	19	22	60	6 / 2
6	50	181	173-215	21	24	56	5 / 1
9	40	272	264-300	32	25	44	4 / 0
12	31	369	348-399	45	24	32	1 / 0
Total	**468**	**-**	**-**	**148**	**145**	**515**	**37 /10**

Table 5.1: Peripheral Blood Samples Collected Post-Allogeneic HSCT

Summary of the blood samples collected and missed by month of follow-up post-alloHSCT. The number of potential samples fell with successive months as either the follow up was insufficient when the study was censored on 31st August 2011 or the patient had died/been re-transplanted.

Using these samples, a preliminary evaluation of leucocyte reconstitution for all the alloHSCT was performed. A more detailed description of lymphocyte and $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell reconstitution will then be provided, taking into account the effect of Alemtuzumab conditioning.

B) LEUCOCYTE RECONSTITUTION (ALL TRANSPLANTS)

To analyse the overall pattern of leucocyte reconstitution in this patient cohort, the total leucocyte count and white cell differential were measured on day zero and at one, two, three, four, five, six, nine and twelve months post-transplant (Table 5.2 & Figure 5.1). At each month, there was wide variation in the counts for all leucocyte subtypes, with coefficients of variation ranging for 0.39 to 2.97. For most leucocyte counts, the widest variability occurred early post-transplant, greatest at one month for total leucocytes, neutrophils, lymphocytes, basophils and at three months for monocytes. However, eosinophil counts also showed wide variation throughout the first year post-transplant with a CV of 2.97 at one year.

Taking the entire study population as a single group, the median leucocyte and neutrophil counts initially increased, falling within the normal healthy control range at one month post-transplant. However, both median counts were subsequently lower for the next three to four months before gradually increasing again at five and six months respectively. In general, lymphocytes showed a much slower recovery with the median lymphocyte count progressively increasing throughout the first year post-transplant. The median lymphocyte count did not reach the normal range until nine months and still remained within the bottom quartile of the normal range at one year post-transplant. The median monocyte, eosinophil and basophil counts all increased within the first month post-transplant to within their normal ranges. However, all three median counts were lower again in the second month, although still remaining within the normal range. The median monocyte and eosinophil counts gradually increased over the next nine months while the median basophil count was relatively static for the first year.

Table 5.2: Peripheral blood leucocyte counts post-allogeneic HSCT (Each box shows the median, range and coefficient of variation)

	Time post-transplant (months)									
	Control	**Zero**	**One**	**Two**	**Three**	**Four**	**Five**	**Six**	**Nine**	**Twelve**
Cells	N = 12	N = 101	N = 91	N = 82	N = 67	N = 55	N = 52	N = 50	N = 40	N = 31
WCC	6.35	3.11	5.34	4.34	4.43	4.37	4.60	5.08	5.84	5.79
	4.52 – 7.78	0.02 – 12.7	0.05 – 34.0	0.17 - 25.7	0.19 – 16.0	0.76 - 10.8	0.63 - 13.5	0.92 - 13.3	0.34 - 12.3	2.88 - 14.3
	0.17	0.80	0.88	0.74	0.56	0.51	0.54	0.46	0..48	0.39
Neut	3.57	2.51	3.22	2.66	2.66	2.51	2.44	2.90	3.32	3.11
	2.69 – 5.12	0.00 – 12.2	0.00 - 25.6	0.10 - 16.6	0.12 - 11.3	0.07 - 8.99	0.39 - 12.3	0.60 - 10. 2	0.05 - 10.8	(1.77 - 7.69
	0.19	0.86	1.02	0.87	0.63	0.68	0.77	0.64	67%	0.44
Lym	2.11	0.04	0.58	0.70	0.79	0.88	0.95	1.04	1.09	1.30
	1.07 – 2.62	0.00 – 2.87	0.01 - 5.77	0.05 - 4.63	0.05 - 5.76	0.14 - 2.79	0.06 - 2.74	0.13 - 4.06	0.08 - 3.78	0.35 - 4.04
	0.27	2.73	1.06	0.85	0.84	0.64	0.60	0.71	0.69	0.58
Mon	0.45	0.05	0.55	0.42	0.42	0.39	0.44	0.48	0.50	0.48
	0.26 – 0.75	0.00 – 2.11	0.00 - 4.55	0.01 - 4.30	0.02 - 6.09	0.04 - 1.07	0.06 - 1.19	0.00 - 1.48	0.05 - 1.09	0.06 - 1.47
	0.31	2.10	1.06	1.06	1.35	0.53	0.47	0.54	0.47	0.56
Eos	0.14	0.00	0.15	0.09	0.10	0.11	0.13	0.16	0.18	0.13
	0.06 – 0.46	0.00 – 0.78	0.00 - 3.46	0.00 - 2.16	0.00 - 0.59	0.04 - 0.84	0.00 - 6.79	0.00 - 1.66	0.00 - 3.22	0.00 - 5.82
	0.71	5.06	1.58	1.55	1.00	1.04	2.42	1.27	1.87	2.97
Bas	0.03	0.00	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	0.01 – 0.05	0..00 – 0.27	0.00 - 0.43	0.00 - 0.07	0.00 - 0.09	0.00 - 0.08	0.00 - 0.04	0.00 - 0.07	0.00 - 0.08	0.00 - 0.06
	0.43	5.54	1.74	0.86	1.11	0.91	0.82	0.89	0.95	0.77

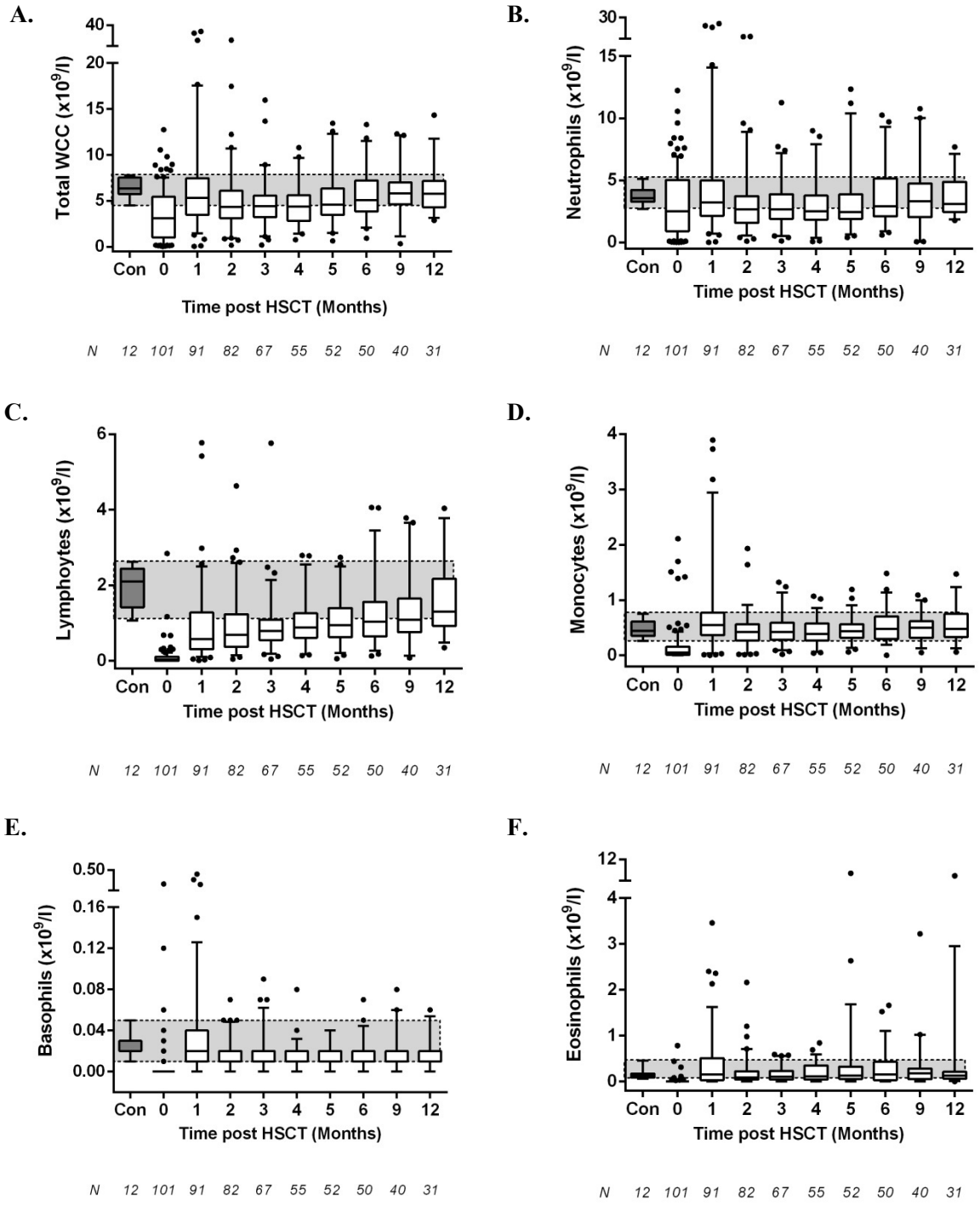


Figure 5.1: Peripheral Blood Leucocyte Reconstitution Post-Allogeneic HSCT

Total leucocyte counts and differentials were measured in 569 peripheral blood samples, collected at 0, 1, 2, 3, 4, 5, 6, 9 and 12 months post-alloHSCT. Blood samples were collected in EDTA and analysed using an automated Sysmex FBC analyser. Box-and-whiskers plots of **(A)** Total white cell count; **(B)** Neutrophil count; **(C)** Lymphocyte count; **(D)** Monocyte count; **(E)** Eosinophil count; **(F)** Basophil count. *N* = number of blood samples analysed at each time; Grey boxes show the normal range measured in EDTA blood samples from 12 healthy volunteer donors (Con).

C) LYMPHOCYTE RECONSTITUTION (BY TRANSPLANT TYPE)

Although the analysis above provides a simple description of haemopoietic reconstitution post-alloHSCT, the heterogeneity of the transplants involved in this study should be taken into consideration. As the main focus of this project is $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell reconstitution, lymphocyte reconstitution and analysis of the lymphocyte subpopulations was assessed in more detail. However, further analysis of the other main leucocyte groups will not be performed within this project.

In preliminary analysis, the effect of the main transplant conditioning variables, i.e. the intensity of the conditioning, Alemtuzumab and the source of the HSC, on the lymphocyte counts was assessed at one, three and six months post-transplant (Table 5.3 & Figure 5.2). In univariate analysis, transplants using Alemtuzumab conditioning had significantly lower lymphocyte counts at one ($0.34x10^9$/l ($0.26-0.44x10^9$/l) v $1.33x10^9$/l ($1.06-1.66x10^9$/l), $P<0.0001$) and three months ($0.58x10^9$/l ($0.46-0.73x10^9$/l) v $1.08x10^9$/l ($0.85-1.37x10^9$/l), $P=0.0007$), although there was no significant difference observed by six months. In addition, RIC ($0.52x10^9$/l ($0.41-0.66x10^9$/l) v $1.05x10^9$/l ($0.61-1.83x10^9$/l), $P=0.02$) and BM harvests ($0.17x10^9$/l ($0.0.01-2.23x10^9$/l) v $0.61x10^9$/l ($0.49-0.76x10^9$/l), $P=0.04$) were both associated with significantly lower lymphocyte counts at one month although there was no significant difference at three and six months (Table 5.3). Using multivariate linear regression, all three variables remained significant at one month (Table 5.4). Taken together, these three variables explained a considerable proportion of the variation in the lymphocyte counts early post-transplant ($R^2=0.46$).

Time (Months)	Variable		*N*	Mean ($x10^9$/l)	95% CI ($x10^9$/l)	*P*
One	Intensity	MAC	14	1.05	0.61 - 1.83	**0.02**
		RIC	77	0.52	0.41 - 0.66	
	Alemtuzumab	No	36	1.33	1.06 - 1.66	**<0.0001**
		Yes	55	0.34	0.26 - 0.44	
	Source HSC	BM	3	0.17	0.01 - 2.23	**0.04**
		PBSCH	88	0.61	0.49 - 0.76	
Three	Intensity	MAC	11	0.62	0.40 - 0.96	0.46
		RIC	56	0.74	0.61 - 0.91	
	Alemtuzumab	No	24	1.08	0.85 - 1.37	**0.0007**
		Yes	43	0.58	0.46 - 0.73	
	Source HSC	BM	1	-	-	-
		PBSCH	66	-	-	
Six	Intensity	MAC	7	1.26	0.66 - 2.40	0.36
		RIC	43	0.96	0.77 - 1.20	
	Alemtuzumab	No	20	1.03	0.70 - 1.52	0.80
		Yes	30	1.26	0.77 - 1.25	
	Source HSC	BM	0	-	-	-
		PBSCH	50	-	-	

Table 5.3: Univariate Analysis of the Peripheral Blood Lymphocyte Counts Post-Allogeneic HSCT

Peripheral blood lymphocyte counts were measured in 91, 67 and 50 alloHSCT at one, three and six months post-transplant respectively. Blood samples were collected in EDTA and analysed using an automated Sysmex FBC analyser. Table shows the mean lymphocyte count and 95% confidence intervals ($x10^9$/l) according to the intensity of the transplant conditioning, *in vivo* T-cell depletion with Alemtuzumab and the source of the HSC at each time point. Counts were normalised using $\log_{10}$ transformation and analysed using independent t-test. Table shows back-transformed data.

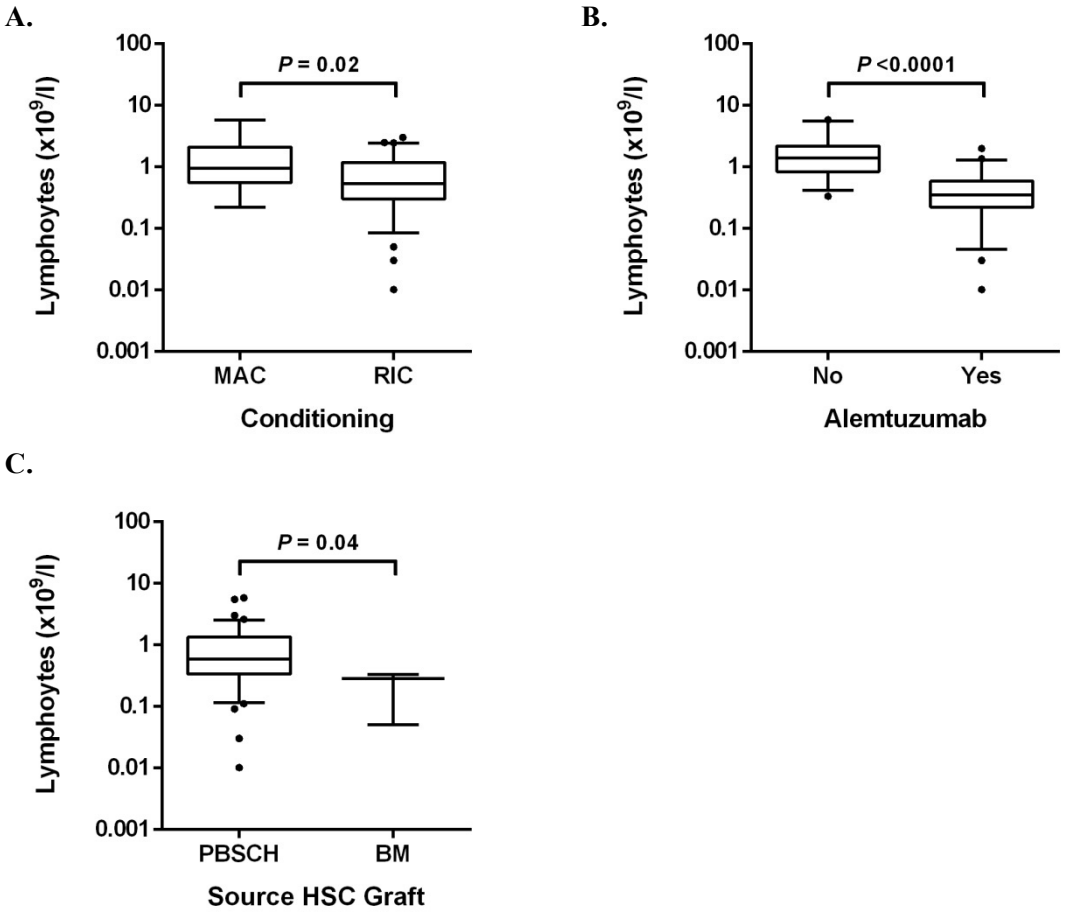


Figure 5.2: One Month Post-Transplant Peripheral Blood Lymphocyte Counts

Peripheral blood lymphocyte counts were measured in 91 alloHSCT at one month post-transplant. Blood samples were collected in EDTA and analysed using an automated Sysmex FBC analyser. Box-and-whiskers plots comparing the PB lymphocyte counts according to **(A)** Intensity of transplant conditioning; **(B)** Alemtuzumab conditioning; **(C)** Source of HSC grafts. Data were initially normalised using a log10 transformation and analysed using independent t-test.

Variable	B	SE B	β	P	R^2
Constant	6.09	0.06			**0.46**
Intensity (MAC)	0.22	0.10	0.17	**0.04**	
Alemtuzumab (Yes)	-0.56	0.08	-0.72	**<0.0001**	
Source (BM)	-0.57	0.21	-0.22	**0.007**	

Table 5.4: Multivariate Linear Regression of the Variables Associated with the One Month Post-Transplant Peripheral Blood Lymphocyte Counts

Peripheral blood lymphocyte counts were measured in 91 alloHSCT at one month post-transplant. Blood samples were collected in EDTA and analysed using an automated Sysmex FBC analyser. Multivariate linear regression was performed to ascertain which transplant variables are independently associated with the lymphocyte count at one month post-transplant. Variables considered in this analysis were intensity of conditioning, *in vivo* T-cell depletion with Alemtuzumab and the source of the HSC (all significant ($P<0.05$) in univariate analysis). Data were transformed using a $\log_{10}$ transformation. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value. Data shown are the transformed data ($\log_{10}$).

D) LYMPHOCYTE RECONSTITUTION

In view of the significant difference in the total lymphocyte counts in the first three months between patients treated with and without Alemtuzumab, the reconstitution of $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cells in the PB was analysed in these two groups separately (Tables 5.5-5.7).

In the non-Alemtuzumab transplants, $CD3^+$ counts initially recovered quickly, with the median $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ counts all being within the normal range by one month post-transplant (Figure 5.3A & 5.4A&C). However, beyond one month, the median $CD3^+CD4^+$ count fell and remained relatively low until twelve months (Figure 5.4A). In contrast, the median $CD3^+CD8^+$ count rose steadily, being within or above the normal range for the entire first year (Figure 5.4C). $CD19^+$ cells recovered slowly, only reaching the normal range at nine months (Figure 5.3C). However, $CD3^-CD56^+$ cells recovered early and the median $CD3^-CD56^+$ count was within the normal range by one month post-transplant. The median count then fell over the next two months but still remained above the lower end of the normal range (Figure 5.3E).

As expected from the multivariate analysis above, the Alemtuzumab-conditioned patients showed a substantially different pattern of lymphocyte reconstitution. In this group, $CD3^+$ cells recovered slowly, gradually increasing over the first nine months post-transplant. Although the median $CD3^+CD8^+$ count reached the normal range by six months, the median $CD3^+$ and $CD3^+CD4^+$ counts remained below the normal range for the entire first year (Figure 5.3B & 5.4B&D). $CD19^+$ cells recovered faster, increasing throughout the first year post-transplant and reaching the normal range by three months (Figure 5.3D). $CD3^-CD56^+$ cells also recovered rapidly, with the median $CD3^-CD56^+$ count being within the normal range from one month onwards (Figure 5.3F). In both groups, the $CD3^+CD4^+$ to $CD3^+CD8^+$ ratios remained below the normal range for most of the first year (Figure 5.4E-F).

In summary, these data suggest that T ($CD3^+$), B ($CD19^+$) and NK ($CD3^-CD56^+$) cell reconstitution post-alloHSCT takes many months and patients may remain immunocompromised for long periods. Furthermore, Alemtuzumab conditioning significantly impairs T-cell reconstitution, particular $CD3^+CD4^+$ cells, and may delay recovery of T-cell mediated immunity.

Table 5.5: Peripheral blood $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cell counts post-allogeneic HSCT (No Alemtuzumab conditioning) (Each box shows the median, range and coefficient of variation)

	Time post-transplant (months)								
	Control	**One**	**Two**	**Three**	**Four**	**Five**	**Six**	**Nine**	**Twelve**
Cells	N = 8	N = 36	N = 31	N = 24	N = 18	N = 20	N = 20	N = 17	N = 13
$CD3^+$	1.38	0.89	0.77	0.84	0.81	0.78	0.75	0.79	1.12
	0.67 – 1.60	0.22 – 4.24	0.23 – 3.90	0.29 – 2.94	0.10 – 1.95	0.04 – 2.20	0.08 – 3.43	0.04 – 2.97	0.51 – 2.89
	0.24	0.81	0.75	0.92	0.64	67.4	0.86	0.83	0.55
$CD3^+CD4^+$	0.93	0.49	0..34	0.30	0.35	0.32	0.36	0.34	0.46
	0.38 – 1.15	0.05 - 2.78	0.04 - 1.16	0.05 - 1.17	0.07 - 0.67	0.03 - 1.14	0.05 - 1.56	0.03 - 0.77	0.29 - 1.17
	0.28	0.87	0.56	0.61	0.50	0.69	0.90	0.60	0.48
$CD3^+CD8^+$	0.35	0.26	0.27	0.29	0.39	0.27	0.37	0.30	0.65
	0.20 – 0.46	0.07 - 1.82	0.04 - 3.23	0.07 - 3.55	0.03 – 1.20	0.01 - 1.59	0.02 - 1.99	0.01 - 2.05	0.15 - 1.82
	0.28	0.89	1.18	1.36	0.82	1.00	1.02	1.05	0.72
CD4:CD8	2.75	1.57	1.24	1.04	0.70	0.92	0.87	1.12	1.02
	1.60 - 3.80	0.42 – 8.97	0.16 – 5.23	0.19 – 5.81	0.36 – 5.63	0.22 – 9.21	0.22 – 4.23	0.20 – 2.77	0.44 – 2.25
	0.32	0.76	0.87	0.95	1.06	1.37	0.84	0.70	0.50
$CD19^+$	0.30	0.01	0.04	0.03	0.05	0.06	0.09	0.18	0.20
	0.12 - 0.52	0.00 – 0.94	0.00 – 0.28	0.00 – 0.58	0.00 – 0.36	0.00 – 0.35	0.00 – 2.06	0.00 – 0.70	0.00 – 1.01
	0.43	2.79	1.29	1.99	1.33	1.18	2.53	1.03	1.07
$CD3^-CD56^+$	0.24	0.33	0.29	0.21	0.19	0.17	0.18	0.22	0.30
	0.11 – 0.65	0.11 – 1.18	0.06 – 0.69	0.03 – 0.87	0.04 – 0.54	0.01 – 0.52	0.04 – 0.61	0.03 – 0.47	0.05 – 0.54
	0.61	0.66	0.49	0.78	0.67	0.66	0.68	0.58	0.60

Table 5.6: Peripheral blood $CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cell counts post-allogeneic HSCT (Alemtuzumab conditioning) (Each box shows the median, range and coefficient of variation)

	Time post-transplant (months)								
	Control	**One**	**Two**	**Three**	**Four**	**Five**	**Six**	**Nine**	**Twelve**
Cells	N= 8	N = 55	N = 51	N = 43	N = 37	N = 32	N = 30	N = 23	N = 18
$CD3^+$	1.38	0.08	0.12	0.19	0.37	0.41	0.57	0.42	0.54
	0.67 – 1.60	0.00 – 1.13	0.00 – 1.56	0.02 – 1.49	0.01 – 2.05	0.02 – 1.85	0.11 – 2.20	0.09 – 1.97	0.16 – 1.62
	0.24	1.37	1.17	0.99	0.91	0.87	0.89	0.80	0.66
$CD3^+CD4^+$	0.93	0.06	0.09	0.10	0.14	0.17	0.21	0.22	0.22
	0.38 – 1.15	0.00 – 0.22	0.00 – 0.60	0.01 – 0.55	0.01 - 0.71	0.01 – 0.80	0.06 – 0.75	0.03 - 0.62	0.09 – 0.51
	0.28	0.87	1.07	0.98	0.85	0.83	0.68	0.56	0.50
$CD3^+CD8^+$	0.35	0.01	0.05	0.06	0.09	0.15	0.24	0.17	0.26
	0.20 – 0.46	0.00 - 1.04	0.00 – 0.95	0.00 – 0.82	0.00 - 1.29	0.00 - 1.02	0.01 - 1.68	0.01 – 1.25	0.02 – 1.23
	0.28	2.42	1.56	1.40	1.31	1.09	1.23	1.12	0.94
CD4:CD8	2.75	3.81	1.45	1.65	1.64	1.61	1.29	1.30	0.85
	1.60 - 3.80	0.06 - 392	0.06 - 59.7	0.07 – 2906	0.11 - 266	0.13 - 30.9	0.23 - 18.4	0.27 - 19.3	0.26 - 6.29
	0.32	3.01	1.97	5.91	3.90	1.65	1.48	1.65	1.04
$CD19^+$	0.30	0.00	0.06	0.13	0.14	0.22	0.23	0.37	0.27
	0.12 – 0.52	0.00 – 0.28	0.00 – 0.93	0.00 – 0.71	0.00 – 0.90	0.00 – 0.54	0.00 – 0.61	0.00 – 0.80	0.00 – 0.95
	0.43	1.94	1.37	0.98	1.04	0.68	0.73	0.69	0.74
$CD3^-CD56^+$	0.24	0.23	0.17	0.21	0.21	0.21	0.22	0.19	0.21
	0.11 – 0.65	0.00 – 1.17	0.03 – 1.10	0.02 – 0.87	0.07 – 0.97	0.08 – 0.92	0.06 – 1.00	0.04 – 2.21	0.04 – 1.48
	0.61	0.82	0.87	0.74	0.86	0.74	0.70	1.56	1.19

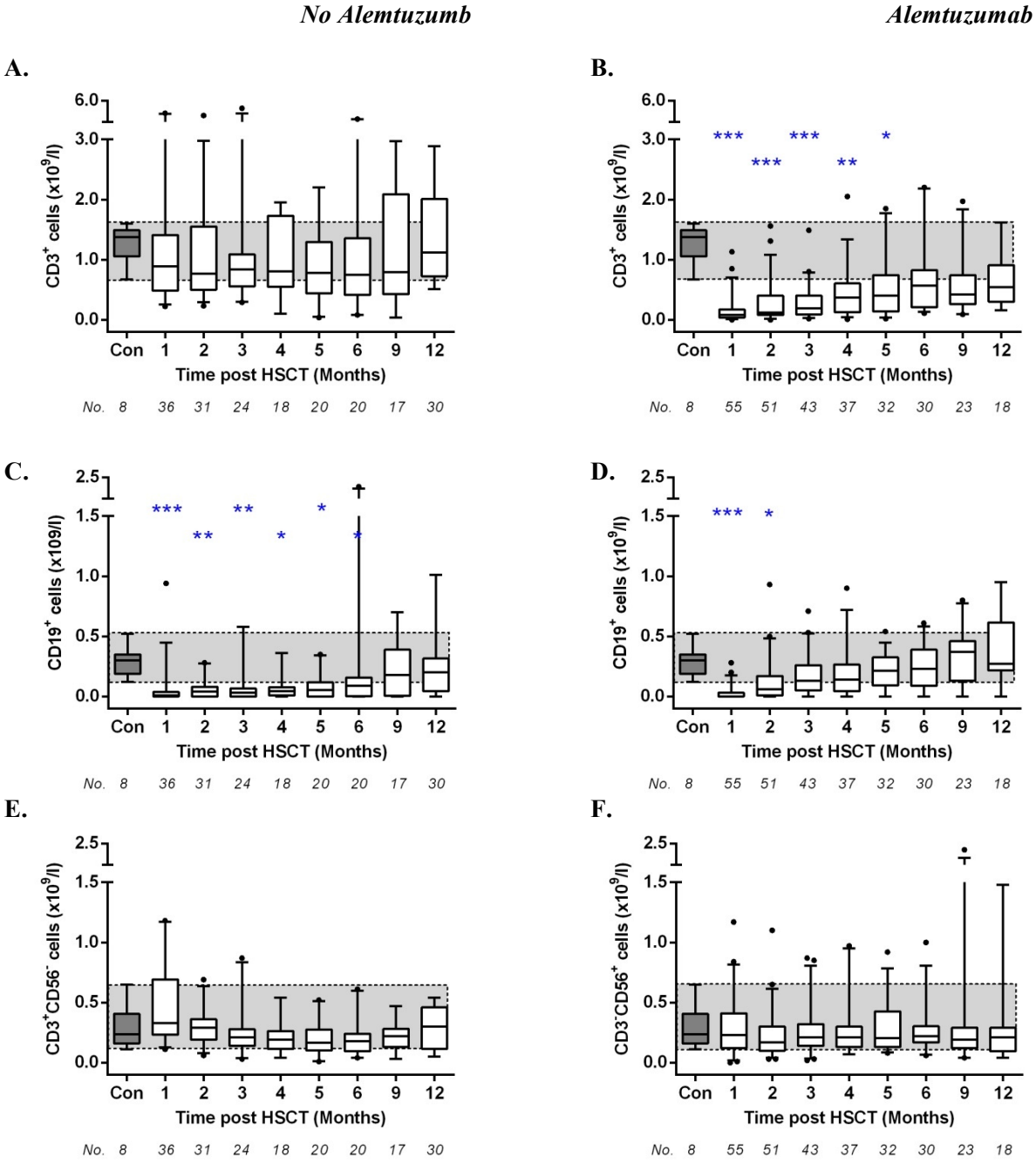


Figure 5.3: Peripheral Blood Lymphocyte Reconstitution Post-Allogeneic HSCT

$CD3^+$, $CD19^+$ and $CD3^-CD56^+$ cells were measured in 468 peripheral blood samples, collected at 1, 2, 3, 4, 5, 6, 9 and 12 months post-alloHSCT. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots of **(A&B)** $CD3^+$ cells; **(C&D)** $CD19^+$ cells; **(E&F)** $CD3^-CD56^+$ cells. **(A-C)** No Alemtuzumab; **(D-F)** Alemtuzumab. *No.* = number of blood samples analysed at each time point; Grey boxes show the normal range measured in EDTA blood samples from eight healthy volunteer donors (Con). *P<0.05, **P<0.01, ***P<0.001 when compared to control using the Kruskall-Wallis Test with Dunns post-hoc test.

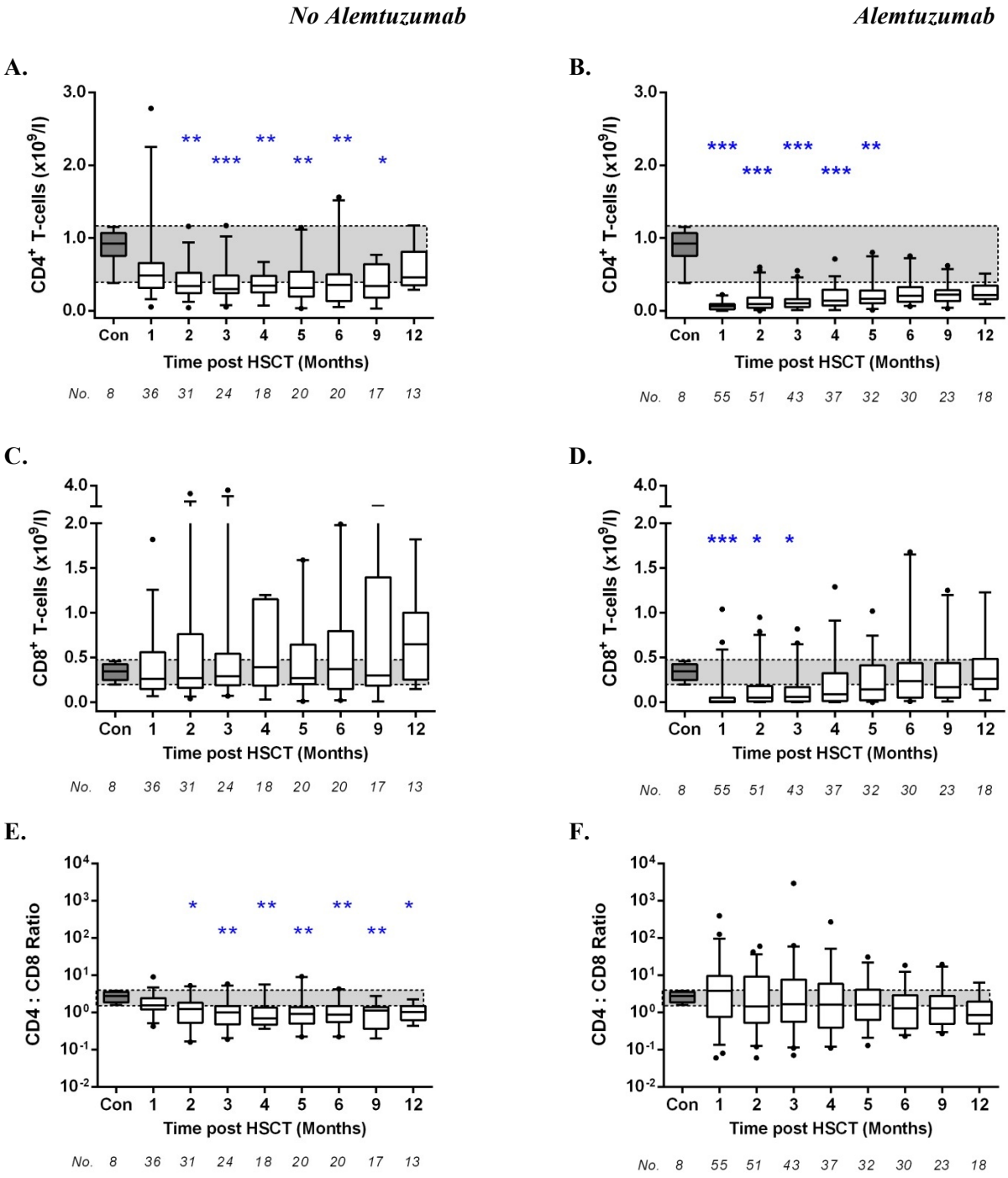


Figure 5.4: Peripheral Blood T-cell Reconstitution Post-Allogeneic HSCT

$CD4^+$ T cells and $CD8^+$ T cells were measured in 468 peripheral blood samples collected at 1, 2, 3, 4, 5, 6, 9 and 12 months post-alloHSCT. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots of **(A&B)** $CD4^+$ T cells; **(C&D)** $CD8^+$ T cells; **(E&F)** CD4:CD8 ratio. **(A-C)** No Alemtuzumab; **(D-F)** Alemtuzumab. *No.* = number of blood samples analysed at each time point; Grey boxes show the normal range measured in EDTA blood samples from eight healthy volunteer donors (Con). *P<0.05, ** P<0.01, ***P<0.001 when compared to control using the Kruskall-Wallis Test with Dunns post-hoc test.

Time (Month)	Cell type	No Alemtuzumab Median	Range	Alemtuzumab Median	Range	*P*
One	$CD3^+$	0.89	0.22 - 4.24	0.08	0.00 - 1.13	**<0.0001**
	$CD3^+CD4^+$	0.49	0.05 - 2.78	0.06	0.00 - 0.22	**<0.0001**
	$CD3^+CD8^+$	0.26	0.07 - 1.82	0.01	0.00 - 1.04	**<00001**
	CD4:CD8	1.57	0.42 - 8.97	3.81	0.06 - 392	**0.01**
	$CD19^+$	0.01	0.00 - 0.94	0.00	0.00 - 0.28	0.32
	$CD3^-CD56^+$	0.33	0.11 - 1.18	0.23	0.00 - 1.17	**0.003**
Three	$CD3^+$	0.84	0.29 - 2.94	0.19	0.02 - 1.49	**<0.0001**
	$CD3^+CD4^+$	0.30	0.05 - 1.17	0.10	0.01 - 0.55	**<0.0001**
	$CD3^+CD8^+$	0.29	0.07 - 3.55	0.06	0.00 - 0.82	**<0.0001**
	CD4:CD8	1.04	0.19 - 5.81	1.65	0.07 - 2906	0.17
	$CD19^+$	0.03	0.00 - 0.58	0.13	0.00 - 0.71	**0.003**
	$CD3^-CD56^+$	0.21	0.03 - 0.87	0.21	0.02 - 0.87	0.90
Six	$CD3^+$	0.75	0.08 - 3.43	0.41	0.11 - 2.20	0.15
	$CD3^+CD4^+$	0.36	0.05 - 1.56	0.17	0.06 - 0.75	0.18
	$CD3^+CD8^+$	0.37	0.02 - 1.99	0.15	0.01 - 1.68	0.13
	CD4:CD8	0.87	0.22 - 4.23	1.61	0.23 - 18.4	0.29
	$CD19^+$	0.09	0.00 - 2.06	0.22	0.00 - 0.61	**0.004**
	$CD3^-CD56^+$	0.18	0.04 - 0.61	0.21	0.06 - 1.00	0.08
Twelve	$CD3^+$	1.12	0.51 - 2.89	0.54	0.16 - 1.62	**0.003**
	$CD3^+CD4^+$	0.46	0.29 - 1.17	0.22	0.09 - 0.51	**0.0005**
	$CD3^+CD8^+$	0.65	0.15 - 1.82	0.26	0.02 - 1.23	**0.02**
	CD4:CD8	1.02	0.44 - 2.25	0.85	0.26 - 6.29	0.83
	$CD19^+$	0.20	0.00 - 1.01	0.27	0.00 - 0.95	0.13
	$CD3^-CD56^+$	0.30	0.05 - 0.54	0.21	0.04 - 1.48	0.26

Table 5.7: Lymphocyte Counts in the Non-Alemtuzumab and Alemtuzumab Transplants

Peripheral blood $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$ and $CD3^-CD56^+$ cell counts were measured from 91, 67, 50 and 31 alloHSCT at one, three, six and 12 months post-transplant respectively. Blood samples were collected in EDTA and analysed using flow cytometry. Table shows the median counts and range ($x10^9/l$) in the No-Alemtuzumab and Alemtuzumab alloHSCT at each time point assessed. Statistics were performed using Mann-Whitney test.

E) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-CELL RECONSTITUTION

As it was hypothesised that putative Tregs influence the outcome of alloHSCT, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell reconstitution was studied in all the transplants together and then in more detail in non-Alemtuzumab and Alemtuzumab transplants separately.

$CD4^+CD25^+FOXP3^+$ Tregs account for approximately 1-10% of total $CD4^+$ T cells in healthy individuals (Rezvani *et al*., 2006; Bloom *et al.,* 2008; Sakaguchi *et al.,* 2010). In keeping with these findings, the median proportion of peripheral blood $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) from healthy donors in this project was 3.15% (2.70-4.30%, *N*=8). However, to ascertain whether this range would still be observed following allogeneic HSCT, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts (absolute counts and %$CD4^+$ T cells) from all 468 post-transplant samples were first examined. $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at different time points post-transplant will then be examined in more detail later.

In this study, the median PB $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell count from all 468 post-transplant samples was $12.9x10^6$/l ($0.02\text{-}110x10^6$/l) or 6.14% $CD4^+$ T cells (0.29-48.1%). Both $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) showed wide variability post-transplant (CV 0.85 and 1.02) and had a positively skewed distribution (Figure 5.5A&C). $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were significantly lower in the post-transplant samples compared to the healthy controls ($12.9x10^6$/l (0.02-110) v $29.6x10^6$/l (14.5-38.7); *P*=0.008). Conversely, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) was significantly greater in the post-transplant samples (6.14% (0.29-48.08) v 3.15% (2.70-4.30%; *P*=0.02) (Figure 5.5B&D).

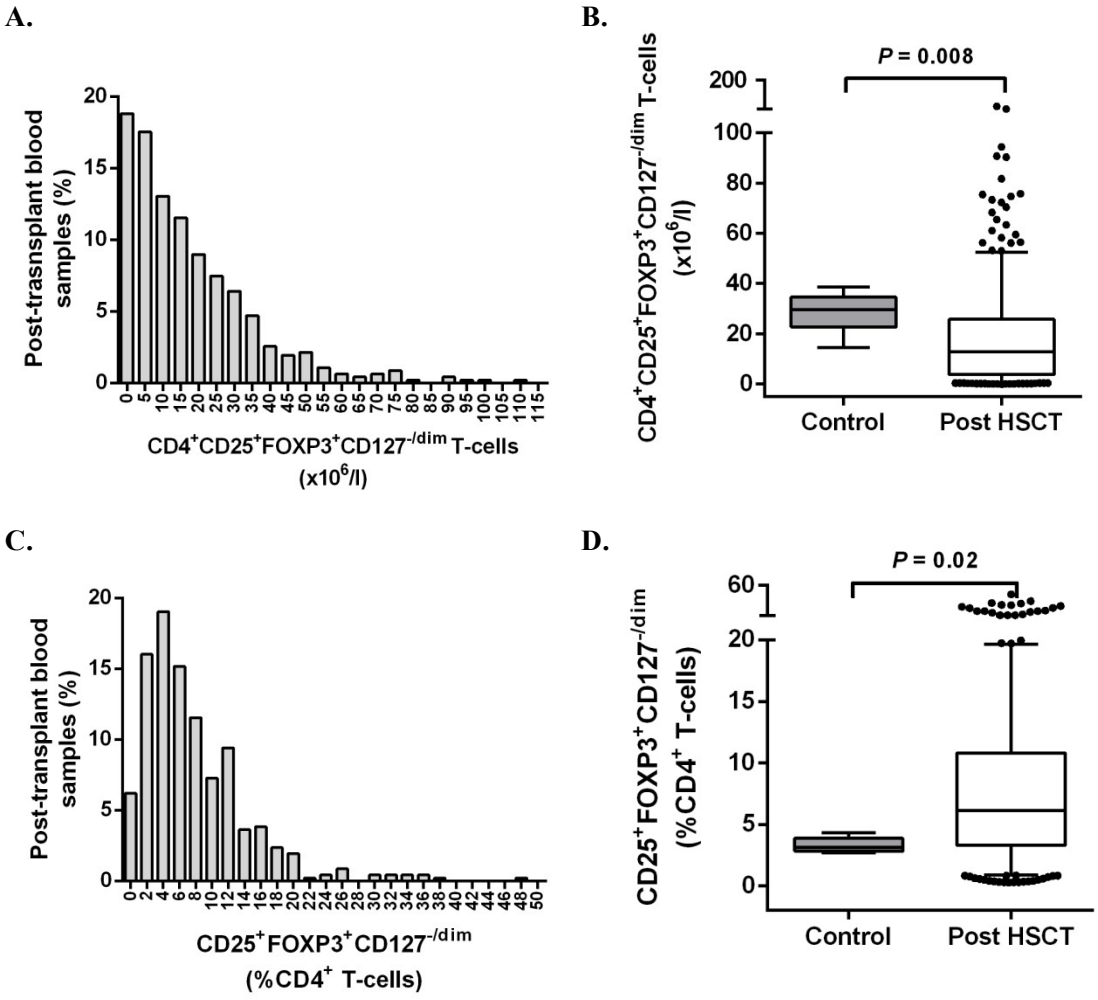


Figure 5.5: Post-alloHSCT Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were measured in all 468 peripheral blood samples collected within the first year post-alloHSCT. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. **(A-B)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l); **(C-D)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells)). **(A&C)** Frequency distribution plots showing the percentage (%) of the post-transplant blood samples that fall within the ranges specified on the x-axis; **(B&D)** Box-and-whiskers plots comparing the post-transplant $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells with the values obtained in eight healthy volunteer donors. Statistics were performed using Mann-Whitney test.

As with $CD3^+$ cells above, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) from the non-Alemtuzumab and Alemtuzumab transplants were analysed separately.

In the non-Alemtuzumab transplants, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts recovered early, being within the normal range by one month post-transplant ($23.1x10^6$/l (2.66–81.78)) (Figure 5.6A). Although these counts fell and were lower at two and three months, the median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell count then showed a gradual increase over the first year. $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ cells expressed as a %$CD3^+$ cells were within the normal range by one month and remained stable over the first year (Figure 5.6C). In contrast, the median proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) was above the normal range at one month post-transplant and increased over the first six months (Figure 5.6E; Table 5.8).

In the Alemtuzumab transplants, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell recovery was slower, with the counts progressively increasing over the first six months but not reaching the normal range until five months (Figure 5.6B). $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts in Alemtuzumab transplants were significantly lower than the non-Alemtuzumab transplants in the early months ($1.14x10^6$/l (0.02-25.2) v $23.1x10^6$/l (2.66-81.8), $P<0.0001$ at one month; $5.71x10^6$/l (0.21-63.5) v $13.5x10^6$/l (1.71-43.9), $P=0.001$ at three months)) but this was not observed from six months onwards ($22.0x10^6$/l (2.91-90.8) v $21.5x10^6$/l (0.15-70.4), $P=0.61$) (Figure 5.6A&B and Tables 5.9-5.10). $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD3^+$ cells and %$CD4^+$ T cells) also progressively increased over the first six months (Figure 5.6D&F). However, the median proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) increased to above the upper limit of the normal range from three months and remained above the normal range for the first year post-transplant (Tables 5.10).

Table 5.8: Peripheral $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts post-allogeneic HSCT (No Alemtuzumab conditioning)
Absolute counts ($x10^6$/l) and relative Treg counts (expressed as %Lymphocytes, $\%CD3^+$ and % $CD3^+CD4^+$ cells). Each box shows the median, range and coefficient of variation. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells

	Time post-transplant (months)								
	Control	**One**	**Two**	**Three**	**Four**	**Five**	**Six**	**Nine**	**Twelve**
Cells	N = 8	N = 36	N = 31	N = 24	N = 18	N = 20	N = 20	N = 17	N = 13
Tregs	29.6	23.1	16.8	13.5	20.2	20.0	21.5	24.3	38.8
	15.4 – 38.7	2.66 – 81.8	3.17 - 75.5	1.71 – 43.9	0.79 – 56.3	0.35 - 110	0.15 – 70.4	0.08 – 90.4	5.93 - 72.4
	0.27	0.70	0.79	0.70	0.69	1.05	0.81	0.91	0.59
% Lym	1.60	1.63	1.57	1.66	1.67	1.83	1.90	0.94	1.33
	1.30 – 1.70	0.01 - 4.74	0.01 - 16.7	0.07 - 6.10	0.08 – 3.54	0.19 - 8.29	0.12 – 4.85	0.11 – 3.91	0.34 – 5.07
	0.09	0.69	1.36	0.66	0.57	0.83	0.74	0.84	0.74
% $CD3^+$	2.20	2.47	2.19	2.09	2.45	2.62	2.32	1.70	2.36
	1.87 – 2.60	0.25 – 8.46	0.13 - 22.1	0.11 – 5.73	0.09 – 5.22	0.22 – 10.2	0.19 – 10.7	0.21 – 7.60	0.96 – 5.78
	0.10	0.74	1.27	0.67	0.52	0.76	0.81	0.84	0.54
% $CD3^+CD4^+$	3.15	4.49	4.48	4.01	6.11	6.91	6.78	5.52	6.52
	2.70 - 4.30	0.58 – 13.4	0.82 – 31.3	0.94 – 15.1	0.34 – 11.7	0.84 - 18.3	0.29 – 15.6	0.31 – 15.6	1.63 – 14.5
	0.18	0.71	0.88	0.69	0.52	0.55	0.62	0.71	0.56

Table 5.9: Peripheral $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts post-allogeneic HSCT (Alemtuzumab conditioning)

Absolute counts ($x10^6$/l) and relative Treg counts (expressed as %Lymphocytes, %%$CD3^+$ and % $CD3^+CD4^+$ cells). Each box shows the median, range and coefficient of variation. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells

	Time post-transplant (months)								
	Control	**One**	**Two**	**Three**	**Four**	**Five**	**Six**	**Nine**	**Twelve**
Cells	N= 8	N= 55	N= 51	N = 43	N = 37	N = 32	N = 30	N= 23	N = 18
Tregs	29.6	1.14	2.76	5.71	13.0	17.1	22.0	17.4	19.3
	15.42 – 38.7	0.02 – 25.2	0.06 - 26.6	0.21 – 63.5	0.45 – 75.9	0.90 – 46.8	2.91 – 90.8	3.15 – 94.5	2.91 – 65.5
	0.27	1.93	1.14	1.22	1.03	0.63	0.64	0.88	0.70
% Lym	1.60	0.27	0.94	0.91	1.28	1.73	2.20	1.87	1.62
	1.30 – 1.70	0.01 – 3.86	0.01 – 6.60	0.07 – 6.12	0.13 – 9.86	0.60 – 5.30	0.93 – 5.86	0.65 – 18.9	0.51 – 7.81
	0.09	1.21	1.07	0.96	1.04	0.66	0.48	1.27	0.83
% $CD3^+$	2.20	1.37	2.03	2.96	6.55	4.70	5.16	4.20	3.02
	1.87 – 2.60	0.02 – 17.3	0.05 – 22.4	0.18 – 19.5	0.28 – 16.9	1.20 – 21.7	1.52 – 15.5	1.10 – 44.4	1.09 – 27.5
	0.10	1.21	1.22	0.98	0.88	0.77	0.64	1.34	1.20
% $CD3^+CD4^+$	3.15	2.05	4.20	7.87	8.87	11.29	10.84	10.37	6.90
	2.70 - 4.30	0.30 – 36.6	0.36 – 38.8	0.91 – 33.5	1.11 – 24.6	2.77 – 29.9	5.13 - -23.4	2.82 – 48.1	3.20 – 33.8
	0.18	1.37	1.17	0.82	0..58	0.56	0.40	0.83	0.79

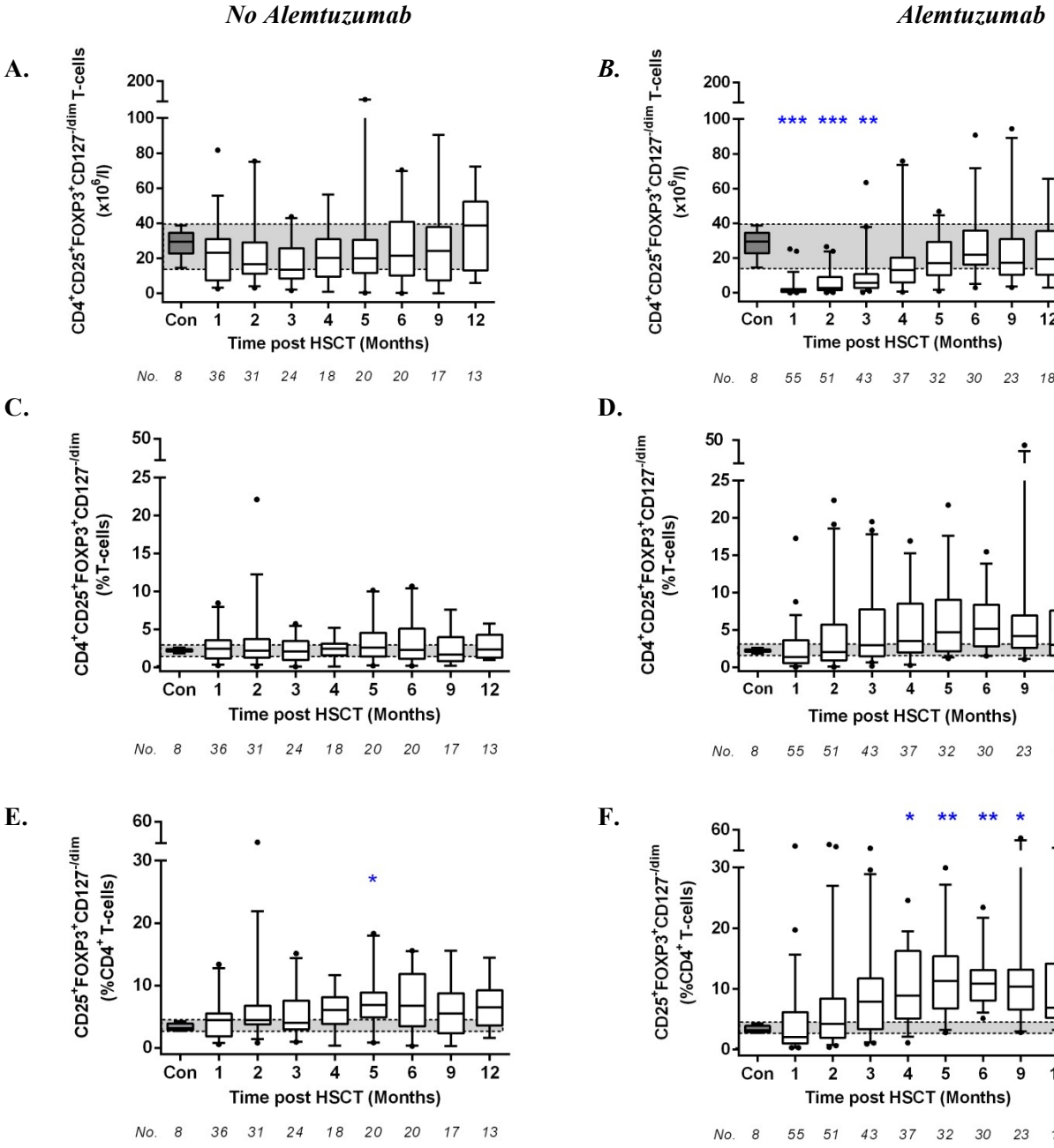


Figure 5.6: Peripheral Blood CD4+CD25+FOXP3+CD127-/dim T-Cell Reconstitution Post-Allogeneic HSCT

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were measured using flow cytometry in 468 post-alloHSCT blood samples, collected at 1, 2, 3, 4, 5, 6, 9 and 12 months post-transplant. Box-and-whiskers plots of (A&B) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (x10^6/l); (C&D) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD3^+$ cells); (E&F) $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). (A-C) No Alemtuzumab; (D-F) Alemtuzumab. *No*. = Number of blood samples analysed at each time point; Grey boxes show the normal range measured in EDTA blood samples from eight healthy volunteer donors (Con). *P<0.05, **P<0.01, ***P<0.001 when compared to control using the Kruskall-Wallis Test with Dunns post-hoc test.

Time (Month)	Cell type	No Alemtuzumab Median	Range	Alemtuzumab Median	Range	*P*
One	Tregs	23.1	2.66 - 81.8	1.14	0.02 - 25.2	**<0.0001**
	%Tregs	4.49	0.58 - 13.4	2.05	0.30 - 36.6	0.06
Three	Tregs	13.5	1.71 - 43.9	5.71	0.21 - 63.5	**0.001**
	%Tregs	4.01	0.94 - 15.1	7.87	0.91 - 33.5	**0.03**
Six	Tregs	21.5	0.15 - 70.4	22.0	2.91 - 90.8	0.61
	%Tregs	6.78	0.29 - 15.6	10.8	5.13 - 23.4	**0.006**
Twelve	Tregs	38.8	5.93 - 72.4	19.3	2.91 - 65.5	0.10
	%Tregs	6.52	1.63 - 14.5	6.90	3.20 - 33.8	0.27

Table 5.10: $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells in the Non-Alemtuzumab and Alemtuzumab Transplants

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l) ('Tregs') and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) ('%Tregs') were measured from 91, 67, 50 and 31 alloHSCT at one, three, six and 12 months post-transplant respectively. Blood samples were collected in EDTA and analysed using flow cytometry. Table shows the median values and range in the No-Alemtuzumab and Alemtuzumab alloHSCT at each time point. Statistics were performed using Mann-Whitney test.

Taken together, these data suggest that the absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts take several months to return to normal values post-alloHSCT. However, the relative proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) recover within the first few months post-transplant and can then exceed the normal values seen in healthy control donors. In light of these findings, it is hypothesised that the post-alloHSCT conditions may promote preferential $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell expansion over that of conventional T cells, possibly due to homeostatic expansion of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in a lymphopenic environment (Matsuoka *et al.,* 2009). Furthermore, use of Alemtuzumab enhances this response. This may be due to the pronounced lymphopenia associated with Alemtuzumab conditioning and/or through the conversion of $CD4^+CD25^-$ conventional T cells to $FOXP3^+$ cells (Bloom *et al.,* 2008).

5.3.2 FACTORS AFFECTING $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T-CELL EXPANSION POST-TRANSPLANT

Despite recent advances in our understanding of Tregs, it remains unclear which other factors affect Treg reconstitution post-alloHSCT. Given the previous data showing the influence of Alemtuzumab on post-transplant $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells, exploratory univariate and multivariate data analysis was performed to ascertain which other transplant variables are associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ($x10^6$/l) and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at one, three and six months post-transplant. Later times were not examined due to the limited number of samples available.

Within this section, the distribution of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell data at each time point was assessed for normality using histogram plots and the D'Agostino-Pearson normality test. When normal distribution could not be assumed, data were transformed using either log10, √ or log (x/(1-x)) transformation (specified within the text). Univariate analysis of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells was then performed using the variables: recipient and donor age (</> median), gender (M/F) and CMV IgG status (pos/neg); conditioning (RIC/MAC); Alemtuzumab (Yes/No); HSC donor and source (sibling/unrelated, PBSCH/BM); HSC graft dose ($CD34^+$, $CD3^+$, $CD3^+CD4^+$, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ($x10^6$/kg), proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) (</> median)); systemic immunosuppression with Prednisolone (Yes/No); acute GvHD (grades 0-I/II-IV) and CMV reactivation (CMV PCR </>1000 copies/ml). The presence of aGvHD and CMV reactivation were assessed both on the day the blood sample was collected and prior to sample collection. Univariate analysis was performed using the independent t-test on the normally distributed data. Multivariate analysis was then performed using multivariate linear regression including only those variables with $P<0.1$ on initial univariate testing.

A) ONE MONTH

One month post-transplant PB samples were collected and analysed from 91 alloHSCT. Samples were not available from ten transplants (seven deaths, one missed sample, one processing error, one transplant with less than one month follow-up). The median proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells) at one month post-transplant was 2.89% $CD4^{+}$ T cells (0.30-36.6%) with a median $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell count of $3.05x10^{6}$/l ($0.02\text{-}81.8x10^{6}$/l). As the one month $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell data were positively skewed, data analysis was performed following transformation i.e. $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells: $\log_{10}(x)$; $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells): $\log_{10}(x/(1-x))$. The frequency distribution of the original and transformed data is shown in Figure 5.7.

In univariate analysis, significantly lower $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts at one month were observed in those alloHSCT involving younger donors; male donors; unrelated donors; Alemtuzumab conditioning; lower HSC graft $CD4^{+}$ T cells, $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and lower proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells); positive CMV both at the time of sampling and within the first month. In multivariate regression, only Alemtuzumab conditioning and a positive CMV (day of sampling) remained significantly associated with one month $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts ($P<0.0001$ and $P=0.008$ respectively; $R^2=0.62$) (Table 5.11 & Figure 5.8).

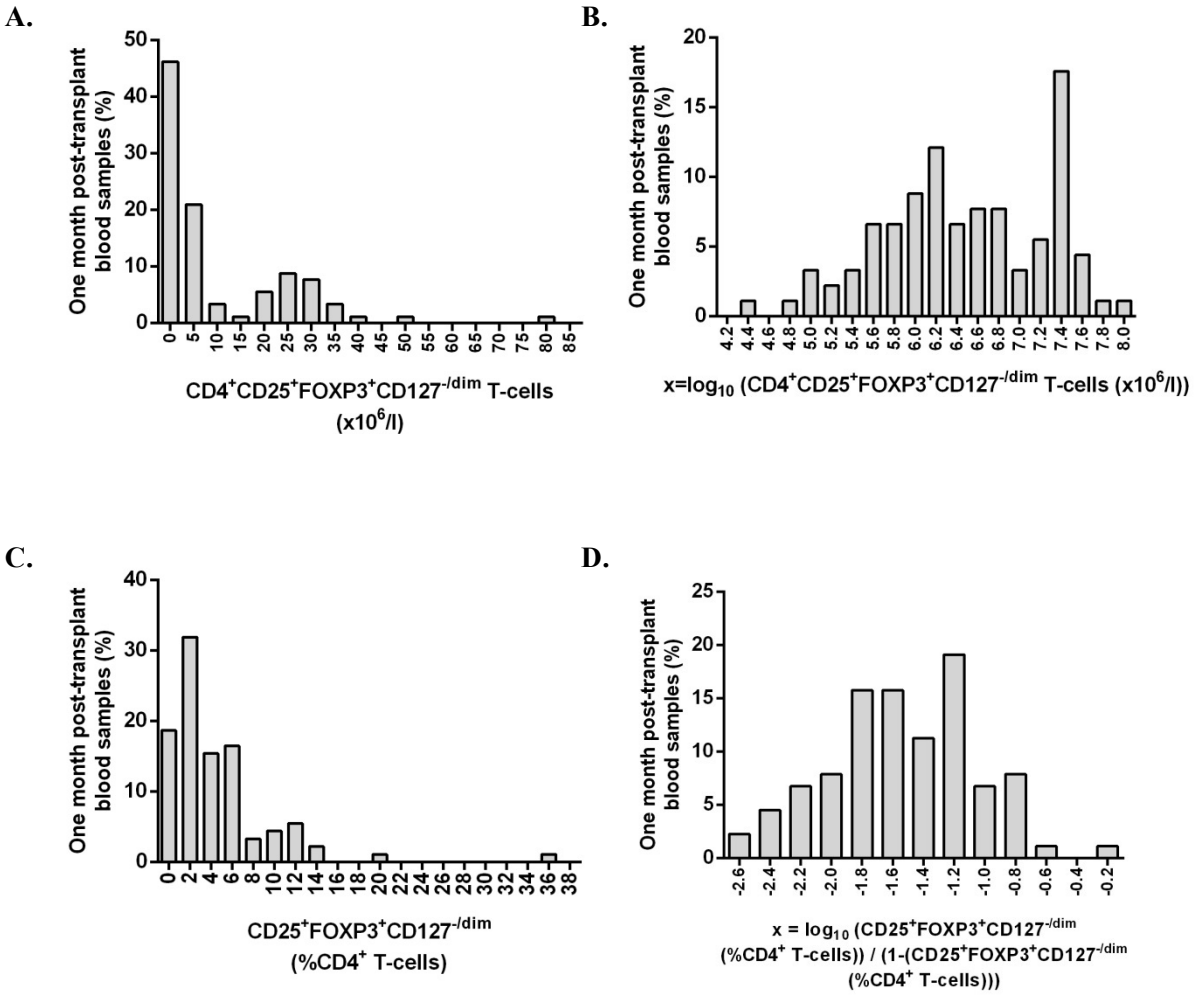


Figure 5.7: Frequency Distribution of the Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells One Month Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were measured by flow cytometry in 91 alloHSCT at one month post-transplant. Figure shows frequency distribution plots showing the percentage (%) of the one month post-transplant blood samples that fall within the ranges specified on the x-axis. **(A-B)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts; **(C-D)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ ($\%CD4^+$ T cells). **(A&C)** Original data; **(B)** Transformed $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell data ($\log_{10}(x)$); **(D)** Transformed $CD25^+FOXP3^+CD127^{-/dim}$ ($\%CD4^+$ T cells) data ($\log_{10}(x/(1-x))$.

Univariate analysis					
Variable	**Group**	***N***	**Mean** ($x10^6$/l)	**95% CI** ($x10^6$/l)	***P***
Donor Age	Low	48	1.60	1.00 - 2.54	**0.0004**
	High	43	6.11	3.44 - 10.8	
Donor Gender	Male	62	2.16	1.37 - 3.40	**0.01**
	Female	29	6.12	3.10 - 12.1	
Donor	Sibling	38	15.5	11.5 - 20.8	**<0.0001**
	Unrelated	53	0.93	0.63 - 1.37	
Alemtuzumab	No	36	17.0	12.9 - 22.5	**<0.0001**
	Yes	55	0.97	0.67 - 1.41	
HSC $CD3^+CD4^+$	Low	44	1.73	0.97 - 3.08	**0.005**
cells	High	47	5.05	3.12 - 8.18	
HSC Tregs	Low	45	5.33	3.15 - 9.04	**0.003**
	High	46	1.72	1.01 - 2.90	
HSC %Tregs	Low	47	4.74	2.72 - 8.30	**0.01**
	High	44	1.85	1.11 - 3.07	
CMV PCR	Neg	67	4.07	2.66 - 6.25	**0.0008**
(0-1 month)	Pos	24	1.29	0.58 - 2.83	
CMV PCR	Neg	77	3.94	2.67 - 5.81	**0.013**
(Day of sample)	Pos	14	0.68	0.23 - 2.04	

Multivariate analysis					
Variable	**B**	**SE B**	**β**	***P***	$\mathbf{R^2}$
Constant	-7.25	0.08			**0.62**
Alemtuzumab (Yes)	-1.18	0.11	-0.72	**<0.0001**	
CMV PCR (Pos) – Day of sample	-0.41	0.15	-0.19	**0.008**	

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Table 5.11: Univariate and Multivariate Analysis of Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Counts One Month Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l) were measured in 91 alloHSCT at one month post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Data were normalised using $log_{10}(x)$ transformation. *(Univariate)* Univariate analysis shows the mean $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts, 95% confidence intervals and *P*-values when analysed according to the main transplant-related variables. Data were analysed using the independent t-test. Table shows back-transformed data. Only variables with $P<0.1$ are shown. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells**;** '%Tregs' = proportion of $CD25^+FOXP3^+CD127^{-/dim}$ T cells (%$CD4^+$ T cells). *(Multivariate)* Multivariate linear regression to ascertain which transplant-related variables are independently associated with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at one month post-transplant. Variables considered in this analysis were variables with $P<0.1$ in univariate analysis. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value. Data shown are the transformed data (log_{10}).

A. **B.**

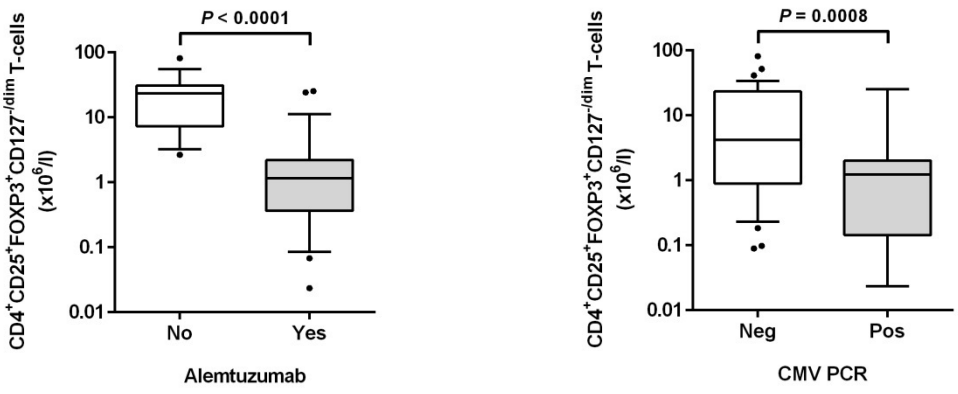


Figure 5.8: Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Counts One Month Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ($x10^6$/l) were measured in 91 alloHSCT at one month post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots analysing the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts according to **(A)** Alemtuzumab conditioning; **(B)** CMV PCR results on the day of blood sampling. Data were normalised using $log_{10}(x)$ transformation and analysed using independent t-test. Figure shows the transformed data.

In univariate analysis, lower proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells) were observed at one month in alloHSCT involving myeloablative conditioning; unrelated donors, Alemtuzumab conditioning; PBSCH; positive CMV both at the time of sampling and within the first month. A non-significant trend towards lower proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells) was also observed in alloHSCT using younger recipients and recipients positive for CMV IgG. As with the absolute $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts, in multivariate linear regression both Alemtuzumab conditioning and positive CMV (day of sampling) were significantly associated with lower proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ (%$CD4^{+}$ T cells) (*P*-=0.045 and *P*=0.001 respectively). In addition, the intensity of the conditioning and the source of the HSC graft were also significant although the number of BM harvests was limited (*P*=0.002 and *P*=0.008 respectively; R^2=0.25) (Table 5.12 & Figure 5.9).

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Table 5.12: Univariate and Multivariate Analysis of Peripheral Blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T Cells) One Month Post-Transplant

Peripheral blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) were measured in 91 alloHSCT at one month post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Data were normalised using $\log_{10}(x/(1-x)$ transformation. *(Univariate)* Univariate analysis shows the mean proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells), 95% confidence intervals and *P*-values when analysed according to the main transplant-related variables. Data were analysed using the independent t-test. Table shows back-transformed data. Only variables with *P*<0.1 are shown. *(Multivariate)* Multivariate linear regression to ascertain which transplant-related variables are independently associated with the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) at one month post-transplant. Variables considered in this analysis were variables with *P*<0.1.in univariate analysis. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value. Data shown are the transformed data $\log_{10}(x/(1-x)$.

Univariate analysis					
Variable	**Group**	***N***	**Mean (%)**	**95% CI (%)**	***P***
Recipient Age	Low	47	2.29	1.70 - 3.09	0.07
	High	44	3.45	2.47 - 4.81	
Recipient CMV	Neg	52	3.34	2.45 - 4.54	0.07
	Pos	39	2.20	1.59 - 3.04	
Conditioning	MAC	14	1.48	0.85 - 2.59	**0.02**
	RIC	77	3.13	2.47 - 3.98	
Donor	Sibling	38	3.63	2.79 - 4.71	**0.04**
	Unrelated	53	2.32	1.65 - 3.23	
Alemtuzumab	No	36	3.63	2.75 - 4.75	**0.04**
	Yes	55	2.35	1.70 - 3.25	
Source	BM	3	10.9	7.83 - 14.9	**<0.0001**
	PBSCH	88	2.66	2.13 - 3.34	
CMV PCR	Neg	67	3.28	2.55 - 2.24	**0.02**
(0-1 month)	Pos	24	1.77	1.14 - 2.73	
CMV PCR	Neg	77	3.21	2.52 - 4.08	**0.003**
(Day of sample)	Pos	14	1.30	0.83 - 2.03	

Multivariate analysis					
Variable	**B**	**SE B**	**β**	***P***	**R^2**
Constant	-1.35	0.76			**0.25**
Conditioning (MAC)	-0.08	0.13	-0.31	**0.002**	
Alemtuzumab (Yes)	-0.19	0.10	-0.20	**0.045**	
Source (BM)	0.69	0.25	0.26	**0.008**	
CMV PCR (Pos) – Day of sample	-0.32	0.13	-0.24	**0.01**	

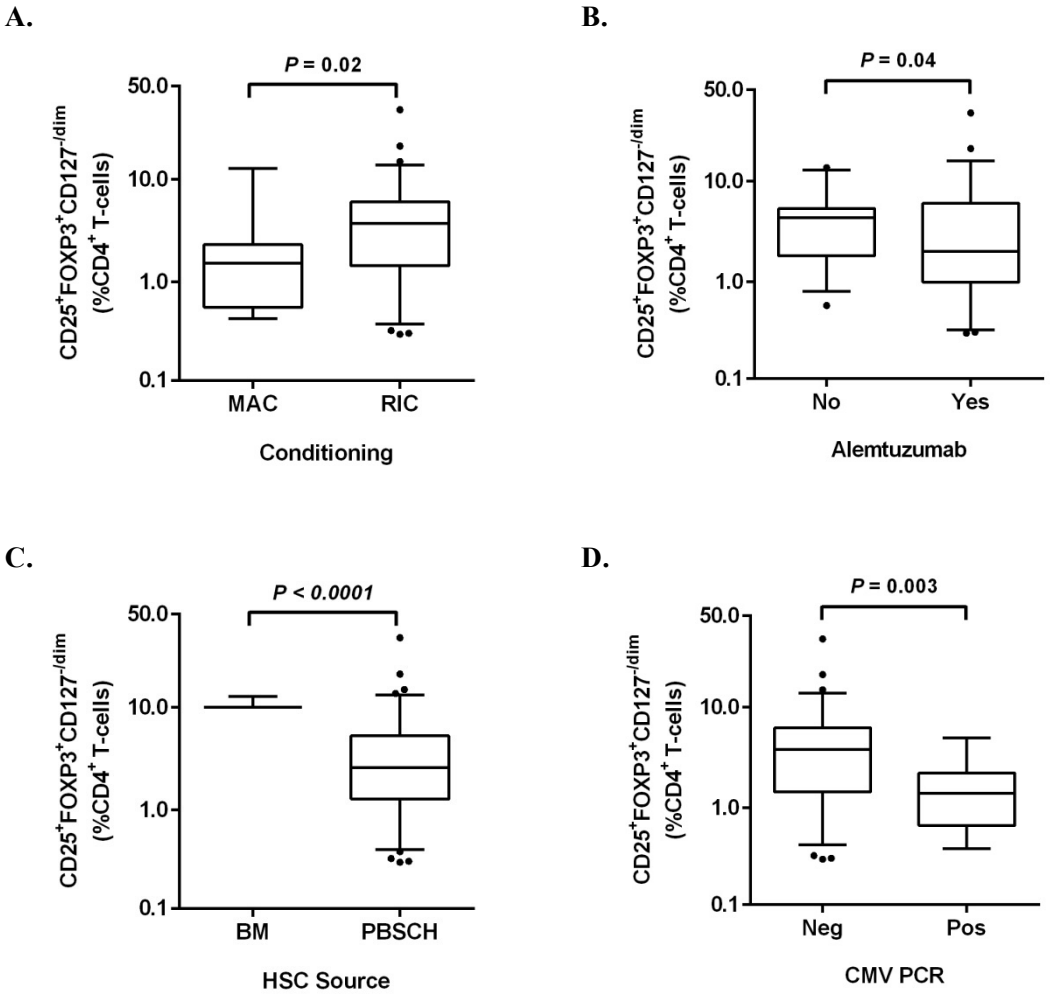


Figure 5.9: Peripheral Blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T Cells) One Month Post-Transplant

Peripheral blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) were measured in 91 alloHSCT at one month post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots analysing the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) according to **(A)** Intensity of the transplant conditioning; **(B)** Alemtuzumab conditioning; **(C)** Source of HSC graft; **(D)** CMV PCR result (on the day of blood sampling). Data were normalised using $\log_{10}$ (x/(1-x)) transformation and analysed using independent t-test. Figures show the transformed data.

Taken together, these data indicate that Alemtuzumab conditioning and CMV reactivation (CMV PCR positive) are significant factors associated with lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells early post-transplant. Alemtuzumab is known to cause profound total T-cell depletion early post-transplant and was, therefore, expected to be associated with lower absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at one month (Chakraverty *et al.,* 2010). With regards to CMV, this data could indicate that CMV reactivation and/or its associated treatment (Ganciclovir), reduces early $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell recovery. Alternatively, it may be hypothesised that alloHSCT with lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells at one month have increased risk of CMV reactivation. In keeping with this hypothesis, Pastore and colleagues (2011) showed a significant correlation between the peripheral $CD4^+CD25^{high}FOXP3^+$ Tregs and the recovery of CMV-specific $CD8^+$ T cells post-alloHSCT.

B) THREE MONTHS

Three month PB samples were available from 67 alloHSCT. Samples were not available from the remaining 34 due to death/2nd transplant (*N*=15), insufficient follow-up (*N*=10), missed sample collection (*N*=7) or laboratory error (*N*=3). The median proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at three months was 5.86% (0.91-33.5%) with a median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell count of $8.47x10^6$/l ($0.21-63.5x10^6$/l). Three month $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell data were positively skewed. Therefore, the data were transformed i.e. $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells: $\log_{10}(x)$; $CD25^+FOXP3^+CD127^{-/dim}$ (%$CD4^+$ T cells): $\log_{10}(x/(1-x))$. Frequency distributions for both the original and transformed data are shown below in Figure 5.10.

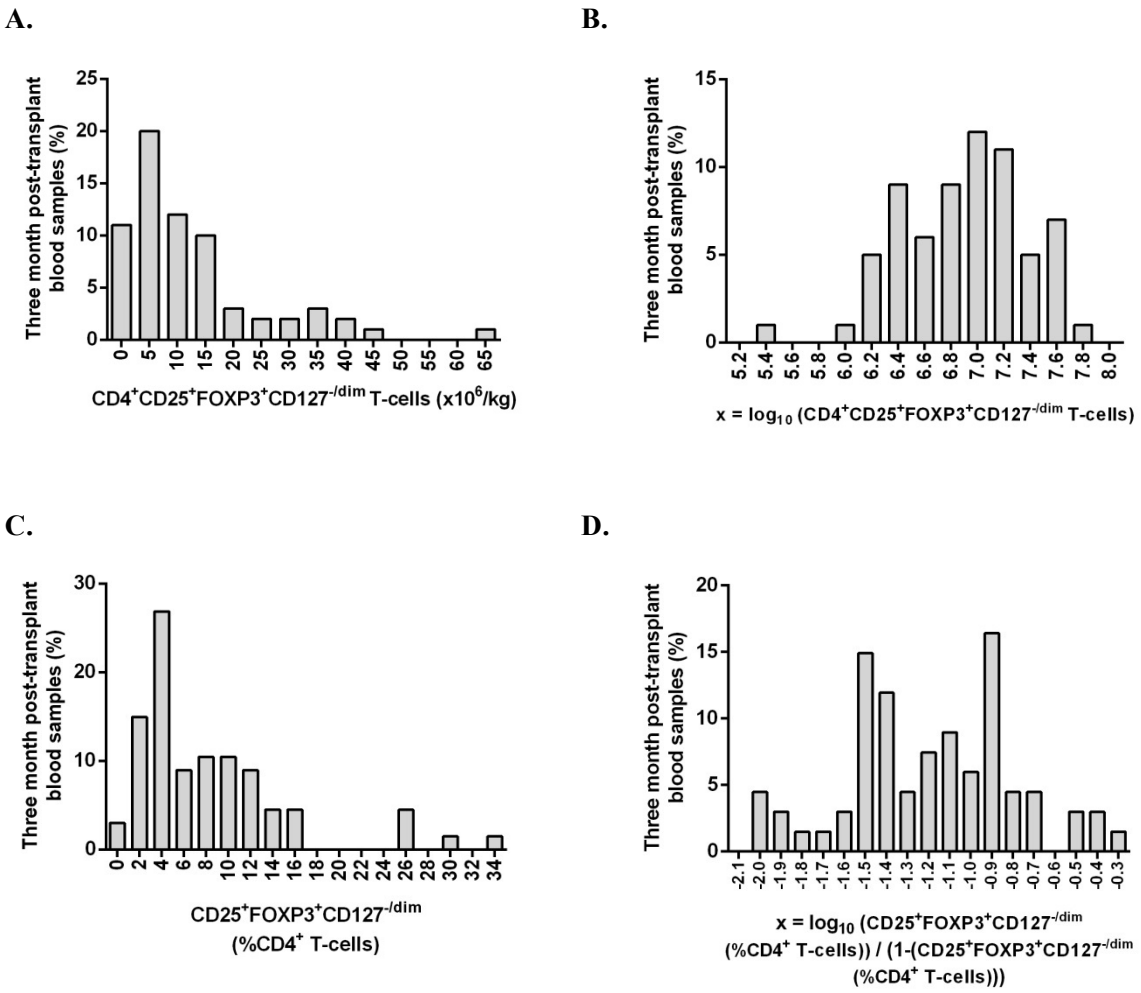


Figure 5.10: Frequency Distribution of the Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells Three Months Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were measured by flow cytometry in 67 alloHSCT at three months post-transplant. Figure shows frequency distribution plots showing the percentage (%) of the three month post-transplant blood samples that fall within the ranges specified on the x-axis. (A-B) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts; (C-D) Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ ($\%CD4^+$ T cells). (A&C) Original data; (B) Transformed $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell data ($\log_{10}(x)$); (D) Transformed $CD25^+FOXP3^+CD127^{-/dim}$ ($\%CD4^+$ T cells) data (log10(x/(1-x)).

By univariate analysis, the variables associated with significantly lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at three months post-transplant were positive recipient CMV IgG; unrelated donors; Alemtuzumab conditioning; grade II-IV acute GvHD (both on the day of sampling and within the first three months); positive CMV PCR titre (day of sampling). There was also a non-significant trend towards lower absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts with younger donor age, concurrent corticosteroids and positive CMV DNA (within first three months post-transplant). On multivariate linear regression, only Alemtuzumab conditioning, grade II-IV acute GvHD (day of sampling) and positive CMV DNA (day of sampling) remained significantly associated with the three month $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts (P=0.02, P=0.001 and P=0.002 respectively; R^2=0.39) (Figure 5.11 & Table 5.13).

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Table 5.13: Univariate and Multivariate Analysis of Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell counts Three Months Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l) were measured in 67 alloHSCT at three months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Data were normalised using $\log_{10}(x)$ transformation. *(Univariate)* Univariate analysis shows the mean $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts, 95% confidence intervals and *P*-values when analysed according to the main transplant-related variables. Data were analysed using the independent t-test. Table shows back-transformed data. Only variables with P<0.1 are shown. *(Multivariate)* Multivariate linear regression to ascertain which transplant-related variables are independently associated with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at three months post-transplant. Variables considered in this analysis were variables with P<0.1 in univariate analysis. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value. Data shown are the transformed data ($\log_{10}$).

Univariate analysis					
Variable	**Group**	***N***	**Mean** ($x10^6$/l)	**95% CI** ($x10^6$/l)	***P***
Recipient CMV	Neg	39	9.73	7.14 - 13.3	**0.047**
	Pos	28	5.78	3.71 - 9.02	
Donor Age	< Median	37	6.46	4.78 - 8.73	0.099
	> Median	30	9.93	6.34 - 15.6	
Donor	Sibling	26	11.7	8.17 - 16.8	**0.01**
	Unrelated	41	6.07	4.31 - 8.57	
Alemtuzumab	No	24	13.4	9.59 - 18.6	**0.002**
	Yes	43	5.81	4.16 - 8.11	
GvHD	0-I	44	10.4	7.94 - 13.7	**0.02**
(0-3 months)	II-IV	23	4.54	2.75 - 7.48	
GvHD	0-I	59	9.29	7.31 - 11.8	**0.0002**
(Day of sample)	II-IV	8	2.21	0.81 - 6.01	
Corticosteroids	No	50	9.04	6.73 - 12.2	0.06
	Yes	17	8.77	3.00 - 8.77	
CMV PCR	Neg	45	9.95	7.57 - 13.1	0.05
(0-3 months)	Pos	22	4.79	2.84 - 8.09	
CMV PCR	Neg	54	9.79	7.66 - 12.6	**0.0002**
(Day of sample)	Pos	13	3.08	1.52 - 6.25	

Multivariate analysis					
Variable	**B**	**SE B**	**β**	***P***	**R^2**
Constant	7.18	0.08			0.39
Alemtuzumab (Yes)	-0.24	0.10	-0.25	**0.02**	
GvHD (II-IV) – Day of sample	-0.48	0.14	-0.34	**0.001**	
CMV PCR (Pos) – Day of sample	-0.38	0.12	-0.33	**0.002**	

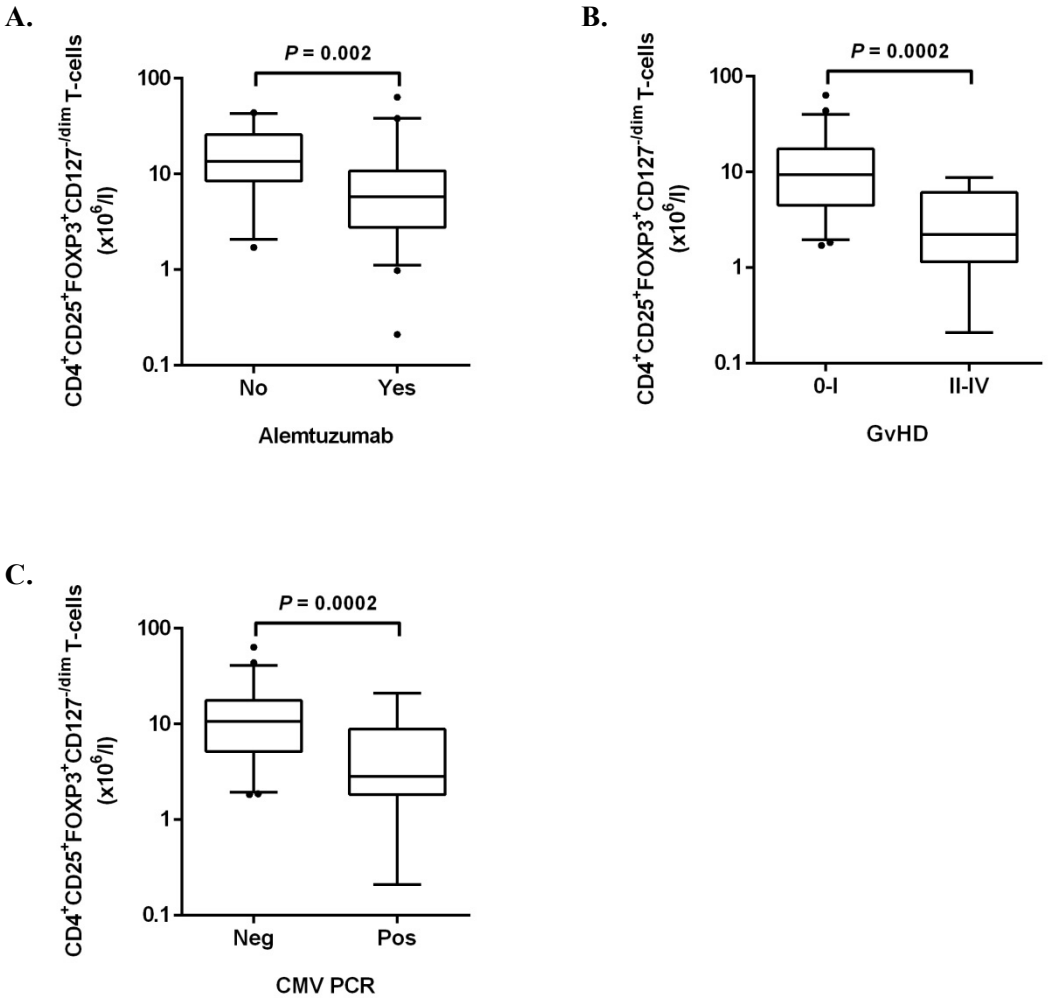


Figure 5.11: Peripheral Blood CD4+CD25+FOXP3+CD127-/dim T-Cell Counts Three Months Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ($x10^6$/l) were measured in 67 alloHSCT at three months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots analysing the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts according to **(A)** Alemtuzumab conditioning; **(B)** Presence of grade II-IV aGvHD (on the day of blood sampling); **(C)** CMV PCR results (on the day of blood sampling). Data were normalised using $\log_{10}(x)$ transformation and analysed using independent t-test. Figure shows the transformed data.

In relation to the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells), variables significantly associated with lower proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) on univariate analysis were older donors; sibling donors; no Alemtuzumab. There were also non-significant trends towards lower proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) with positive recipient CMV IgG; low HSC $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells and low proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells); acute GvHD (first three months). Only positive recipient CMV IgG and Alemtuzumab (*P*=0.03 and *P*=0.01; R^2=0.14) remained significant on multivariate analysis (Table 5.14 & Figure 5.12).

Univariate analysis					
Variable	**Group**	**N**	**Mean** (%Treg)	**95% CI** (%Treg)	***P***
Recipient CMV	Neg	39	7.05	5.35 - 9.25	0.07
	Pos	28	4.76	3.40 - 6.63	
Donor Age	< Median	37	7.41	5.49 - 9.93	**0.03**
	> Median	30	4.59	3.43 - 6.10	
Donor	Sib	26	2.24	3.17 - 5.67	**0.01**
	Un	46	7.42	5.59 - 9.78	
Alemtuzumab	No	24	4.37	3.18 - 5.97	**0.03**
	Yes	43	7.13	5.40 - 9.33	
HSC Tregs	Low	37	4.96	3.85 - 6.40	0.05
	High	30	7.51	5.25 - 10.6	
HSC %Tregs	Low	37	4.98	3.68 - 6.71	0.05
	High	30	7.50	5.59 - 10.0	
GvHD	0-I	44	6.89	5.48 - 8.63	0.07
(0-3 months)	II-IV	23	4.57	2.91 - 7.10	

Multivariate analysis					
Variable	**B**	**SE B**	**β**	***P***	**R^2**
Constant	-1269	0.08			0.14
Recipient CMV (Pos)	-0.21	0.09	-0.26	**0.03**	
Alemtuzumab (Yes)	0.25	0.10	0.30	**0.01**	

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Table 5.14: Univariate and Multivariate Analysis of Peripheral Blood CD25⁺FOXP3⁺CD127⁻/ᵈⁱᵐ cells (%CD4⁺ T Cells) Three Months Post-Transplant

Peripheral blood $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were measured in 67 alloHSCT at three months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Data were normalised using $\log_{10}(x/(1-x)$ transformation. *(Univariate)* Univariate analysis shows the mean proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), 95% confidence intervals and *P*-values when analysed according to the main transplant-related variables. Data were analysed using the independent t-test. Table shows back-transformed data. Only variables with $P<0.1$ are shown. 'Tregs' = $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells**;** '%Tregs' = proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). *(Multivariate)* Multivariate linear regression to ascertain which transplant-related variables are independently associated with the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at three months post-transplant. Variables considered in this analysis were variables with $P<0.1$.in univariate analysis. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value. Data shown are the transformed data $\log_{10}(x/(1-x)$.

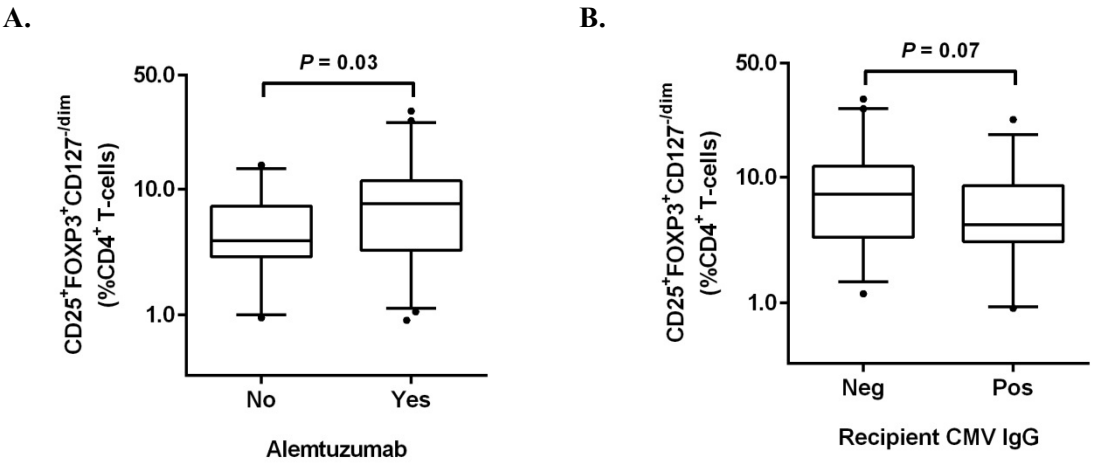


Figure 5.12: Peripheral Blood CD25⁺FOXP3⁺CD127⁻/ᵈⁱᵐ Cells (%CD4⁺ T Cells) Three Months Post-Transplant

Peripheral blood $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were measured in 67 alloHSCT at three months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots analysing the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) according to **(A)** Alemtuzumab conditioning; **(B)** Recipients CMV IgG antibody status (i.e. previous exposure to CMV). Data were normalised using $\log_{10}(x/(1-x))$ transformation and analysed using independent t-test. Figure shows the transformed data.

These data again indicate that Alemtuzumab conditioning and CMV are significant factors associated with reconstitution of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells post-alloHSCT. However, although Alemtuzumab continues to be associated with lower absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts, it was associated with significantly higher proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at three months. It is hypothesised that the lymphopenic environment produced by Alemtuzumab conditioning may preferentially expand the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) by homeostatic expansion over that of conventional T cells (Matsuoka *et al.,* 2010). Alternatively, it has been suggested that Alemtuzumab may induce $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells from $CD4^+CD25^-$ conventional T cells (Bloom *et al.,* 2008). The presence of grade II-IV GvHD was also a significant predictor of lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts. It is uncertain whether this is directly due to GvHD or the associated treatment with corticosteroids. Alternatively, it may be hypothesised that alloHSCT patients with lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells at three months have an increased risk of GvHD i.e. this data shows an association but does not demonstrate whether it is cause or effect.

C) SIX MONTHS

At six months post-transplant, samples were available from 50 alloHSCT. Samples were unavailable from 51 transplants due to death/2^{nd} transplant (*N*=24), insufficient follow-up (*N*=21), missed sample collection (*N*=5) or laboratory error (*N*=1). The median proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at six months was 9.27% (0.29-23.4%) with a median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell count of $21.9x10^6$/l ($0.15-90.8x10^6$/l). The six month $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were not normally distributed and required transformation using the formula $\sqrt{(x)}$. However, the six month $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) data did not require transformation. Frequency distributions for both the original and transformed data are shown below in Figure 5.13**.**

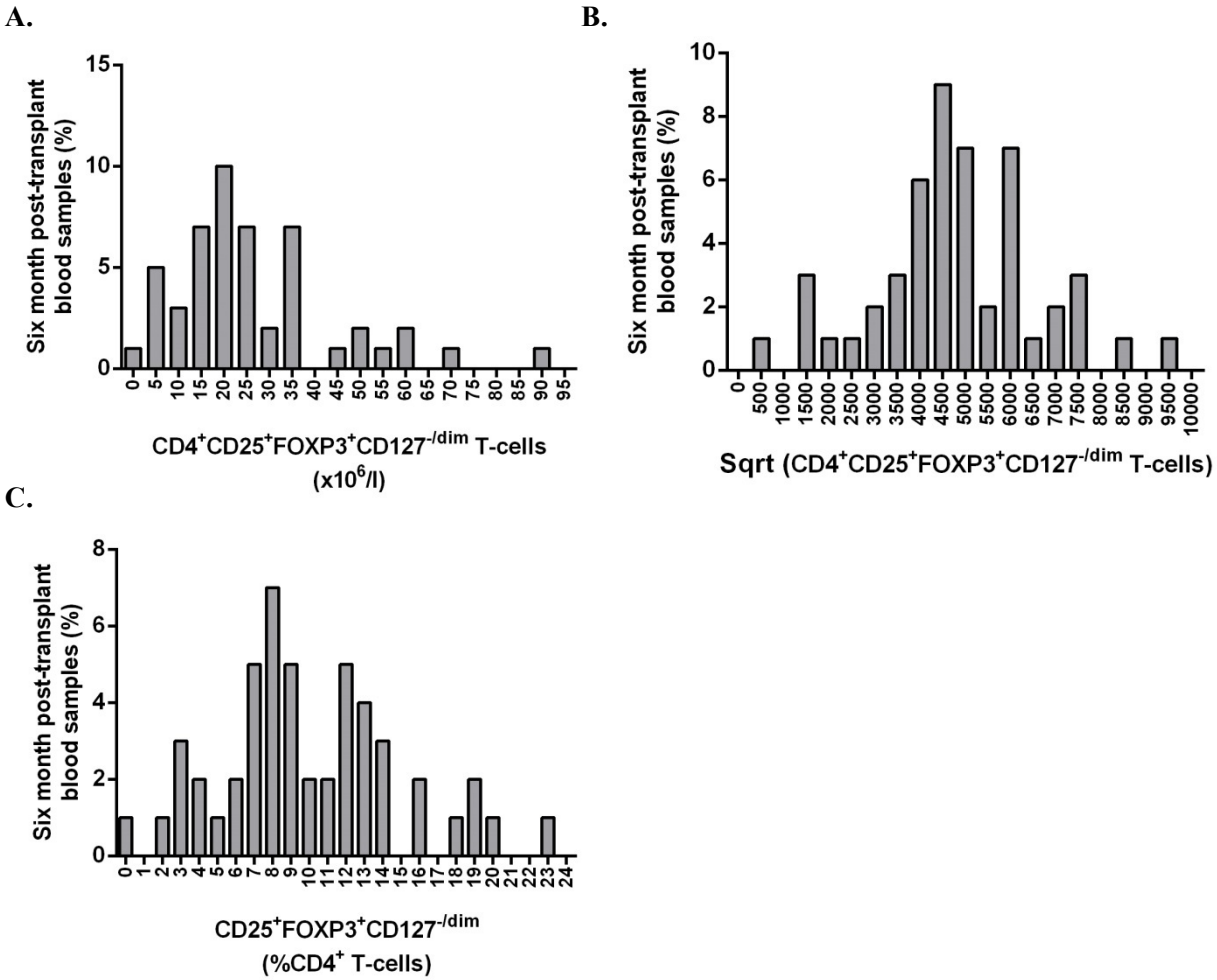


Figure 5.13: Frequency Distribution of the Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells Six Months Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were measured by flow cytometry in 50 alloHSCT at six months post-transplant. Figure shows frequency distribution plots showing the percentage (%) of the six month post-transplant blood samples that fall within the ranges specified on the x-axis. (A-B) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts; (C) Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). (A&C) Original data; (B) Transformed $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell data (√x);

In univariate analysis of the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at six months, only two variables were significantly associated with low counts: high HSC $CD34^+$ dose and the use of corticosteroids on the day of sampling. In multivariate linear regression, only the use of corticosteroids remained significant (*P*=0.002; R^2=0.18) (Table 5.15 & Figure 5.14). Of note, the R^2 value (0.18) is lower than at one and three months (0.62 and 0.39 respectively), suggesting that the variables examined in this data analysis account for less of the variation in the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at six months compared to the earlier time points.

Univariate analysis					
Variable	**Group**	***N***	**Mean** ($x10^6$/l)	**95% CI** ($x10^6$/l)	***P***
HSC $CD34^+$	< Median	23	29.5	21.4 - 38.8	**0.02**
	> Median	27	18.3	13.2 - 24.3	
Corticosteroids	No	36	32.8	22.5 - 33.7	**0.002**
	Yes	14	13.0	6.23 - 22.1	

Multivariate analysis					
Variable	**B**	**SE B**	**β**	***P***	**R^2**
Constant	5725	278			0.18
Corticosteroids (Yes)	-1676	525	-0.42	**0.002**	

Table 5.15: Univariate and Multivariate Analysis of Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Counts Six Months Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l) were measured in 50 alloHSCT at six months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Data were normalised using √(x) transformation. *(Univariate)* Univariate analysis shows the mean $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts, 95% confidence intervals and *P*-values when analysed according to the main transplant-related variables. Data were analysed using the independent t-test. Table shows back-transformed data. Only variables with *P*<0.1 are shown. *(Multivariate)* Multivariate linear regression to ascertain which transplant-related variables are independently associated with the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at six months post-transplant. Variables considered in this analysis were variables with *P*<0.1 in univariate analysis. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value. Data shown are the transformed data (√).

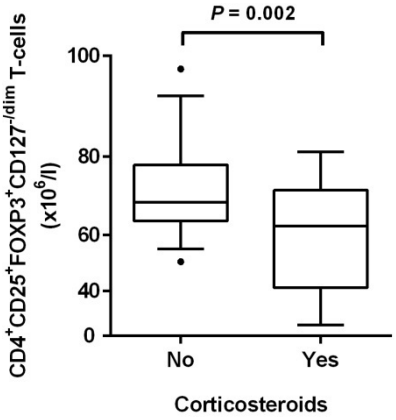


Figure 5.14: Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Counts Six Months Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ($x10^6$/l) were measured in 50 alloHSCT at six months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. Box-and-whiskers plots analysing the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts according to the use of corticosteroids (on the day of blood sampling). Data were normalised using $\sqrt{(x)}$ transformation and analysed using independent t-test. Figure shows the normalised data.

In univariate analysis of the $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) as six months, the variables significantly associated with lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were positive donor CMV IgG; sibling donor; non-Alemtuzumab conditioning; clinical GvHD and concurrent use of corticosteroids. There was also a non-significant trend towards lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) with positive CMV PCR (day of collection) and clinical GvHD within the first six months. In multivariate linear regression, positive CMV PCR and concurrent use of corticosteroids were significantly associated with the six month proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) (P=0.04 and P<0.001; R^2=0.31) (Table 5.16).

Univariate analysis					
Variable	**Group**	***N***	**Mean** (%)	**95% CI** (%)	***P***
Donor CMV	Neg	34	11.0	9.46 - 12.5	**0.03**
	Pos	16	7.77	4.73 - 10.8	
Donor	Sib	21	7.55	5.48 - 9.62	**0.003**
	Un	29	11.7	9.93 - 13.5	
Alemtuzumab	No	20	7.42	5.25 - 9.58	**0.003**
	Yes	30	11.7	9.94 - 13.4	
CMV PCR	Neg	47	10.3	8.85 - 11.8	0.06
(Day of sample)	Pos	3	4.61	1.73 - 11.0	
GvHD	No	17	11.6	9.37 - 13.8	0.10
(0-6 months)	Yes	33	9.12	727 - 11.0	
GvHD	No	32	11.2	9.55 - 12.9	**0.02**
(Day of sample)	Yes	18	7.77	5.21 - 10.3	
Corticosteroids	No	36	11.5	9.94 - 13.1	**0.0003**
	Yes	14	6.01	3.81 - 8.22	

Multivariate analysis					
Variable	**B**	**SE B**	**β**	***P***	**R^2**
Constant	11.8	0.72			0.31
CMV PCR (Pos)	-5.39	2.54	-0.26	**0.04**	
Corticosteroids (Yes)	-5.40	1.34	-0.49	**<0.0001**	

Table 5.16: Univariate and Multivariate Analysis of Peripheral Blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ Cells (%$CD4^{+}$ T Cells) Six Months Post-Transplant

Peripheral blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) were measured in 50 alloHSCT at six months post-transplant. Blood samples were collected in EDTA and analysed using multi-colour flow cytometry. *(Univariate)* Univariate analysis shows the mean proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells), 95% confidence intervals and *P*-values when analysed according to the main transplant-related variables. Data were analysed using the independent t-test. Only variables with $P<0.1$ are shown. *(Multivariate)* Multivariate linear regression to ascertain which transplant-related variables are independently associated with the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) at six months post-transplant. Variables considered in this analysis were variables with $P<0.1$ in univariate analysis. Table shows the b-values (B) and standard error (SE), standardised beta (β), P-value and the R^2 value.

Taken together, these data indicate that at six months post-transplant, corticosteroid therapy was a significant predictor of low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute counts and $\%CD4^+$ T cells). Corticosteroids are lymphotoxic and may therefore reduce the total T-cell count, including $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. However, the GvHD for which the steroids are being used could also contribute towards the low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. CMV reactivation was also a significant predictor of lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) at six months. As before, this could indicate that CMV reactivation and/or its treatment reduces $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell recovery. Alternatively, alloHSCT with lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) may have increased CMV reactivation. The earlier effect of Alemtuzumab on $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells no longer appears to be significant by six months post-transplant.

The following table summarises which variables were significantly associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the blood (absolute counts and $\%CD4^+$ T cells) in the first six months post-transplant using multivariate linear regression (Table 5.17). In keeping with the observations above (Chapter 5.3.1), Alemtuzumab was associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the first three months. Also, of note, CMV reactivation was independently associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (counts and $\%CD4^+$ T cells) throughout the first six months post-alloHSCT.

Time	**$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells**			**$CD25^+FOXP3^+CD127^{-/dim}$ ($\%CD4^+$ T cells)**		
(Months)	Variable	*P*	R^2	Variable	*P*	R^2
One	Alemtuzumab	<0.0001	0.62	Alemtuzumab	0.045	0.25
	CMV PCR Pos	0.008		CMV PCR Pos	0.01	
				RIC	0.002	
				PBSCH	0.008	
Three	Alemtuzumab	0.02	0.39	No Alemtuzumab	0.01	0.14
	CMV PCR Pos	0.002		R. CMV IgG Pos	0.03	
	GvHD	0.001				
Six	Corticosteroids	0.002	0.18	Corticosteroids	<0.0001	0.31
				CMV PCR Pos	0.04	

Table 5.17: Summary of the Variables Associated with Low Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells

Summary of the transplant-related variables that were significantly associated with low PB $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute counts and $\%CD4^+$ T cells) post-transplant using multivariate linear regression.

5.3.3 PERIPHERAL BLOOD $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS AND TRANSPLANT EVENTS

Having explored which variables are associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells post-alloHSCT, this work then examined in further detail whether $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were reduced in GVHD. It has been previously proposed that human $CD4^+CD25^+FOXP3^+$ Tregs are lower in the setting of GvHD (Rezvani *et al.,* 2006, Magenau *et al.,* 2010). However, this observation has not been demonstrated in several other studies (Meignin *et al.,* 2005; Arimoto *et al.,* 2007).

A) ACUTE GVHD

To test this hypothesis, all the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) measured from patients with and without aGvHD were compared, recording possible confounding characteristics that are known to influence $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and/or $CD4^+$ T cells (Tables 5.18-5.19). Of the 240 samples analysed in the first three months post-transplant, 56 were collected from patients with clinical evidence of aGvHD at the time of collection. Absolute $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were significantly lower in those patients with grade I-IV aGvHD ($3.17x10^6$/l (0.02-$63.47x10^6$/l) v $7.84x10^6$/l (0.06-$81.8x10^6$/l); P=0.02) (Figure 5.15A). No significant difference was observed in the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) between these two groups (3.38% (0.30-38.8%) v 4.31% (0.30-36.6%), P=0.87) (Figure 5.15B & Table 5.18). Patients with aGvHD at the time of sample collection also had significantly lower lymphocyte counts, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD4^+CD25^-FOXP3^-$, $CD3^+CD8^+$, $CD19^+$ and $CD3^-CD56^+$ cell counts. These samples also had significantly higher CD4:CD8 T-cell ratios (Table 5.18).

Acute GvHD		**No** (*N*=184)	**Yes** (*N*=56)	***P***
Sample Time	Median (days)	61 (27 - 110)	62 (33 - 110)	0.61
Cell Counts				
Treg	x 10^6/l	7.84 (0.06 - 81.8)	3.17 (0.02 - 63.5)	**0.02**
Treg (%Lym)	%	0.97 (0.01 - 16.7)	0.96 (0.01 - 6.60)	0.98
Treg (%$CD3^+$)	%	2.11 (0.02 - 22.2)	2.24 (0.18 - 22.4)	0.60
Treg (%$CD3^+CD4^+$)	%	4.31 (0.30 - 36.6)	3.38 (0.30 - 38.8)	0.87
$CD25^-$ Tcon	x 10^6/l	126 (2.30 – 2690)	62.3 (0.60 – 509)	**0.006**
$CD25^-$ (%$CD3^+CD4^+$)	%	74.3 (21.9 – 96.9)	71.6 (9.40 – 92.6)	0.14
$CD25^+$ Tcon	x 10^6/l	28.0 (0.10 - 905)	20.0 (0.20 – 301)	0.12
$CD25^+$ (%$CD3^+CD4^+$)	%	17.0 (1.30 – 65.7)	18.9 (2.70 – 71.0)	0.29
Lymphocytes	x 10^9/l	0.78 (0.01 - 5.77)	0.44 (0.05 - 2.25)	**<0.0001**
$CD3^+$	x 10^9/l	0.41 (0.00 - 4.94)	0.15 (0.00 - 1.54)	**0.001**
$CD19^+$	x 10^9/l	0.03 (0.00 - 0.94)	0.00 (0.00 - 0.53)	**0.001**
$CD3^-CD56^+$	x 10^9/l	0.26 (0.00 - 1.18)	0.17 (0.02 - 0.81)	**0.001**
$CD3^+CD4^+$	x 10^9/l	0.18 (0.00 - 2.78)	0.11 (0.00 - 0.66)	**0.01**
$CD3^+CD8^+$	x 10^9/l	0.15 (0.00 - 3.55)	0.04 (0.00 - 0.95)	**0.001**
CD4 : CD8	Ratio	1.43 (0.06 - 2906)	2.24 (0.07 - 126)	**0.045**

Table 5.18: Lymphocyte Counts in Blood Samples Collected in the Presence and Absence of Grade I-IV Acute GvHD

Total lymphocyte, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD56^+$, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell ('Treg'), $CD4^+CD25^-FOXP3^-$ T-cell ('$CD25^-$ Tcon') and $CD4^+CD25^+FOXP3^-$ T-cell ('$CD25^+$ Tcon') counts were measured in 240 peripheral blood samples collected within the first three months post-transplant. Table shows the median values and range analysed according to the absence or presence of acute GvHD (all grades) on the day of sampling. Data were analysed using the Mann-Whitney test.

Acute GvHD		No (*N*=184)	Yes (*N*=56)	*P*
Immunosuppression				
Prednisolone	No/Yes	164 / 20	26 / 30	**<0.0001**
CMV Reactivation				
PCR	Neg / Pos	146 / 38	41 / 15	0.36
Recipient				
Age	Median (years)	48 (18 - 74)	46 (18 - 66)	0.48
Gender	Male/Female	109 / 75	32 / 24	0.88
CMV	IgG Neg/Pos	105 / 79	33 / 23	0.88
Donor				
Age	Median (years)	40 (19 - 68)	36 (20 - 68)	**0.045**
Gender	Male/Female	127 / 57	41 / 15	0.62
CMV	IgG Neg/Pos	120 / 64	42 / 14	0.19
Transplant				
Conditioning	RIC/MAC	152 / 32	51 / 5	0.14
Source	PBSCH/BM	182 / 2	54 / 2	0.23
Donor	Sib/Un	86 / 98	11 / 45	**0.0003**
Alemtuzumab	No/Yes	81 / 103	10 / 46	**0.0005**
HLA mismatch	No/Yes	158 / 26	43 / 13	0.15
Gender mismatch	FM/Other	151 / 33	52 / 4	0.06
ABO mismatch	No/Yes	148/36	48/8	0.44

Table 5.19: Baseline Characteristics of the Blood Samples Collected in the Presence and Absence of Grade I-IV Acute GvHD

240 peripheral blood samples were collected within the first three months post-alloHSCT and divided into two groups according to the absence (*N*=184) or presence (*N*=56) of acute GvHD (all grades) at the time of sampling. Table shows the baseline transplant characteristics of the two groups. Data were analysed using the Fisher's test.

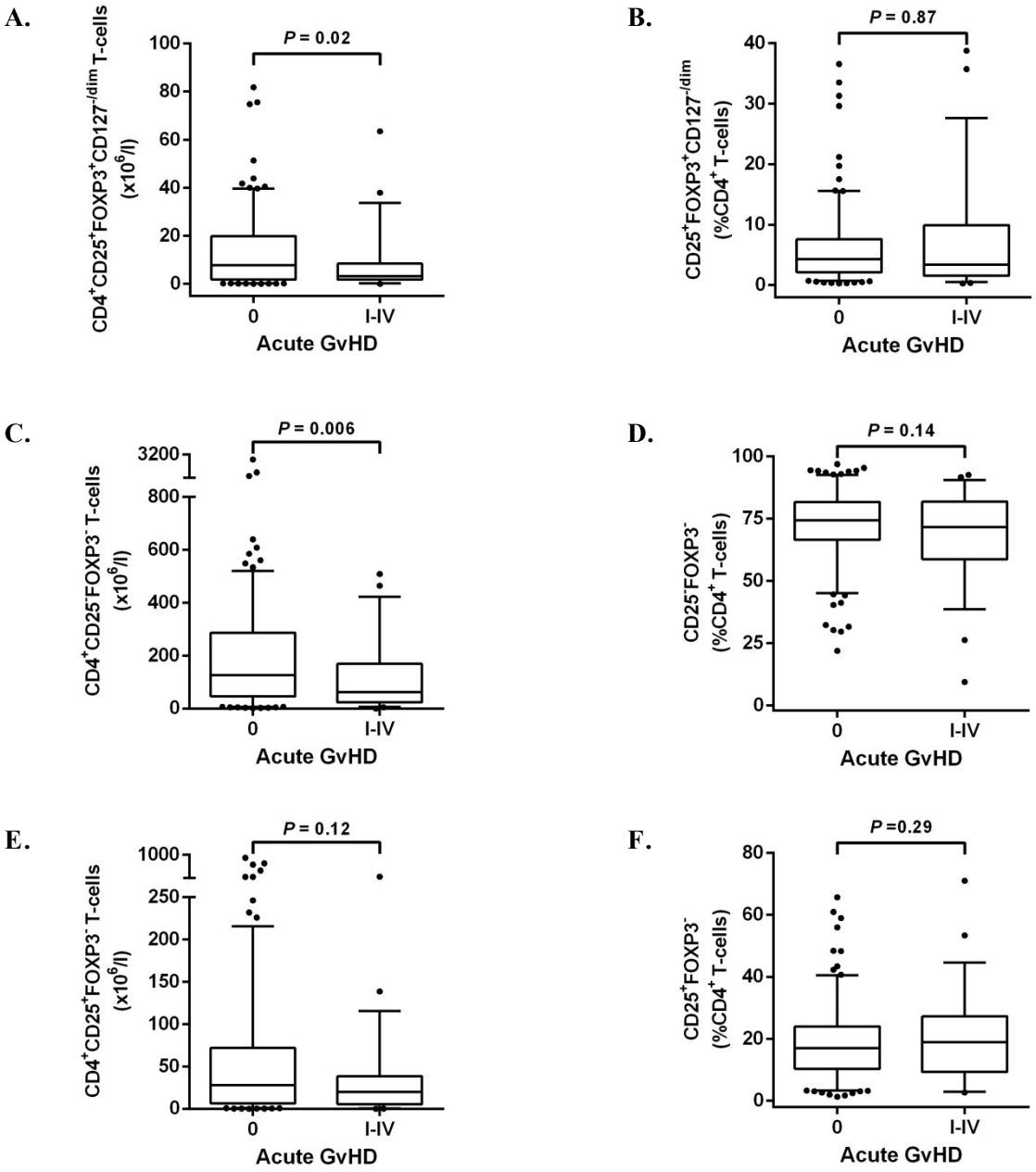


Figure 5.15: Peripheral Blood CD4+ T Cells in Acute GvHD (Grade I-IV)

$CD4^+$ T cells were measured in 240 peripheral blood samples collected within the first three months post-transplant. Box-and-whiskers plots analysing **(A)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l), **(B)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), **(C)** $CD4^+CD25^-FOXP3^-$ T-cell counts ($x10^6$/l), **(D)** Proportion of $CD25^-FOXP3^-$ cells (%$CD4^+$ T cells); **(E)** $CD4^+CD25^+FOXP3^-$ T-cell counts ($x10^6$/l), **(E)** Proportion of $CD25^+FOXP3^-$ cells (%$CD4^+$ T cells) according to the absence or presence of acute GvHD (grades I-IV) on the day of sampling. Data were analysed using the Mann-Whitney test.

Although the results were significant, comparison of the baseline characteristics of the two groups identified two important confounding factors that could contribute towards these findings (Table 5.19). A greater proportion of the aGvHD samples were from transplants using unrelated donors (80.4% v 53.6%, *P*=0.0003) and therefore received Alemtuzumab. As previously shown, Alemtuzumab conditioning is associated with lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts. In addition, patients had already commenced corticosteroids for the GvHD by the time the samples were collected. As such, a significantly greater proportion of the GvHD samples were collected from patients receiving corticosteroids (53.6% v 10.9%, *P*<0.0001). Since Alemtuzumab and corticosteroids are toxic to lymphocytes, both these factors could also reduce the lymphocyte and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts.

In view of these possible confounding factors, subgroup analysis was repeated on the Alemtuzumab and non-Alemtuzumab transplants separately, including only those samples that were collected before the patients started corticosteroids (Figure 5.16 & Table 5.20). In the Alemtuzumab transplants, no significant difference was observed in either the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell count ($2.67x10^6$/l (0.06-37.7) v 2.68 $x10^6$/l (0.02-63.5), *P*=0.49) or the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) (4.51% (0.30-36.6) v 2.36% (0.30-25.6%), *P*=0.58) between those samples taken in the presence and absence of aGvHD. However, the aGvHD samples had significantly higher $CD4^+CD25^+FOXP3^-$ T-cell counts ($19.6x10^6$/l (0.20-301) v $7.95x10^6$/l (0.10-159), *P*=0.03). For the non-Alemtuzumab transplants, the analysis could not be performed as there were insufficient samples in the GvHD group (*N*=2).

In summary, these findings suggest that aGvHD is associated with higher numbers of activated conventional T cells ($CD4^+CD25^+FOXP3^-$ T cells) but with no difference in either the absolute numbers or relative proportions of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (putative Tregs). Hence, aGvHD could be associated with a relative imbalance in the pro-inflammatory T-cell (effector) to regulatory T-cell (Treg) responses post-alloHSCT. However, this was not a randomised study and, therefore, it must be considered that other confounding factors may account for these data.

Alemtuzumab Transplants only		No GvHD (*N*=92)	GvHD (*N*=24)	*P*
Sample Time	Median (days)	61 (27 - 110)	48 (33 - 98)	0.57
Cell Counts				
Treg	x 10^6/l	2.67 (0.06 - 37.4)	2.68 (0.02 - 63.5)	0.49
Treg (%Lym)	%	0.59 (0.01 - 5.13)	0.91 (0.01 - 6.12)	0.23
Treg (%$CD3^+$)	%	1.72 (0.02 - 18.4)	1.70 (0.18 - 19.5)	0.96
Treg (%$CD3^+CD4^+$)	%	4.51 (0.30 - 36.6)	2.36 (0.30 - 25.6)	0.58
$CD25^-$ Tcon	x 10^6/l	53.7 (2.30 - 479)	66.4 (5.80 - 399)	0.24
$CD25^-$ (%$CD3^+CD4^+$)	%	77.6 (21.9 - 95.4)	75.1 (9.40 - 92.6)	0.25
$CD25^+$ Tcon	x 10^6/l	7.95 (0.10 - 159)	19.6 (0.20 - 301)	**0.03**
$CD25^+$ (%$CD3^+CD4^+$)	%	12.1 (1.30 - 33.6)	16.2 (2.70 - 71.0)	0.29
Lym	x 10^9/l	0.54 (0.01 - 2.73)	0.49 (0.12 -1.63)	0.51
$CD3^+$	x 10^9/l	0.13 (0.00 - 1.56)	0.17 (0.00 - 0.93)	0.43
$CD19^+$	x 10^9/l	0.06 (0.00 - 0.93)	0.04 (0.00 - 0.53)	0.25
$CD3^-CD56^+$	x 10^9/l	0.22 (0.00 - 1.10)	0.17 (0.03 - 0.77)	0.37
$CD3^+CD4^+$	x 10^9/l	0.07 (0.00 - 0.60)	0.12 (0.01 - 0.55)	0.07
$CD3^+CD8^+$	x 10^9/l	0.04 (0.00 - 1.04)	0.03 (0.00 - 0.79)	0.60
CD4 : CD8	Ratio	1.34 (0.06 - 2906)	3.23 (0.17 - 126)	0.11

Table 5.20: Comparison of the Peripheral Blood Lymphocyte Counts in the Presence and Absence of Acute GvHD (Alemtuzumab Conditioned Transplants Only)

Total lymphocyte, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD56^+$ and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell ('Treg'), $CD4^+CD25^-FOXP3^-$ T-cell ('$CD25^-$ Tcon') and $CD4^+CD25^+FOXP3^-$ T-cell ('$CD25^+$ Tcon') counts were measured in 116 peripheral blood samples collected within the first three months post-transplant from Alemtuzumab-conditioned alloHSCT that had not received corticosteroid therapy. Table shows the median values and range analysed according to the absence or presence of acute GvHD (all grades) on the day of sampling. Data were analysed using the Mann-Whitney test.

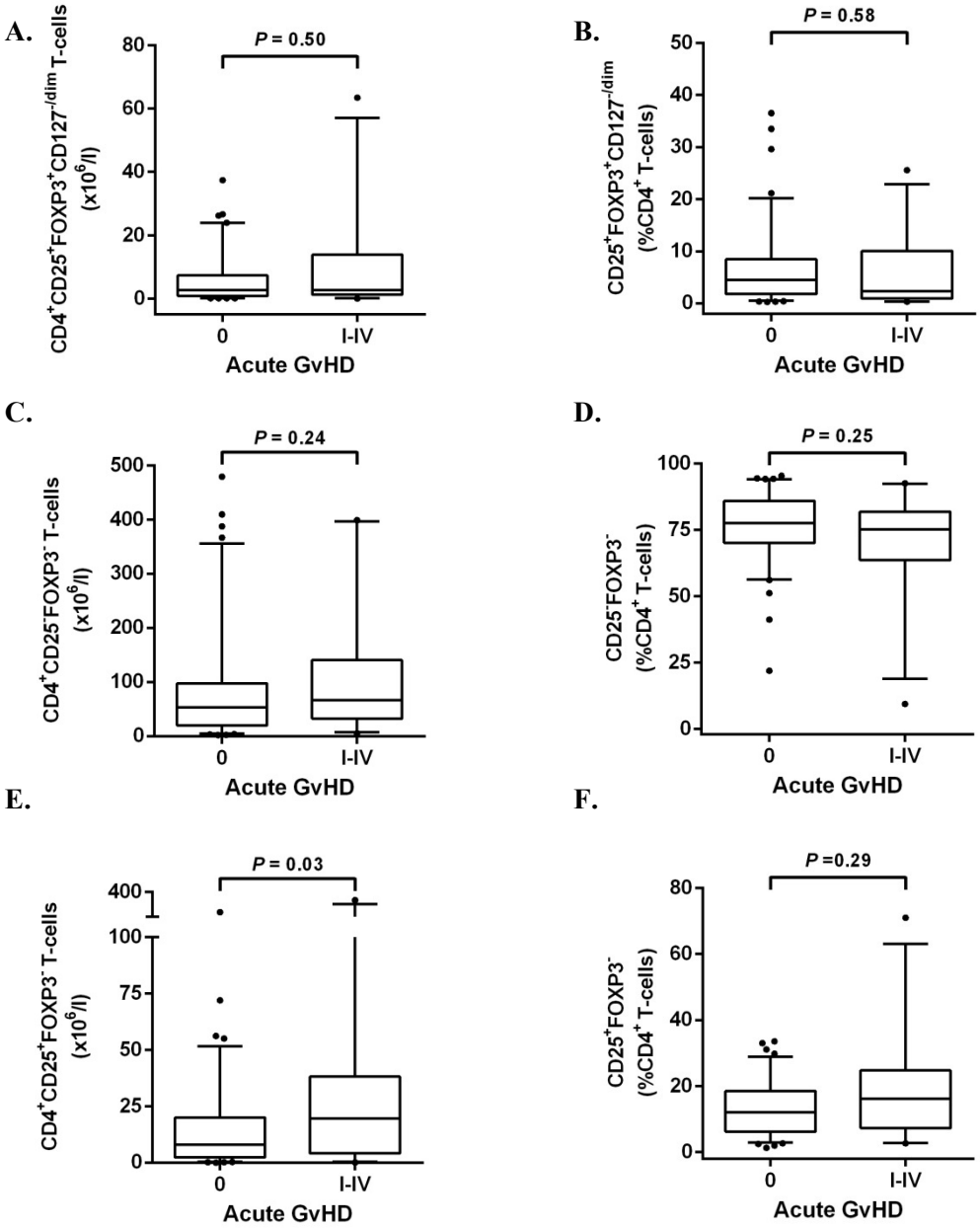


Figure 5.16: Peripheral Blood CD4+ T Cells in Acute GvHD (Grade I-IV) from Alemtuzumab Conditioned Transplants Only

$CD4^+$ T cells were measured in 116 peripheral blood samples collected within the first three months post-transplant from Alemtuzumab-conditioned alloHSCT that had not received corticosteroid therapy. Box-and-whiskers plots analysing **(A)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l), **(B)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), **(C)** $CD4^+CD25^-FOXP3^-$ T-cell counts ($x10^6$/l), **(D)** Proportion of $CD25^-FOXP3^-$ cells (%$CD4^+$ T cells), **(E)** $CD4^+CD25^+FOXP3^-$ T-cell counts ($x10^6$/l), **(F)** Proportion of $CD25^+FOXP3^-$ cells (%$CD4^+$ T cells) according to the absence or presence of acute GvHD (all grades) on the day of sampling according to the absence or presence of grade I-IV aGvHD (on the day of sampling). Data were analysed using the Mann-Whitney test.

B) CHRONIC GVHD

As well as aGvHD, this project also tested the hypothesis that cGvHD is associated with reduced $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells. Blood samples collected beyond three months were divided according to the presence (*N*=106) or absence (*N*=122) of cGvHD at the time the samples were collected. In keeping with the hypothesis, the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) were significantly lower in cGvHD (7.51% (0.29-48.1%) v 8.71% (0.34-29.9%); *P*=0.04) although there was no significant difference in the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts (Figure 5.17 & Table 5.21). However, as before, the two groups were not matched for other important confounding variables. The cGvHD group contained a greater proportion of samples from patients receiving prednisolone (44.3% v 13.3%; *P*<0.0001) and had a lower proportion of samples from Alemtuzumab treated patients (70.5% v 50.9%; *P*=0.003) (Table 5.22)

Chronic GvHD		No (*N*=122)	Yes (*N*=106)	*P*
Sample Time	Median (days)	167 (117 - 439)	177 (117-390)	0.44
Cell Counts				
Treg	x 10^6/l	18.3 (0.71 - 110)	20.8 (0.08 - 101)	0.80
Treg (%Lym)	%	1.78 (0.08 - 8.29)	1.78 (0.11 - 18.9)	0.86
Treg (%$CD3^+$)	%	3.40 (0.09 - 21.7)	3.07 (0.19 - 44.4)	0.15
Treg (%$CD3^+CD4^+$)	%	8.71 (0.34 - 29.9)	7.51 (0.29 - 48.1)	**0.04**
$CD25^-$ Tcon	x 10^6/l	145 (9.30 - 646)	172 (5.50 - 1390)	0.26
$CD25^-$ (%$CD3^+CD4^+$)	%	69.1 (36.4 - 89.3)	70.5 (2.90 - 93.2)	0.83
$CD25^+$ Tcon	x 10^6/l	37.3 (2.50 - 347)	38.4 (1.40 - 390)	0.15
$CD25^+$ (%$CD3^+CD4^+$)	%	16.1 (3.70 - 55.4)	17.6 (4.30 - 59.5)	0.33
Lymphocytes	x 10^9/l	1.09 (0.14 - 3.78)	0.98 (0.06 - 4.06)	0.57
$CD3^+$	x 10^9/l	0.55 (0.02 - 2.89)	0.59 (0.01 - 3.43)	0.63
$CD19^+$	x 10^9/l	0.18 (0.00 - 0.95)	0.12 (0.00 - 2.06)	**0.01**
$CD3^-CD56^+$	x 10^9/l	0.21 (0.04 - 2.21)	0.20 (0.01 - 0.92)	0.91
$CD3^+CD4^+$	x 10^9/l	0.22 (0.00 - 0.93)	0.28 (0.01 - 1.56)	0.14
$CD3^+CD8^+$	x 10^9/l	0.25 (0.00 - 2.05)	0.22 (0.00 - 1.97)	0.77
CD4 : CD8	Ratio	0.95 (0.11 - 30.9)	1.14 (0.12 - 266)	0.32

Table 5.21: Lymphocyte Counts in the Blood Samples Collected in the Presence and Absence of Chronic GvHD

Total lymphocyte, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD56^+$ and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell ('Treg'), $CD4^+CD25^-FOXP3^-$ T-cell ('$CD25^-$ Tcon') and $CD4^+CD25^+FOXP3^-$ T-cell ('$CD25^+$ Tcon') counts were measured in 228 peripheral blood samples collected beyond the first three months post-transplant (up to one year). Table shows the median counts and range analysed according to the absence or presence of cGvHD (all grades) on the day of sampling. Data were analysed using the Mann-Whitney test.

Chronic GvHD		**No** ***(N=122)***	**Yes** (*N*=106)	***P***
Immunosuppression				
Prednisolone	No/Yes	106 / 16	59 / 47	**<0.0001**
Recipient				
Age	Median (years)	47 (18-74)	48 (18-74)	0.62
Gender	Male/Female	71 / 51	67 / 39	0.50
CMV	IgG -/+	75 / 47	63 / 43	0.79
Donor				
Age	Median (years)	36 (19-62)	41 (19-68)	0.06
Gender	Male/Female	92 / 30	73 / 33	0.30
CMV	IgG -/+	91 / 31	64 / 42	**0.02**
Transplant				
Conditioning	RIC/MAC	108 / 14	88 / 18	0.26
Source	PBSC/BM	122 / 0	106 / 0	**-**
Donor	Sib/Un	41 / 81	55 / 51	**0.007**
Alemtuzumab	No/Yes	36 / 86	52 / 54	**0.003**
HLA mismatch	No/Yes	104 / 18	93 / 13	0.70
Gender mismatch	FM/Other	107 / 13	89 / 15	0.43
ABO mismatch	No/Yes	98 / 24	86 / 20	1.00

Table 5.22: Baseline Characteristics of the Blood Samples Collected in the Presence and Absence of Chronic GvHD

228 peripheral blood samples were collected beyond three months post-alloHSCT (up to one year) and divided into two groups according to the absence (*N*=122) or presence (*N*=106) of chronic GvHD (all grades) at the time of sampling. Table shows the baseline transplant characteristics of the two groups. Data were analysed using the Fisher's test.

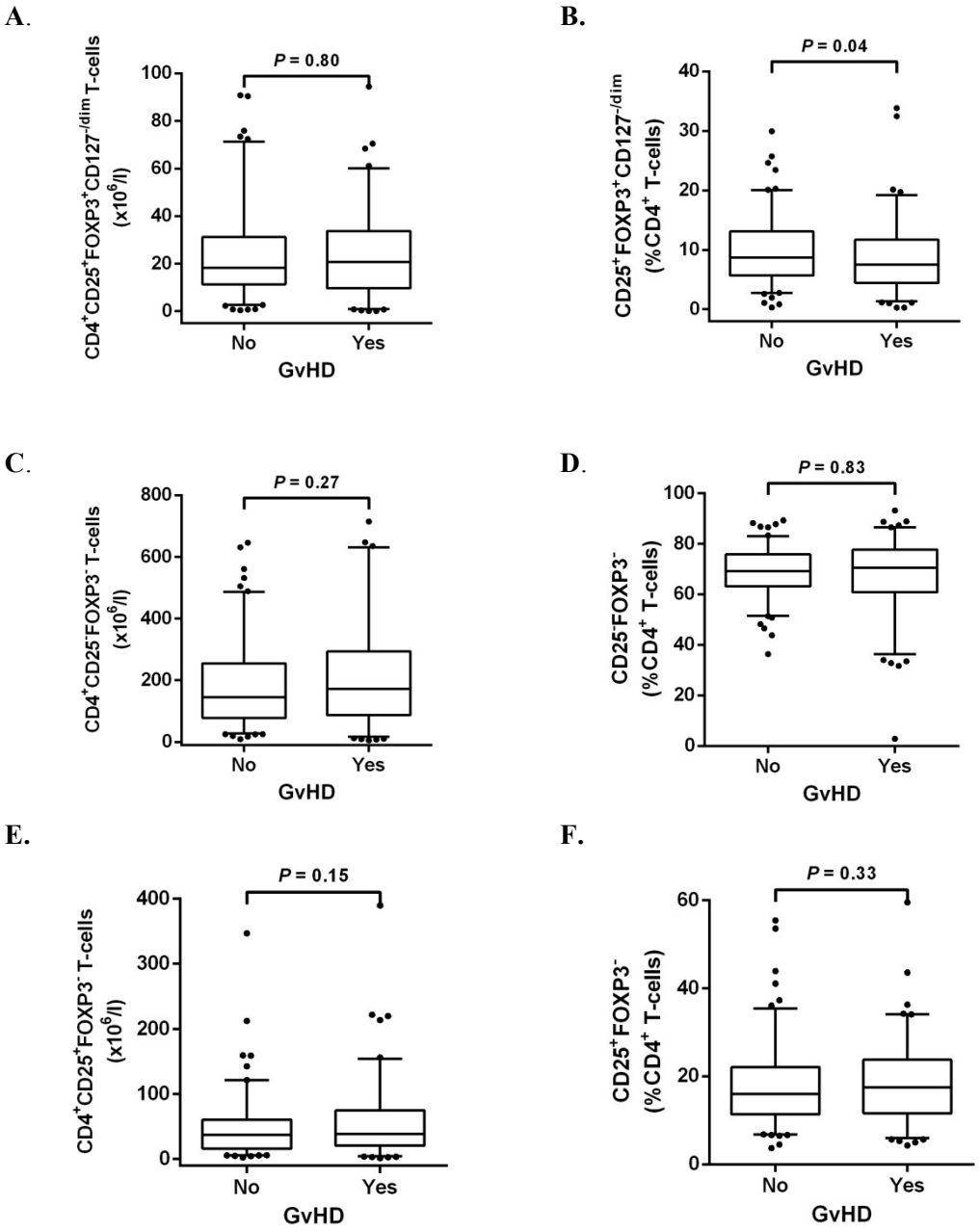


Figure 5.17: Peripheral Blood CD4+ T Cells in Chronic GvHD

$CD4^+$ T cells were measured in 228 peripheral blood samples collected beyond three months post-transplant (up to one year). Box-and-whiskers plots analysing **(A)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l), **(B)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), **(C)** $CD4^+CD25^-FOXP3^-$ T-cell counts ($x10^6$/l), **(D)** Proportion of $CD25^-FOXP3^-$ cells (%$CD4^+$ T cells), **(E)** $CD4^+CD25^+FOXP3^-$ T-cell counts ($x10^6$/l), **(F)** Proportion of $CD25^+FOXP3^-$ cells (%$CD4^+$ T cells) according to the absence or presence of cGvHD (all grades) on the day of sampling. Data were analysed using the Mann-Whitney test.

On subgroup analysis of the Alemtuzumab and non-Alemtuzumab transplants separately, using only samples taken from patients not taking corticosteroids, there was no significant difference observed in either the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts or the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in association with cGvHD (Table 5.23). However, in the Alemtuzumab conditioned transplants, the absolute number of activated $CD4^{+}CD25^{+}FOXP3^{-}$ T cells was significantly higher in the cGvHD samples ($40.0x10^{6}$/l (3.80-220) v $29.8x10^{6}$/l (4.60-212), P=0.03). This difference was not observed in the relative proportions of $CD4^{+}CD25^{+}FOXP3^{-}$ T cells (%$CD4^{+}$ T cells) or in the non-Alemtuzumab conditioned transplants.

In summary, in Alemtuzumab conditioned transplants, cGvHD is associated with higher numbers of $CD4^{+}CD25^{+}FOXP3^{-}$ T cells (activated conventional T cells) but with no significant difference in either the absolute numbers or relative proportions of $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells (putative Tregs). In keeping with the aGvHD data, this indicates that cGvHD could also be associated with a relative imbalance in pro-inflammatory T cells to regulatory T cells post-alloHSCT. However, once again, this was not a randomised study and, therefore, other confounding variables may account for these findings. Furthermore, this pattern was not observed with non-Alemtuzumab alloHSCT. Although the number of samples was smaller in this cohort, it may be hypothesised that cGvHD seen in T-replete alloHSCT (no Alemtuzumab) is associated with a different pattern of T-cell mediated responses.

Chronic GvHD		No	Yes	*P*
No Alemtuzumab		*N*=31	*N*=18	
Treg	x 10^6/l	26.0 (4.96 -110)	22.9 (5.93-70.4)	0.44
Treg (%$CD3^+CD4^+$)	%	8.68 (1.97 - 18.3)	6.89 (1.63 - 15.6)	0.24
$CD25^-$ Tcon	x 10^6/l	227 (102 - 631)	253 (138 - 458)	0.66
$CD25^-$ (%$CD3^+CD4^+$)	%	69.8 (50.8 - 79.8)	72.0 (56.0 - 82.3)	0.52
$CD25^+$ Tcon	x 10^6/l	72.5 (19.1 - 347)	69.9 (20.7 - 143)	1.00
$CD25^+$ (%$CD3^+CD4^+$)	%	18.4 (7.80 - 37.3)	18.5 (6.60 - 36.3)	0.89
Alemtuzumab		*N*=75	*N*=41	
Treg	x 10^6/l	17.3 (2.29 - 90.8)	25.6 (1.29 - 94.5)	0.90
Treg (%$CD3^+CD4^+$)	%	9.76 (2.82 - 29.9)	11.6 (2.24 - 48.1)	0.06
$CD25^-$ Tcon	x 10^6/l	115 (25.0 - 646)	153 (5.50 - 625)	0.30
$CD25^-$ (%$CD3^+CD4^+$)	%	68.8 (36.4 - 89.3)	65.5 (29.0 - 87.3)	0.09
$CD25^+$ Tcon	x 10^6/l	29.8 (4.60 - 212)	40.0 (3.80 - 220)	**0.03**
$CD25^+$ (%$CD3^+CD4^+$)	%	14.2 (3.70 - 55.4)	17.6 (5.30 - 59.50	0.06

Table 5.23: Comparison of Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-Cell Counts in the Presence and Absence of Chronic GvHD

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ('Treg'), $CD4^+CD25^-FOXP3^-$ T-cell ('$CD25^-$ Tcon') and $CD4^+CD25^+FOXP3^-$ T-cell ('$CD25^+$ Tcon') were analysed in 165 peripheral blood samples collected from three months to one year post-transplant from patients that not taking corticosteroids. Table shows the median values and ranges for the absolute counts (x10^6/l) and the relative proportion of the cells (%$CD4^+$ T cells) in the absence or presence of chronic GvHD symptoms (all grades) on the day of sampling. Data were analysed separately for the Alemtuzumab and non-Alemtuzumab conditioned transplants using the Mann-Whitney test.

C) CMV REACTIVATION

Given the earlier findings that CMV reactivation was significantly associated with low PB $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (Chapter 5.3.2), this work also investigated whether $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were reduced in the presence of CMV reactivation. To test this hypothesis, post-transplant samples were separated according to the presence or absence of detectable CMV DNA at the time of sample collection. Of note, only samples collected in the first three months were analysed as this was the period in which regular CMV PCR data were available. Of the 240 samples analysed, 53 were positive for CMV.

In keeping with the previous findings, blood samples collected from patients positive for CMV had lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($2.48x10^6$/l (0.02-25.4) v $8.28x10^6$/l (0.09-81.8); $P<0.0001$) and lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) (2.67% (0.36-35.7) v 4.60% (0.30-38.8); $P<0.0001$) (Figure 5.18 & Table 5.24). In addition, these samples also had significantly lower absolute $CD4^+$ T-cell, $CD4^+CD25^-FOXP3^-$ T-cell and $CD4^+CD25^+FOXP3^-$ T-cell counts; lower proportions of $CD25^+FOXP3^-$ cells (%$CD4^+$ T cells); lower CD4:CD8 T-cell ratios. In contrast, the relative proportion of $CD25^-FOXP3^-$ cells (%$CD4^+$ T cells) was significantly higher in the CMV positive blood samples (Table 5.24).

CMV		CMV PCR Neg (*N*=187)	CMV PCR Pos (*N*=53)	*P*
Sample Time	Median (days)	61 (27 - 110)	62 (27 - 98)	0.18
Cell Counts				
Treg	x 10^6/l	8.28 (0.09 - 81.8)	2.48 (0.02 - 25.4)	**<0.0001**
Treg (%Lym)	%	1.19 (0.04 - 16.7)	0.39 (0.01 - 2.14)	**<0.0001**
Treg (%$CD3^{+)}$	%	2.50 (0.21 - 22.4)	0.99 (0.02 - 19.1)	**<0.0001**
Treg (%$CD3^+CD4^+$)	%	4.60 (0.30 - 38.8)	2.67 (0.36 - 35.7)	**<0.0001**
$CD25^-$ Tcon	10^6/l	135 (2.31 - 2690)	79.9 (0.60 - 437)	**0.006**
$CD25^-$ (%$CD3^+CD4^+$)	%	72.2 (9.40 - 96.9)	80.5 (30.3 - 95.4)	**<0.0001**
$CD25^+$ Tcon	x 10^6/l	31.6 (0.10 - 905)	12.1 (0.20 - 226)	**<0.0001**
$CD25^+$ (%$CD3^+CD4^+$)	%	18.3 (1.70 - 71.0)	12.9 (1.30 - 65.7)	**0.0001**
Lymphocytes	x 10^9/l	0.70 (0.01 - 5.77)	0.63 (0.05 - 4.63)	0.56
$CD3^+$	x 10^9/l	0.34 (0.00 - 4.94)	0.25 (0.00 - 3.90)	0.53
$CD19^+$	x 10^9/l	0.03 (0.00 - 0.94)	0.03 (0.00 - 0.58)	0.87
$CD3^-CD56^+$	x 10^9/l	0.22 (0.00 - 1.18)	0.23 (0.02 - 1.10)	0.61
$CD3^+CD4^+$	x 10^9/l	0.20 (0.00 - 2.78)	0.10 (0.00 - 0.61)	**0.001**
$CD3^+CD8^+$	x 10^9/l	0.11 (0.00 - 3.55)	0.13 (0.00 - 3.23)	0.30
CD4 : CD8	Ratio	1.73 (0.11 - 2906)	0.53 (0.06 - 37.3)	**<0.0001**

Table 5.24: Lymphocyte Counts in the Blood Samples Collected in the Presence and Absence of CMV Reactivation

Total lymphocyte, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, $CD3^-CD56^+$ and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell ('Treg'), $CD4^+CD25^-FOXP3^-$ T-cell ('$CD25^-$ Tcon') and $CD4^+CD25^+FOXP3^-$ T-cell ('$CD25^+$ Tcon') counts were measured in 240 peripheral blood samples collected within the first three months post-transplant. Table shows the median counts and range analysed according to CMV PCR results on the day of sampling (Pos >1000copies/ml). Data were analysed using the Mann-Whitney test.

CMV		CMV PCR Neg (*N*=187)	CMV PCR Pos (*N*=53)	*P*
Immunosuppression				
Prednisolone	No/Yes	153 / 34	37 / 16	0.08
GvHD				
GvHD 0-IV	No/Yes	146 / 41	38 /15	0.36
GvHD II-IV	No/Yes	171 / 16	46 / 7	0.30
Recipient				
Age	Median (years)	47 (18-74)	48 (18-74)	0.85
Gender	Male/Female	109 / 78	32 / 21	0.87
CMV	IgG -/+	134 / 53	4 / 49	**<0.0001**
Donor				
Age	Median (years)	39 (21-68)	36 (19-68)	0.05
Gender	Male/Female	134 / 53	34 / 19	0.31
CMV	IgG -/+	134 /53	28 / 25	**0.01**
Transplant				
Conditioning	RIC/MAC	158 / 29	45 / 8	1.00
Source	PBSCH/BM	184 / 3	52 / 1	1.00
Donor	Sib/Un	87 / 100	10 / 43	**0.0002**
Alemtuzumab	No/Yes	83 / 104	8 / 45	**<0.0001**
HLA mismatch	No/Yes	159 / 28	42 / 11	0.30
Gender mismatch	FM/Other	156 / 31	47 / 6	0.40
ABO mismatch	No/Yes	148 / 39	48 / 5	0.07

Table 5.25: Baseline Characteristics of the Blood Samples Collected in the Presence and Absence of CMV Reactivation

240 peripheral blood samples were collected within the first three months post-alloHSCT and divided into two groups according to the CMV PCR results on the day of sampling (Pos >1000copies/ml). Table shows the baseline transplant characteristics of the two groups. Data were analysed using the Fisher's test.

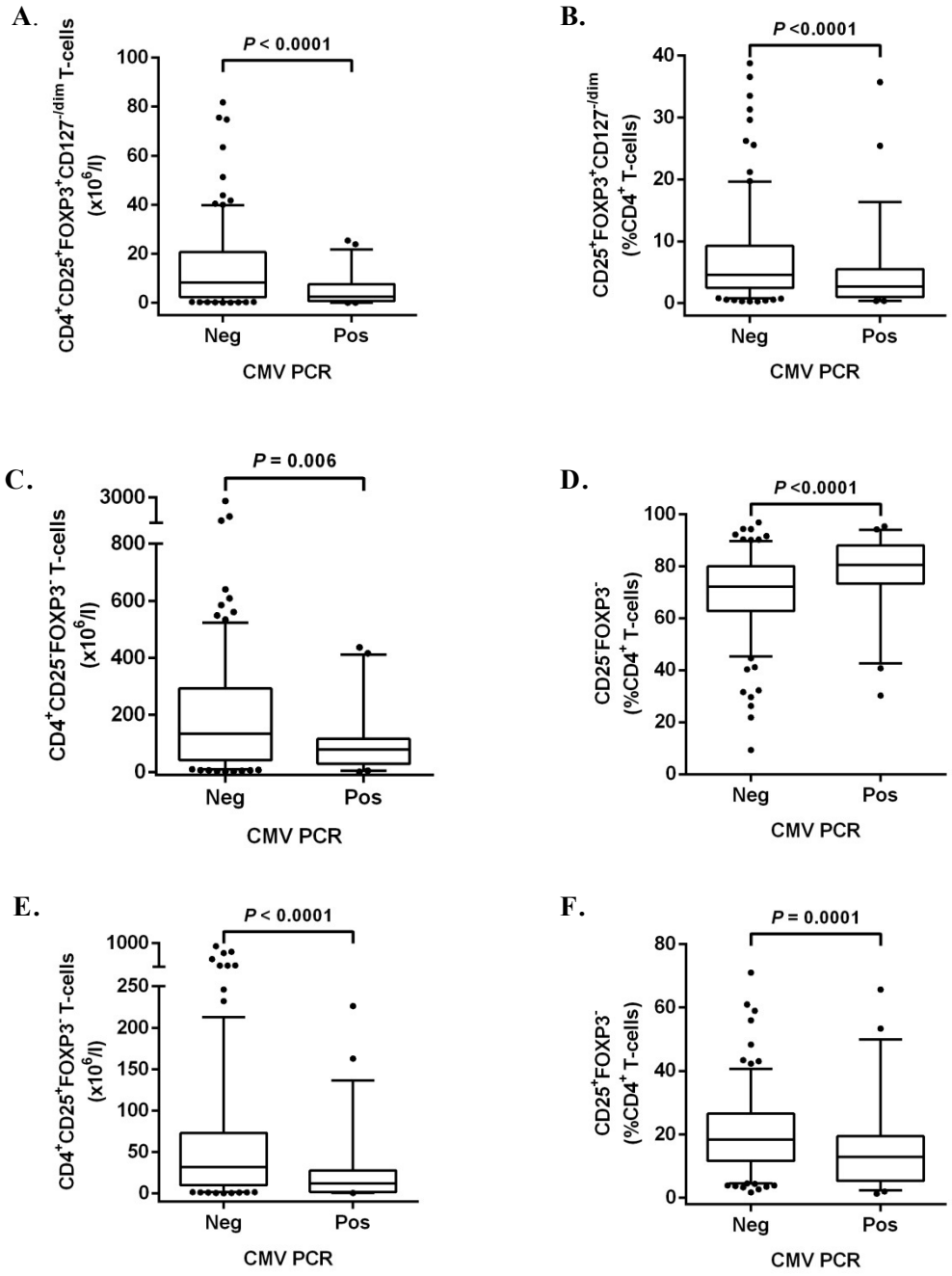


Figure 5.18: Peripheral Blood CD4+ T Cells in CMV Reactivation

$CD4^+$ T cells were measured in 240 peripheral blood samples collected within the first three months post-transplant. Box-and-whiskers plots analysing **(A)** $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l), **(B)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells), **(C)** $CD4^+CD25^-FOXP3^-$ T-cell counts ($x10^6$/l), **(D)** Proportion of $CD25^-FOXP3^-$ cells (%$CD4^+$ T cells), **(E)** $CD4^+CD25^+FOXP3^-$ T-cell counts ($x10^6$/l), **(F)** Proportion of $CD25^+FOXP3^-$ cells (%$CD4^+$ T cells) according to the CMV PCR results on the day of sampling (Pos >1000copies/ml). Data were analysed using the Mann-Whitney test.

As with GvHD, analysis of the baseline characteristics of these two groups, however, demonstrated that a greater proportion of the CMV positive samples were from transplants using Alemtuzumab conditioning (84.9% v 55.6%; $P<0.0001$) (Table 5.25). Subgroup analysis of the Alemtuzumab and non-Alemtuzumab transplants separately still demonstrated lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the CMV positive samples, although the difference in the $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the non-Alemtuzumab group was not statistically significant (Figure 5.19 & Table 5.26). In the Alemtuzumab conditioned transplants, the absolute numbers and relative proportions of $CD4^+CD25^+FOXP3^-$ T cells (activated conventional $CD4^+$ T cells) were lower in the CMV positive samples, although the absolute counts only showed a non-significant trend ($P=0.07$). In contrast, the relative proportion of $CD4^+CD25^-FOXP3^-$ T cells was significantly higher in these samples but there was no significant difference in the absolute $CD4^+CD25^-FOXP3^-$ T-cell counts. In the non-Alemtuzumab conditioned transplants, there were no significant difference observed in either $CD4^+CD25^+FOXP3^-$ T cells or $CD4^+CD25^-FOXP3^-$ T cells (absolute counts and relative proportions). However, there were only eight CMV-positive samples available for analysis within this subgroup.

CMV		No	Yes	*P*
No Alemtuzumab		*N*=83	*N*=8	
Treg	x 10^6/l	19.1 (2.66 - 81.8)	8.48 (1.71 - 25.4)	**0.02**
Treg (%$CD3^+CD4^+$)	%	4.54 (0.58 - 31.3)	3.16 (0.82 - 6.36)	0.11
$CD25^-$ Tcon	x 10^6/l	286 (16.1 - 2690)	171 (38.8 - 437)	0.08
$CD25^-$ (%$CD3^+CD4^+$)	%	70.9 (29.7 - 96.9)	72.0 (30.3 - 86.7)	0.95
$CD25^+$ Tcon	x 10^6/l	78.8 (4.20 - 905)	70.8 (12.1 - 226)	0.66
$CD25^+$ (%$CD3^+CD4^+$)	%	21.0 (1.70 - 61.0)	24.6 (9.20 - 65.7)	0.58
Alemtuzumab		*N*=104	*N*=45	
Treg	x 10^6/l	3.09 (0.09 - 63.5)	1.94 (0.02 - 23.9)	**0.02**
Treg (%$CD3^+CD4^+$)	%	4.88 (0.30 - 38.8)	2.15 (0.36 - 35.7)	**0.001**
$CD25^-$ Tcon	x 10^6/l	48.8 (2.30 - 479)	67.9 (0.60 - 410)	0.44
$CD25^-$ (%$CD3^+CD4^+$)	%	73.0 (9.40 - 94.4)	80.5 (40.8 - 95.4)	**<0.0001**
$CD25^+$ Tcon	x 10^6/l	10.3 (0.10 - 301)	7.50 (0.20 - 55.1)	0.07
$CD25^+$ (%$CD3^+CD4^+$)	%	16.3 (2.70 - 71.0)	11.6 (1.30 - 33.1)	**0.001**

Table 5.26: Comparison of Peripheral Blood $CD4^+$ T-Cell Counts in the Presence and Absence of CMV Reactivation

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell ('Treg'), $CD4^+CD25^-FOXP3^-$ T-cell ('$CD25^-$ Tcon') and $CD4^+CD25^+FOXP3^-$ T-cell ('$CD25^+$ Tcon') counts were measured in 240 peripheral blood samples collected within the first three months post-transplant. Table shows the median values and range for the absolute counts (x10^6/l) and the relative proportions (%$CD4^+$ T cells) according to the CMV PCR results on the day of sampling (Pos>1000copies/ml). Data were analysed separately for the Alemtuzumab and non-Alemtuzumab conditioned transplants using the Mann-Whitney test.

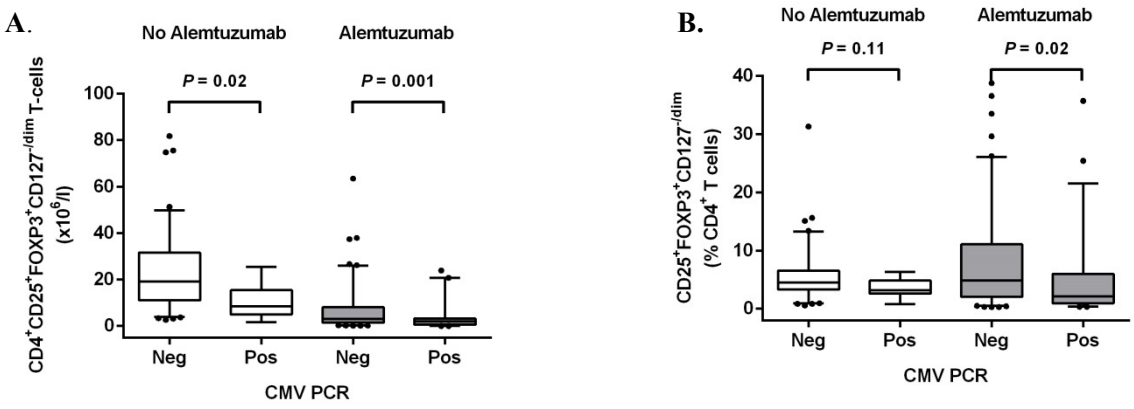


Figure 5.19: Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells in CMV Reactivation

$CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were measured in 240 peripheral blood samples collected within the first three months post-transplant. Box-and-whiskers plots analysing the (A) $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$/l); **(B)** Proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) according to the CMV PCR results on the day of sampling (Pos >1000copies/ml). Data were analysed separately for the Alemtuzumab and non-Alemtuzumab conditioned transplants using the Mann-Whitney test.

Taken together, these data indicate that CMV reactivation post-alloHSCT is associated with lower absolute numbers and relative proportions of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (putative Tregs) in the peripheral blood. In the Alemtuzumab-conditioned transplants, CMV reactivation is also associated with lower absolute numbers and proportions of $CD4^+CD25^+FOXP3^-$ T cells ('activated' T cells) but higher proportions of $CD4^+CD25^-FOXP3^-$ T cells. These data may suggest that CMV reactivation reduces $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and/or activation of conventional $CD4^+$ T cells. However, these observations could also be due to the Ganciclovir treatment, rather than CMV reactivation *per se*. Alternatively, it may be hypothesised that alloHSCT with lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and/or lower $CD4^+CD25^+FOXP3^-$ T cells have increased risk of CMV reactivation. In keeping with this hypothesis, Pastore and colleagues (2011) showed a significantly lower peripheral $CD4^+CD25^{high}FOXP3^+$ Tregs in patients with CMV infection. In turn, it was proposed that $CD4^+CD25^{high}FOXP3^+$ Tregs prevent GvHD, allowing immune reconstitution and promote the recovery of CMV-specific $CD8^+$ T cells post-alloHSCT.

5.3.4 PREDICTING TRANSPLANT OUTCOMES

In view of the earlier findings that low proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) in the graft were associated with poor engraftment, increased NRM and worse OS (Chapter 4), it was hypothesised that poor $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell recovery post-alloHSCT may increase the risk of GvHD, NRM and reduce OS. To examine this hypothesis, the transplants were divided into two groups according to the median $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells (counts or %$CD4^{+}$ T cells) in the PB at one and three months post-transplant. The cumulative incidence of transplant related events (aGvHD (NIH grade II-IV), cGvHD (NIH all grades), CMV reactivation, disease relapse, NRM and OS) in these two groups were compared. Since this analysis was investigating the prospective development of these outcomes, any patient that had already had that event by the time of sampling was excluded from analysis. Therefore, the patients assessed for the development of aGvHD or CMV were not on corticosteroids or CMV therapy (Val/ganciclovir) at the time the blood samples were collected.

A) ONE MONTH

91 transplants were assessed at one month post-transplant. Eighteen patients had already developed aGvHD and seventeen patients had CMV reactivation and were therefore excluded from their respective analyses. In univariate cumulative incidence analysis, patients with low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts at one month post-transplant had no significant difference in the incidence of aGvHD, cGvHD, CMV reactivation, disease relapse or NRM by one year. However, in contrast to the hypothesis, patients with low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts had a non-significant trend towards improved overall survival at one year (80.2% (67.8-94.9) v 54.7% (40.7-73.6), P=0.053). There was no difference in one year overall survival when using the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) at one month (Table 5.27).

Therefore, these data indicate that the peripheral blood $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell numbers (absolute counts or relative proportions) at one month post-transplant do not significantly predict the subsequent development of transplant-related complications or overall survival.

Outcome	Cell type	Group	*N*	Probability (%)	95% CI (%)	*P*
Acute GvHD (Grade II-IV)	Tregs	Low	36	22.5	9.64 - 38.7	0.49
		High	37	34.4	18.3 - 51.0	
	%Tregs	Low	36	32.6	16.6 - 49.7	0.37
		High	37	25.5	11.7 - 41.9	
Chronic GvHD (All grades)	Tregs	Low	45	41.3	23.3 - 58.5	0.90
		High	46	48.0	29.8 - 64.0	
	%Tregs	Low	45	48.1	29.2 - 64.8	0.38
		High	46	43.3	25.7 - 59.6	
CMV	Tregs	Low	37	22.0	10.2 - 36.7	0.85
		High	37	25.5	11.6 - 41.9	
	%Tregs	Low	37	24.2	10.9 - 40.3	0.88
		High	37	23.7	10.9 - 39.3	
Relapse	Tregs	Low	45	25.0	11.0 - 41.9	0.97
		High	46	22.8	11.1 - 37.0	
	%Tregs	Low	45	17.3	6.62 - 32.3	0.28
		High	46	28.6	15.1 - 43.7	
NRM	Tregs	Low	45	8.03	1.92 - 20.0	0.19
		High	46	21.2	9.66 - 35.7	
	%Tregs	Low	45	13.7	4.73 - 27.2	0.95
		High	46	16.5	6.42 - 30.7	
Overall Survival	Tregs	Low	45	80.2	67.8 - 94.9	0.05
		High	46	54.7	40.7 - 73.6	
	%Tregs	Low	45	74.8	61.5 - 91.0	0.32
		High	46	59.9	45.2 -78.0	

Table 5.27: Cumulative Incidence and Survival Analysis According to the Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells at One Month Post-Transplant

Peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$) ('Tregs') and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) ('%Tregs') were measured in 91 alloHSCT at one month post-transplant *(18 transplants already had aGvHD and 17 transplants already had CMV reactivation by one month and were therefore excluded from their respective analysis).* Blood samples were collected in EDTA and analysed using flow cytometry. The alloHSCT were grouped above ('High') or below ('Low') the one month median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell count or median proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) and the cumulative incidence of aGvHD (grade II-IV), cGvHD (all grades), CMV reactivation, disease relapse and NRM and the probability of overall survival were calculated for the 'High' and 'Low' groups respectively. Table shows the probability (%), 95% confidence intervals (%) and *P*-values at one year post-transplant.

B) THREE MONTHS

Blood samples were collected from 67 transplants at three months. At the time of sampling, 23 patients had already developed grade II-IV aGvHD, two patients had cGvHD, 22 patients had CMV reactivation and two patients had relapsed disease.

In univariate analysis, patients with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute counts or $\%CD4^+$ T cells) three months post-transplant had no significant difference in the incidence of delayed grade II-IV aGvHD, CMV reactivation, relapse, NRM or overall survival (Table 5.28). However, patients with low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) at three months had a significantly higher incidence of developing cGvHD (66.2% (40.2-82.9%) v 33.3% (16.6-51.7%); *P*=0.03) (Figure 5.20A). This difference was not observed when analysing the data using the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts. Since the relative proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) could be altered by changes in the absolute numbers of 'non-Tregs', the cGvHD data were also analysed using the three month absolute $CD4^+CD25^-FOXP3^-$ T-cell counts (conventional $CD4^+$ T cells) and $CD4^+CD25^+FOXP3^-$ T-cell counts (activated $CD4^+$ T cells) (Figure 5.20C&D). As with $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts, patients with low $CD4^+CD25^-FOXP3^-$ T cells or low $CD4^+CD25^+FOXP3^-$ T cells at three months post-transplant had no significant difference in the cumulative incidence of cGvHD (36.1% (17.4-55.3%) v 59.1% (36.3-76.1%), *P*=0.19; 53.7% (30.0-72.5%) v 43.9% (34.2-62.0), *P*=0.23) (Figure 5.20C&D).

Outcome	Cell type	Group	*N*	Probability (%)	95% CI (%)	*P*
Acute GvHD (Delayed II-IV)	Tregs	Low	22	10.0	1.60 - 27.8	0.06
		High	22	33.5	14.4 - 54.0	
	%Tregs	Low	22	23.6	8.19 - 43.5	0.92
		High	22	19.9	5.89 - 40.0	
Chronic GvHD (All grades)	Tregs	Low	32	54.5	30.8 - 73.1	0.22
		High	33	43.3	23.7 - 61.5	
	%Tregs	Low	32	66.2	40.2 - 82.9	**0.03**
		High	33	33.3	16.6 - 51.7	
CMV	Tregs	Low	22	11.9	1.82 - 32.2	0.48
		High	23	4.76	0.30 - 20.2	
	%Tregs	Low	22	15.8	3.72 - 35.5	0.08
		High	23	0.00	-	
Relapse	Tregs	Low	32	14.1	4.30 - 29.7	0.45
		High	33	25.2	10.6 - 42.9	
	%Tregs	Low	32	23.4	10.1 - 34.9	0.26
		High	33	16.6	4.87 - 34.5	
NRM	Tregs	Low	33	16.2	4.70 - 33.7	0.12
		High	34	3.49	0.24 - 15.5	
	%Tregs	Low	33	9.03	1.42 - 25.7	0.66
		High	34	10.2	2.43 - 24.2	
Overall Survival	Tregs	Low	33	75.7	60.3 - 95.1	0.97
		High	34	76.1	61.9 - 93.5	
	%Tregs	Low	33	75.1	59.4 - 94.8	0.72
		High	34	76.2	62.2 - 93.3	

Table 5.28: Cumulative Incidence and Survival Analysis According to the Peripheral Blood $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T Cells at Three Months Post-Transplant

Peripheral blood $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts ($x10^{6}$) ('Tregs') and the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) ('%Tregs') were measured in 67 alloHSCT at three months post-transplant *(23 transplants already had delayed aGvHD, 2 transplants already had cGvHD, 23 transplants already had CMV reactivation and 2 transplants already had disease relapse by three months and were therefore excluded from their respective analysis).* Blood samples were collected in EDTA and analysed using flow cytometry. The alloHSCT were grouped above ('High') or below ('Low') the three month median $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell count or median proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) and the cumulative incidence of aGvHD (grade II-IV), cGvHD (all grades), CMV reactivation, disease relapse and NRM and the probability of overall survival were calculated for the 'High' and 'Low' groups respectively. Table shows the probability (%), 95% confidence intervals (%) and *P*-values at one year post-transplant.

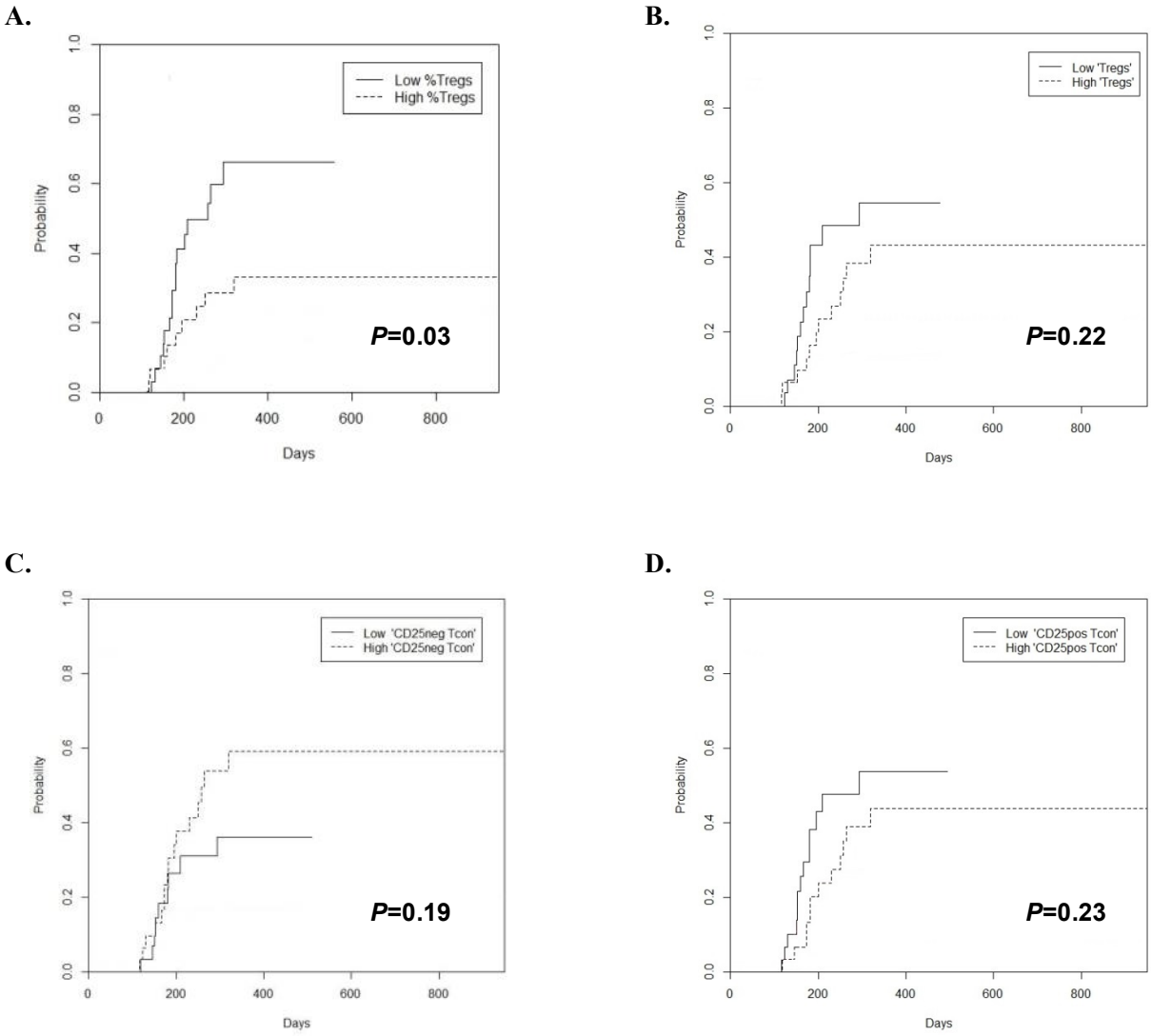


Figure 5.20: Cumulative Incidence of Chronic GvHD According to the Peripheral Blood Cell Counts at Three Months Post-Transplant

Peripheral blood **(A)** $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) ('%Tregs'), **(B)** $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells (x10^{6}/l) ('Tregs'), **(C)** $CD4^{+}CD25^{-}FOXP3^{-}$ T cells (x10^{6}/l) ('CD25neg Tcon') and **(D)** $CD4^{+}CD25^{+}FOXP3^{-}$ T cells (x10^{6}/l) ('CD25pos Tcon') were measured in 65 alloHSCT at three months post-transplant. Blood samples were collected in EDTA and analysed using flow cytometry. The 65 alloHSCT were grouped above ('High') or below ('Low') the median values for each cell population respectively and the cumulative incidence of cGvHD (all grades) analysed.

In light of these findings, it was necessary to determine which other factors were also associated with increased cGvHD in this patient cohort and whether the three month proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) was an independent predictor of cGvHD. Other variables tested in univariate analysis were the recipient and donor age/gender; female-to-male donation; intensity of conditioning; donor type; Alemtuzumab; HLA matching; graft counts ($CD34^{+}$/$CD3^{+}$/$CD19^{+}$/$CD3^{-}CD56^{+}$/$CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts, proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) as per Chapter 4.3.4). Those variables significantly associated with an increased risk of cGvHD in this cohort were male recipients, sibling donors and transplants not using Alemtuzumab conditioning (Table 5.29A). When combined in multivariate analysis, only Alemtuzumab remained independently associated with cGvHD (HR 0.42 (0.20-0.91); *P*=0.03) (Table 5.29B).

Taken together, these data indicate that the peripheral blood $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell numbers (absolute counts or relative proportions) at three months post-transplant do not independently predict the subsequent development of cGvHD or other transplant-related events.

A. Univariate analysis					
Variable	**Group**	***N***	**cGvHD** (%)	**95% CI** (%)	***P***
Recipient Gender	Male	35	65.4	41.5 - 81.4	**0.03**
	Female	30	28.6	12.0 - 47.7	
Donor	Sib	26	68.1	41.6 - 84.5	**0.03**
	Un	39	33.1	16.6 - 50.6	
Alemtuzumab	No	24	69.0	41.6 - 85.4	**0.03**
	Yes	41	34.2	17.9 - 51.3	
3 month %Tregs	Low	32	66.2	40.2 - 82.9	**0.03**
	High	33	33.0	16.0 - 51.7	

B. Multivariate analysis				
Variable	**Group**	**HR**	**95% CI**	***P***
Alemtuzumab	No	1	-	-
	Yes	0.42	0.20 - 0.91	**0.03**

Table 5.29: Univariate and Multivariate Analysis of Chronic GvHD beyond Three Months Post-Transplant

(A) The cumulative incidence of cGvHD from three months onwards in 65 alloHSCT was analysed according to transplant-related variables (only variables with $P<0.1$ are shown). Table shows the cumulative incidence of cGvHD (%) and 95% confidence intervals (%) at one year. '%Tregs' = peripheral blood $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells); 'High' > median, 'Low' < median. Chronic GvHD was assessed using the NIH Consensus Classification (Filipovich *et al.,* 2005). **(B)** Multivariate analysis of cGvHD beyond three months in the 65 alloHSCT. Variables considered in this analysis were variables with $P<0.1$ in univariate analysis. Table shows the hazard ratios (HR), 95% CI and *P*-values using the Fine and Gray regression model.

C) CHANGE OVER TIME

As well as investigating $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells at one and three months, it was hypothesised that examining the change over time may be a better assessment of putative Treg recovery and a more useful predictor of transplant outcomes. Therefore, to examine this hypothesis, the ratio of the three month to one month $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (counts and $\%CD4^+$ T cells) was determined. Patients were divided into two groups based upon increasing (>1) or decreasing (<1) ratios over the first three months. 66 patients were initially available for analysis with $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (counts and $\%CD4^+$ T cells) decreasing over the first three months in 19 patients. 24 patients had already developed aGvHD and 22 already had CMV reactivation, necessitating exclusion for their respective analyses.

In univariate analysis, falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (counts and $\%CD4^+$ T cells) were not associated with any difference in the cumulative incidence of late aGvHD, cGvHD, CMV reactivation or relapse (Table 5.31). However, patients' whose $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts fell from one to three months post-transplant had significantly higher rates of NRM (24.3% v 2.78%; *P*=0.01) and significantly lower overall survival (59.0% v 83.0%; *P*=0.03) at one year post-transplant (Figure 5.21 & Table 5.31). The causes of death in this patient cohort are shown in Table 5.30. Of note, this pattern was not observed when analysing the change in $CD4^+CD25^-FOXP3^-$ T-cell counts (NRM: 11.5% (2.78-27.1%) v 8.22% (1.31-23.7%), *P*=0.51; OS: 77.0% (62.3-95.0%) v 73.9% (58.7-93.0%), *P*=0.90) or $CD4^+CD25^+FOXP3^-$ T-cell counts (NRM: 8.39% (1.37-23.9%) v 10.6% (2.51-25.57%), *P*=0.92; OS: 79.1% (64.3-97.2%) v 72.7% (57.9-91.2%), *P*=0.81). Neither was it observed when analysing $CD4^+$ T-cell counts (OS = 77.0% (62.3-95.0) v 73.3% (57.9-93.0), *P*=0.93) or total lymphocyte counts (OS = 68.0% (51.7-89.5) v 82.2% (69.2-97.8), *P*=0.20).

Outcome	Cell Type	Group	*N*	Probability (%)	95% CI (%)	*P*
Acute GvHD (Delayed II-IV)	Tregs	< 1	12	33.3	9.41 - 60.0	0.33
		> 1	31	16.7	5.94 - 32.2	
	%Tregs	< 1	12	34.4	9.48 - 61.6	0.31
		> 1	31	16.7	5.94 - 32.2	
Chronic GvHD (All)	Tregs	< 1	19	46.4	20.9 - 68.6	0.88
		> 1	45	48.5	30.2 - 64.6	
	%Tregs	< 1	18	54.8	24.2 - 77.5	0.59
		> 1	46	46.0	28.7 - 61.8	
CMV	Tregs	< 1	14	14.3	2.11 - 37.5	0.30
		> 1	30	4.0	0.27 - 17..4	
	%Tregs	< 1	14	14.3	2.11 - 37.5	0.30
		> 1	30	4.0	0.27 - 17.4	
Relapse	Tregs	< 1	19	16.5	3.81 - 36.9	0.77
		> 1	45	21.9	9.76 - 37.0	
	%Tregs	< 1	18	18.2	4.13 - 40.1	0.96
		> 1	46	20.6	9.24 - 35.0	
NRM	Tregs	< 1	19	24.3	6.79 - 47.4	**0.01**
		> 1	47	2.78	0.20 - 12.6	
	%Tregs	< 1	19	14.9	1.95 - 39.8	0.59
		> 1	47	7.70	1.92 - 19.0	
Overall Survival	Tregs	< 1	19	59.0	39.4 - 88.4	**0.03**
		> 1	47	83.0	71.4 - 96.5	
	%Tregs	< 1	19	71.9	51.4 - 100	0.99
		> 1	47	76.8	64.5 - 91.4	

Table 5.30: Cumulative Incidence and Survival Analysis According to the Change in Peripheral Blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T Cells from One to Three Months Post-Transplant

Paired peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ($x10^6$) ('Tregs') and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) ('%Tregs') were measured at one and three months post-transplant in 66 alloHSCT *(23 transplants already had delayed aGvHD, 2 transplants already had cGvHD, 22 transplants already had CMV reactivation and 2 transplants already had disease relapse by three months and were therefore excluded from their respective analyses).* Blood samples were collected in EDTA and analysed using flow cytometry. The alloHSCT were grouped according to increasing ('>1') or decreasing ('<1') $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts or proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) from one to three months post-transplant. The cumulative incidence of delayed aGvHD (grade II-IV), cGvHD (all grades), CMV reactivation, disease relapse and NRM and the probability of overall survival were calculated for the '>1' and '<1' groups respectively. Table shows the probability (%), 95% confidence intervals (%) and *P*-values at one year post-transplant.

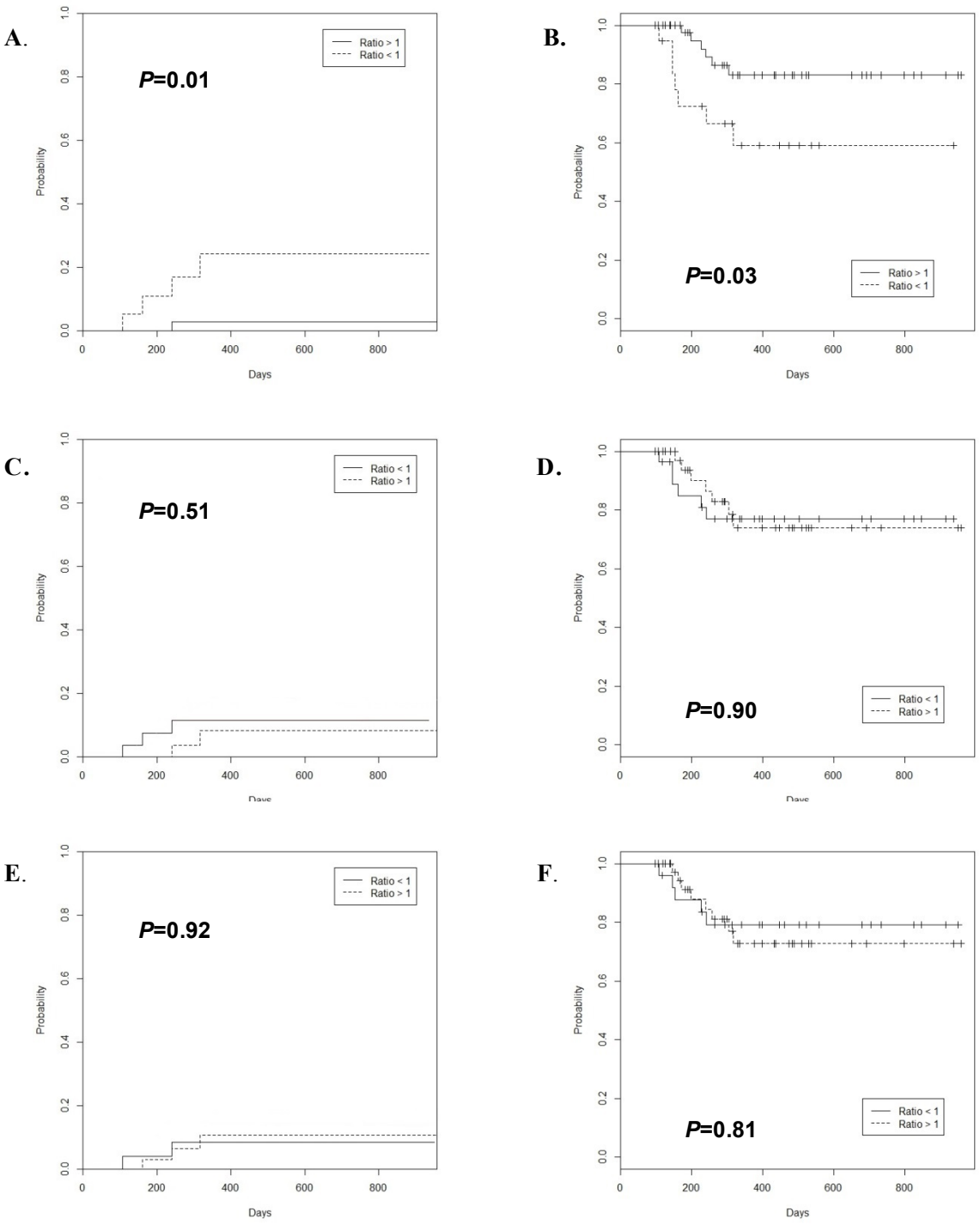


Figure 5.21: Non-Relapse Mortality and Overall Survival

Paired peripheral blood **(A&B)** $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells ($x10^{6}$); **(C&D)** $CD4^{+}CD25^{-}FOXP3^{-}$ T cells ($x10^{6}$/l); **(E&F)** $CD4^{+}CD25^{+}FOXP3^{-}$ T cells ($x10^{6}$/l) were measured at one and three months post-transplant in 66 alloHSCT. Blood samples were collected in EDTA and analysed using flow cytometry. The alloHSCT were grouped according to increasing ('Ratio>1') or decreasing ('Ratio<1') counts from one to three months post-transplant. Figures show **(A/C/E)** Cumulative incidence of NRM; **(B/D/F)** Probability of overall survival calculated for the two groups respectively.

Change in Tregs	*N*	GvHD	Relapse	Other	Total
< 1	19	4	2	1 (SVC obstruction)	7
> 1	47	0	5	1 (Neuropathy)	6

Table 5.31: Cause of Death

Number and cause of death (GvHD, relapse of other) in the 66 alloHSCT divided according to decreasing ('<1') or increasing ('>1') $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts ('Tregs') from one to three months post-transplant.

In view of these results, other transplant variables were considered in univariate analysis of NRM and OS (recipient and donor age, gender and CMV IgG status; female-to-male transplants; disease; status at transplant; HSC source; donor type; intensity of conditioning; Alemtuzumab; HLA matching; CMV reactivation and aGvHD within the first three months; HSC graft CD34, $CD3^+$, $CD19^+$, $CD3^-CD56^+$, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell and proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). Other variables significantly associated with higher one-year NRM were low HSC graft $CD34^+$ and $CD19^+$ cells (26.7% v 5.11%; *P*=0.02 and 26.2% v5.31%; *P*=0.03) (Table 5.32A). There was also a non-significant trend towards increased NRM with low HSC graft $CD3^+$ (24.1% v 6.98%, *P*=0.09) and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell dose (28.0% v 6.85%, *P*=0.06). On multivariate analysis, only low PB $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell ratios (HR 14.3 (1.09-186); *P*=0.04) and low HSC graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses remained independently associated with higher NRM (HR 9.43 (1.87-47.5); *P*=0.007) (Table 5.32B).

A. Univariate analysis					
Variable	**Group**	***N***	**NRM** (%)	**95% CI** (%)	***P***
PB Tregs	< 1	19	24.3	6.79 - 47.4	**0.01**
(3/12:1/12)	> 1	47	2.78	0.20 - 12.6	
HSC $CD34^+$	Low	12	26.7	5.66 - 54.4	**0.02**
	High	54	5.11	0.87 - 15.5	
HSC $CD3^+$	Low	12	24.1	2.14 - 59.0	0.09
	High	54	6.98	1.73 - 17.4	
HSC Tregs	Low	10	28.0	2.09 - 65.8	0.06
	High	56	6.85	1.71 - 17.1	
HSC $CD19^+$	Low	13	26.2	5.44 - 53.9	**0.03**
	High	53	5.31	0.89 - 16.1	

B. Multivariate analysis				
Variable	**Group**	**HR**	**95% CI**	***P***
PB Tregs	< 1	14.3	1.09 - 186	**0.04**
(3/12:1/12)	> 1	1	-	**-**
HSC Tregs	Low	9.43	1.87 - 47.5	**0.007**
	High	1	0.20 - 0.93	**-**

Table 5.32: Univariate and Multivariate Analysis of Non-Relapse Mortality beyond Three Months Post-Transplant

Paired peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells ('Tregs') were measured in 66 alloHSCT at one and three months post-transplant. **(A)** The cumulative incidence of NRM from three months onwards in these 66 alloHSCTs was analysed according to transplant-related variables (only variables with $P<0.1$ are shown). Table shows the cumulative incidence of NRM (%) and 95% confidence intervals (%) at one year. **(B)** Multivariate analysis of NRM beyond three months in the 66 alloHSCT. Variables considered in this analysis were variables with a $P<0.1$.in univariate analysis. Table shows the hazard ratios (HR), 95% CI and *P*-values using the Fine and Gray regression model.

In relation to OS, CMV reactivation in the first three months post-transplant was associated with reduced OS beyond three months (61.2% v 82.3%; *P*=0.04). Positive recipient CMV IgG showed a non-significant trend towards reduced OS (*P*=0.09) (Table 5.33A). On multivariate analysis, both falling $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts (HR 8.69 (2.09-36.2), *P*=0.003) and CMV reactivation (HR 8.44 (2.00-35.7; *P*=0.004) in the first three months post-alloHSCT were independently associated with a significantly worse overall survival (Table 5.33B).

A. Univariate analysis					
Variable	**Group**	***N***	**OS** (%)	**95% CI** (%)	***P***
PB Tregs	< 1	19	59.0	39.4 - 88.4	**0.03**
(3/12:1/12)	> 1	47	83.0	71.4 - 96.5	
Recipient CMV IgG	Neg	39	82.5	69.4 - 98.1	0.08
	Pos	27	65.1	48.1 - 88.1	
CMV reactivation	No	44	82.4	70.4 - 96.5	**0.04**
(0-3 months)	Yes	22	61.2	42.1 - 88.9	

B. Multivariate analysis				
Variable	**Group**	**HR**	**95% CI**	***P***
PB Tregs	< 1	8.69	2.09 - 36.2	**0.003**
(3/12:1/12)	> 1	1	-	-
CMV reactivation	No	1	-	-
(0-3 months)	Yes	8.44	2.00 - 35.7	**0.004**

Table 5.33: Univariate and Multivariate Analysis of Overall Survival beyond Three Months Post-Transplant

Paired peripheral blood $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells ('Tregs') were measured in 66 alloHSCT at one and three months post-transplant. **(A)** Kaplan-Meier estimation of overall survival in these 66 alloHSCTs was analysed according to transplant-related variables (only variables with *P*<0.1 are shown). Table shows the probability of overall survival (%) and 95% confidence intervals (%) at one year. '>1' = increasing, '<1' = decreasing $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts from one to three months post-transplant. **(B)** Multivariate analysis of overall survival in the 66 alloHSCT. Variables considered in this analysis were variables with *P*<0.1 in univariate analysis. Table shows the hazard ratios (HR), 95% CI and *P*-values using the Cox Proportional Hazards.

In summary, a reduction in the peripheral blood absolute $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts from one to three months post-alloHSCT was an independent predictor of higher NRM and inferior overall survival beyond three months. However, this pattern was not observed when analysing $CD4^{+}CD25^{-}FOXP3^{-}$ T-cell or $CD4^{+}CD25^{+}FOXP3^{-}$ T-cell counts. These findings indicate that falling $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts is not simply a measure of a failing graft and/or loss of all T-cell immunity. However, it may be hypothesised that a reduction in $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts over the first three months post-transplant may be reflective of impaired T-cell regulation and increased mortality.

5.3.5 MEASURING POST-TRANSPLANT PUTATIVE TREGS USING THE MSRT-QPCR ASSAY

The final section of work in this chapter was to test the Treg MSRT-qPCR assay using post-alloHSCT blood samples.

A) CORRELATION BETWEEN FLOW CYTOMETRY AND THE MSRT-QPCR ASSAY

To test the feasibility and sensitivity of measuring T cells and putative Tregs post-alloHSCT using the MSRT-qPCR assay, the blood samples with the highest (2.77% (1.19-5.98%), *N*=20) and lowest proportions of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (% all cells) (0.22% (0.05-0.32%), *N*=20) were re-analysed using the MSRT-qPCR assay. An additional group of random samples were also analysed to ensure the tested cohort contained samples throughout the entire post-transplant range (0.74% (0.34-1.15%, *N*=23).

For the quantitation of $CD3^+$ cells, the two assays produced very similar results, showing a significant strong correlation (ρ=0.96, *P*<0.0001, *N*=63) (Figure 5.22A). However, when analysing *FOXP3*, the MSRT-qPCR assay did not detect any unmethylated *FOXP3* in 31 of the 63 samples, although methylated *FOXP3* copies ('non-Tregs') were detected. Of note, these 31 samples generally contained low proportions of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (% all cells) by flow cytometry (0.26% (0.05-1.51%)). Despite this, the two assays still showed a correlation (ρ=0.81, *P*<0.0001) (Figure 5.22B). When analysing the FOXP3 to CD3 ratios, there was a moderate correlation between the results from the two methods (ρ=0.65, *P*<0.0001) (Figure 5.22C). Subgroup analysis of the 32 samples only in which unmethylated *FOXP3* was detected showed a strong correlation (ρ=0.82, *P*<0.0001, *N*=32) (Figure 5.22D).

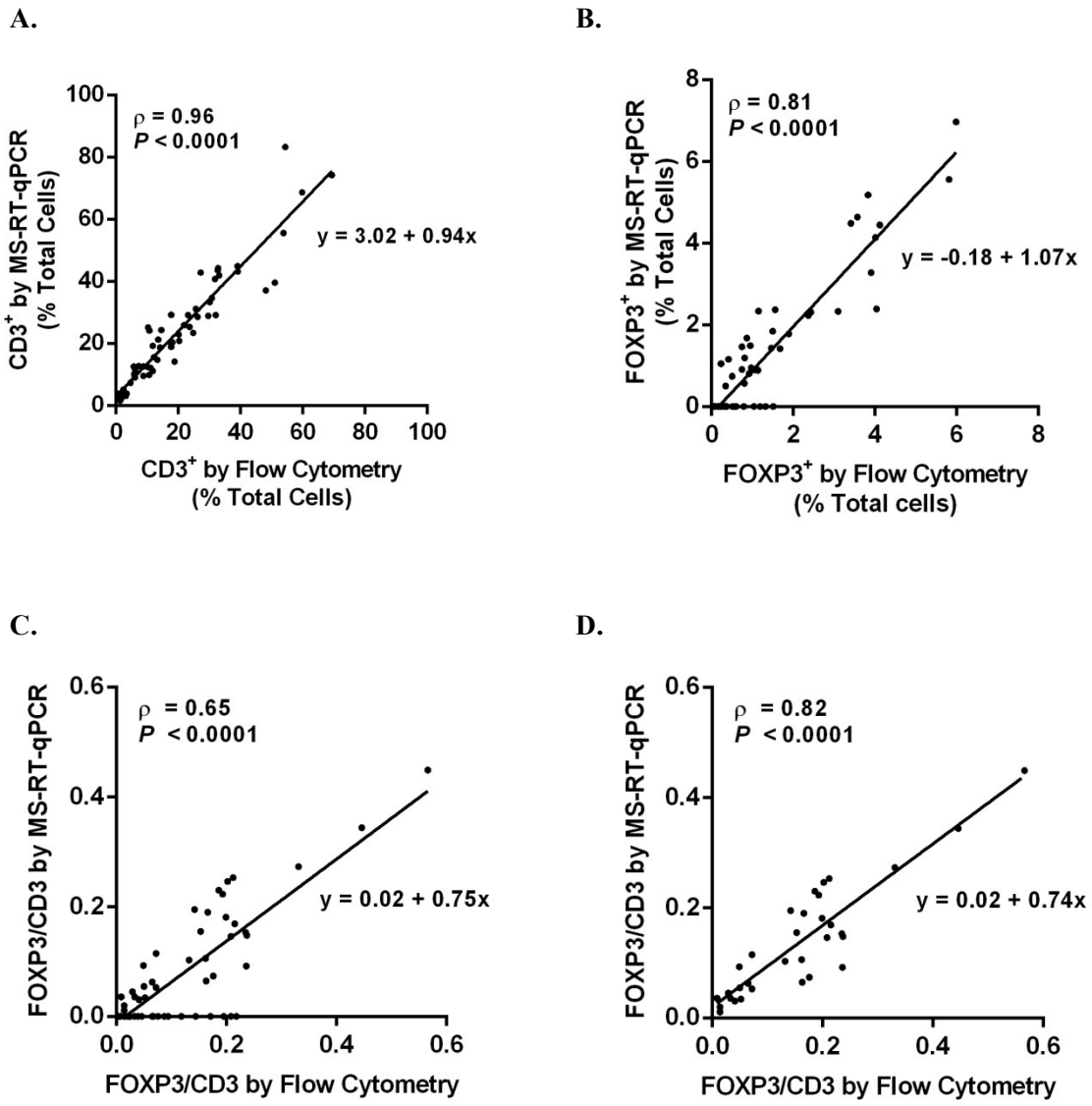


Figure 5.22: Correlation between the CD3, FOXP3 and FOXP3:CD3 Ratios as Determined by Flow Cytometry and the MSRT-qPCR Assay

(A) $CD3^+$ **(B)** $FOXP3^+$ **(C&D)** $FOXP3^+$:$CD3^+$ ratios were measured in 63 post-alloHSCT blood samples using flow cytometry (x-axis) and the MSRT-qPCR assay (y-axis). Each figure shows the Spearman correlation coefficient (ρ), *P*-value and linear regression analysis. Results are expressed as a percentage of all cells. **(A-C)** Analysis of all samples tested (*N*=63), **(D**) Analysis of only those samples in which unmethylated *FOXP3* was detectable (*N*=32).

B) FOXP3 EXPRESSION IN GVHD

In humans, FOXP3 is transiently expressed in activated conventional $CD4^+$ T cells (Allan *et al.,* 2007; Tran *et al.,* 2007). Therefore, measuring FOXP3 expression in samples taken in the setting of inflammation may detect both $FOXP3^+$ Tregs and $FOXP3^+$ activated T cells. Detecting unmethylated *FOXP3* TSDR, however, is specific for $FOXP3^+$ Tregs only (Wieczorek *et al.,* 2009). To examine this hypothesis, the post-transplant samples analysed above were divided according to the presence or absence of GvHD at the time of collection. If the hypothesis is correct, samples collected at the time of GvHD would have lower putative Tregs when measured by the MSRT-qPCR compared to flow cytometry. However, in a non-inflammatory setting with few activated T cells, the two assays should produce similar results.

Of the 32 blood samples with flow cytometry and MSRT-qPCR data, eight were collected when the patient had grade II-IV GvHD. In contrast to the hypothesis, there was no significant difference in either the proportion of putative Tregs (% all cells) (2.31% (0.34-5.81%) v 2.09% (0.51-5.56), P=0.84) or the putative Treg to T-cell ratios (0.18 (0.03-0.57) v 0.17 (0.04-0.45), P=0.64) when analysed by flow cytometry and the MSRT-qPCR assay respectively (Figure 5.23A-B, E-F). In the 24 samples that were collected when patients did not have GvHD, there was no difference in the putative Treg to T-cell ratios (0.16 (0.01-0.33) v 0.10 (0.01-0.27), P=0.14) using the two assays (Figure 5.23D&F). However, there was a non-significant trend towards higher proportions of putative Tregs using the MSRT-qPCR assay compared to flow cytometry (1.47% (0.57-6.96) v 1.31% (0.24-5.98), P=0.07) (Figure 5.23C&E).

These data suggest that either activated $FOXP3^+$ cells were not present or despite concerns regarding the specificity of FOXP3 expression in acute inflammation, the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry assay does not appear to detect significant numbers of $FOXP3^+$ activated T cells.

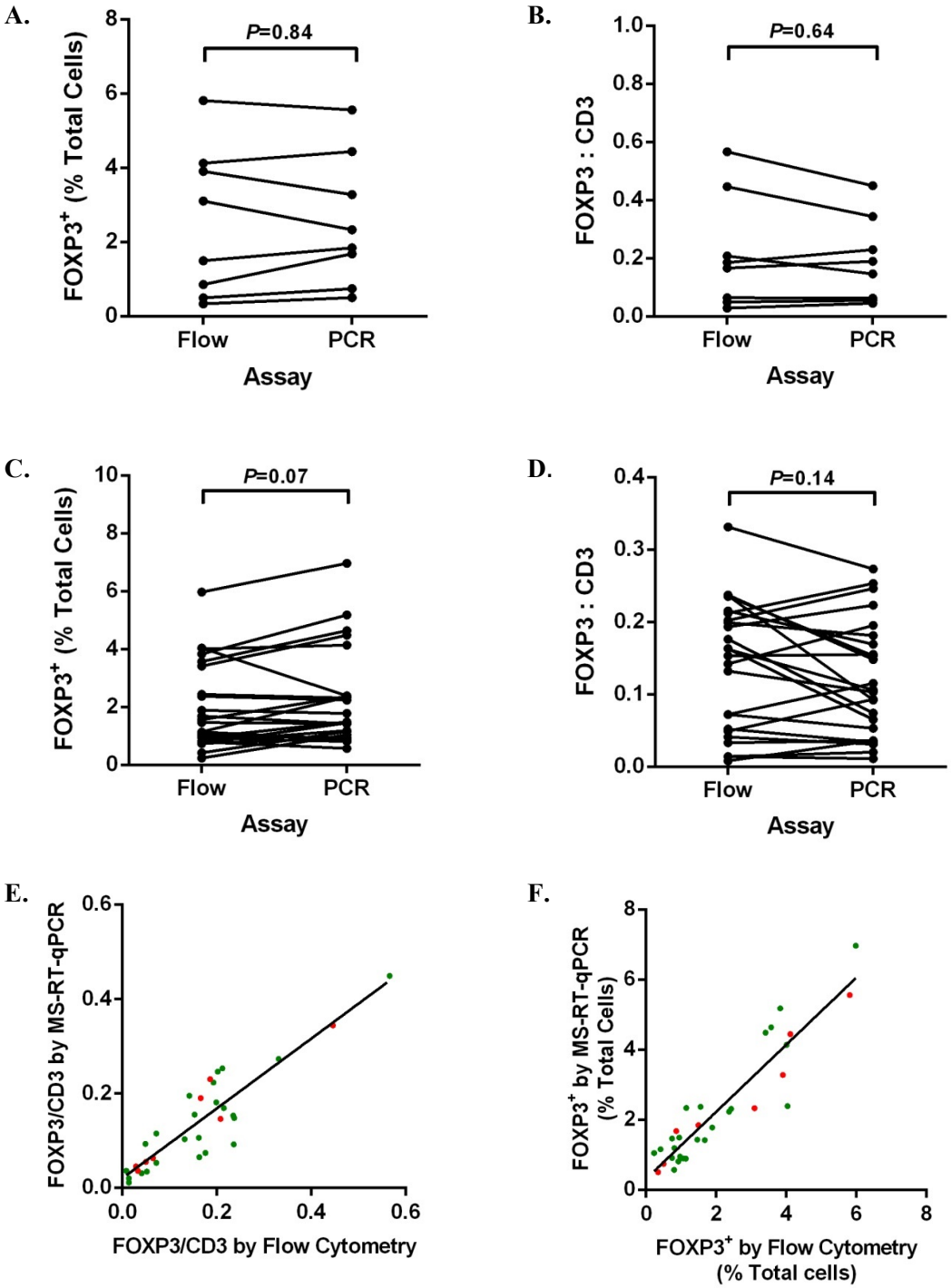


Figure 5.23 FOXP3 and FOXP3:CD3 Ratios as in GvHD and Non-GvHD Samples

Peripheral blood samples were collected in the presence (*N*=8) **(A-B)** and absence (*N*=24) **(C-D)** of GvHD from alloHSCT. The samples were analysed for **(A, C, E)** Proportion of $FOXP3^+$ cells (% all cells); **(B, D, F)** $FOXP3^+$ to $CD3^+$ ratios using flow cytometry (Flow) and the MSRT-qPCR (PCR). Analysed using Wilcoxon matched-pairs signed rank test. **(E-F)** Comparison of the results from the two assays (●) No GvHD samples; (●) GVHD samples.

5.4 CONCLUSIONS

Reconstitution of the immune system is essential to protect the recipient from infection while balancing the GVT response against excessive GvHD. In this chapter, the pattern and kinetics of immune reconstitution were monitored in 101 alloHSCT, analysing $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute counts and $\%CD4^+$ T cells) and lymphocyte reconstitution in 468 post-transplant blood samples. The main conclusions from these data are:

- In the first year post-allogeneic HSCT, there was wide variation in the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and the proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) between patients at all points examined.
- In the non-Alemtuzumab conditioned transplants, the $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells), $CD3^+$ cells, $CD3^+CD8^+$ cells, $CD3^-CD56^+$ cells and lymphocytes recovered to within the normal range by one month post-transplant. Recovery of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells counts, $CD3^+CD4^+$ cells, and $CD19^+$ cells, however, was more delayed. The median proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) exceeded the normal range from two months onwards.
- In the Alemtuzumab conditioned transplants, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells, $CD3^+CD4^+$ cells, $CD3^+CD8^+$ cells and lymphocytes had a much slower recovery, only reaching the normal ranges at six to nine months post-transplant. However, $CD19^+$ cells recovered faster and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) recovered quickly, exceeding the upper limit of the normal range from three months.
- Although $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were significantly lower in the Alemtuzumab conditioned transplants in the first three months, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) were significantly higher from three to six months.
- These data suggest that pre-transplant conditioning with Alemtuzumab significantly alters the pattern and timing of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell reconstitution.

In view of these findings, exploratory analysis was performed to ascertain which transplant variables were significantly associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells post-transplant. In multivariate analysis:

- Alemtuzumab remained significantly associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts in the first three months. It was also associated with lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at one month but higher proportions by three months.
- The presence of CMV at the time of sampling was significantly associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells).
- RIC and PBCSH were independently associated with low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at one month post-transplant.
- GvHD symptoms at the time of sampling were associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells at three months.
- Use of corticosteroids was associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at six months.

This chapter also examined the hypotheses that $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells are reduced in GvHD and CMV reactivation and that low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells post-transplant increase the risk of developing transplant related complications. The conclusions from this analysis were:

- $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were significantly lower in aGvHD, although no difference was observed in the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). However, the GvHD and non-GvHD samples were not evenly matched for other co-variables. In subgroup analysis of the Alemtuzumab transplants only, not taking corticosteroids, aGvHD samples had significantly higher $CD4^+CD25^+FOXP3^-$ T-cell counts but no difference in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. These findings suggest that aGvHD may be associated with a relative imbalance in the pro-inflammatory to regulatory T-cell responses post-alloHSCT.
- The proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were significantly lower in cGvHD. However, the GvHD and non-GvHD were not evenly matched for Alemtuzumab. In subgroup analysis of the Alemtuzumab transplants only, not taking corticosteroids, cGvHD samples had significantly higher $CD4^+CD25^+FOXP3^-$ T-cell counts but no difference in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. These findings suggest that cGvHD may also be associated with a relative imbalance in the pro-inflammatory to regulatory T-cell responses post-alloHSCT.
- $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were significantly lower in CMV reactivation.
- In multivariate analysis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) post-transplant were not associated with an increased risk of developing GvHD, CMV reactivation, disease relapse, NRM or overall survival at one year. A fall in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts from one to three months was, however, associated with a significantly higher risk of NRM and inferior overall survival at one year post-transplant.

Finally, this study also compared two methods (flow cytometry and MSRT-qPCR) of quantifying T cells and putative Tregs in post-transplant blood samples. In particular, this work tested the hypothesis that in the setting of GvHD, some of the $FOXP3^+$ cells may have been activated T cells and would not be detected by the MSRT-qPCR assay. In conclusion:

- The two methods showed a very strong correlation when quantifying T cells.
- The MSRT-qPCR assay did not detect unmethylated *FOXP3* TSDR in many samples, particularly in samples with low proportions of putative Tregs as measured by flow cytometry. However, the two assays still showed a correlation for putative Tregs and putative Treg : T-cell ratios.
- There was no significant difference in the proportion of putative Tregs or the putative Treg : T-cell ratios between the two methods when analysing the samples collected at the time of acute inflammation (GvHD).
- These findings suggests that either activated $FOXP3^+$ cells were not present in the GvHD samples and/or the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell flow cytometry assay does not detect significant numbers of $FOXP3^+$ activated T cells.

Chapter 6

Inducing FOXP3 Expression in $CD4^+$ T Cells

6. INDUCING FOXP3 EXPRESSION IN CD4+ T CELLS

6.1 INTRODUCTION

Producing Treg cellular therapies for human clinical use has remained challenging due to the lack of Treg-specific cell surface markers, low numbers of Tregs in the peripheral blood and HSC harvests, high costs and practical difficulties associated with Good Manufacturing Practice (GMP). There remains considerable interest, therefore, in expanding Tregs *in vitro* or *in vivo* by inducing stable FOXP3 expression and suppressive function in conventional $CD4^+$ T cells.

In mice, Foxp3 expression can be induced in naïve $CD4^+$ T cells through TCR stimulation and TGFβ (Chen *et al.,* 2003b; Zheng *et al.,* 2004; Chen and Konkel, 2010). These induced Tregs (iTregs) have a similar phenotype to the naturally occurring Tregs (nTregs) produced in the thymus and have suppressive function (Chen *et al.,* 2003b; Zheng *et al.,* 2004; Lan *et al.,* 2012a; Lan *et al.,* 2012b). iTregs are important for maintaining mucosal immune tolerance and suppress chronic inflammation (Curotto de Lafaille and Lafaille, 2009; Maloy and Powrie, 2011). However, in humans, induced FOXP3 expression and the functional capabilities of iTregs remain more controversial. In contrast to mice, FOXP3 can be transiently expressed in activated conventional $CD4^+$ T cells without conferring suppressive function (Allan *et al.,* 2007; Tran *et al.,* 2007).

In light of these observations, the initial work in this chapter will verify the hypothesis that CD25 and FOXP3 can be induced in isolated human $CD4^+CD25^-$ conventional T cells by CD3/CD28 stimulation. Preliminary experiments will establish the *in vitro* $CD4^+$ T-cell culture methods and investigate the kinetics of $CD4^+$ T-cell activation, CD25 and FOXP3 expression using cell counts, flow cytometry and CFSE proliferation assays. The experiments will then test the hypothesis that TGFβ can increase CD3/CD28-induced FOXP3 expression.

In view of the observation that demethylation of the FOXP3 TSDR enhancer is specific to nTregs and is required for stable FOXP3 expression, this project will then test the hypothesis that Azacitidine can enhance TCR/TGFβ induced FOXP3 expression in human conventional $CD4^+CD25^-$ T cells. Azacitidine is a cytosine analogue that is incorporated into DNA and RNA during cell division and inhibits the function of DNA methyltransferse 1 (DNMT1) causing passive DNA demethylation (Polansky *et al*., 2008). It will test whether this FOXP3 expression is stable upon removal of the TCR stimulus and will examine whether these cells contain a demethylated *FOXP3* TSDR.

The remaining work presented in this chapter will then assess the phenotype and suppressive function of the TCR/TGFβ/Azacitidine cultured $CD4^+$ T cells. In particular, it will test the ability of these cells to produce pro-inflammatory cytokines and will also test the hypothesis that the combination of TCR stimulation, TGFβ and Azacitidine can induce Treg-like phenotype and function

If successful, these methods could provide a potential strategy to produce functional $FOXP3^+$ cells for human Treg cellular therapy trials and/or to increase $CD4^+FOXP3^+$ Tregs post-alloHSCT. Using Azacitidine post-transplant to increase $CD4^+FOXP3^+$ Tregs would be an attractive option since its use post-HSCT is currently being investigated to reduce relapse from myeloid malignancies (Platzbecker *et al.,* 2012).

6.2 SPECIFIC METHODS

6.2.1 CELL ISOLATION AND CULTURE

Conventional and naïve $CD4^+CD25^-$ T cells were isolated under sterile conditions from human buffy coats and leucocyte cones (NHSBT, Oxford) as per Methods 2.3.2. Cell cultures, CFSE proliferation assays and cytokine staining were performed as per Methods 2.3.3-5. Suppressive function assays were performed as per Methods 2.3.6, testing the ability of the cultured cells to inhibit anti-CD3/CD28 driven proliferation of autologous $CD4^+CD25^-$ T cells. These $CD4^+CD25^-$ T cells were stored at -80^{o}C in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 90% RH50 (50% RPMI-1640 (Lonza), 50% heat inactivated human AB serum (NHSBT, Oxford)) until the day of the experiment. They were thawed by rapidly warming to 37^{o}C, diluted and washed with pre-warmed RH10 culture media and allowed to rest for two hours at 37^{o}C in a tissue culture incubator.

6.2.2 FLOW CYTOMETRY

The isolated and cultured $CD4^+$ T cells were analysed by flow cytometry using Methods 2.2.2-4 and the fluorochrome-conjugated anti-human antibodies used were as per Methods 2.2.2. For all culture experiments, the total staining volume was 50µl/test and 10µl human FcR blocking reagent was also added prior to anti-FOXP3 antibody to minimise background staining. The antibody staining controls used were FMO controls and isotype controls as specified.

6.2.3 MOLECULAR BIOLOGY

DNA was extracted from cultured cells and bisulphite treated as per Methods 2.4.1-2. The MSRT-qPCR assay was run as per Methods 2.4.7.

6.3.1 ESTABLISHING THE *IN VITRO* CD4+ T-CELL CULTURE METHODS

A) CD4+CD25- T-CELL ISOLATION

Human $CD4^+CD25^-$ T cells used in the *in vitro* cell culture experiments were isolated from buffy coats or leucocyte cones as described in Methods 2.3.1. The PBMC separated from these products contained 15.8-54.6% (*N*=11) $CD4^+CD25^-$ T cells (% of nucleated cells). The median purity of the isolated $CD4^+CD25^-$ T cells was 91.9% of all nucleated cells (71.4-97.6%, *N*=25). On average, the isolated $CD4^+$ T cells contained only 1.3% (0.2-3.4%, *N*=25) $FOXP3^+$ cells (Figure 6.1).

B) *IN VITRO* T CELL ACTIVATION AND PROLIFERATION

Before testing the main study hypotheses, a preliminary series of experiments was performed to establish the *in vitro* cell culture methods and verify that $CD4^+$ T cells are activated by anti-CD3/CD28 beads. To start, isolated human $CD4^+CD25^-$ T cells were incubated at 37°C in 96-well U-bottomed plates ($0.5x10^5$ cells/ml) with anti-CD3/CD28 Dynabeads at a cell-to-bead ratio of 1:1, as per the laboratory's *in vitro* Treg/T-cell expansion protocol. Under these conditions, $CD4^+CD25^-$ T-cell proliferation showed an initial lag phase of 48 hours followed by exponential growth over the next three days (Figure 6.2A-B). By day five, cell numbers plateaued at a mean cell density of 3.0 ± $0.1x10^6$ cells/ml. As demonstrated by CFSE staining, 96.9% (94.2-99.0%, *N*=4) of the cells on day five had divided at least once (Figure 6.2C-E). As measured by Trypan blue staining, cell viability was 98.1 ± 0.68% and 83.0 ± 2.96% on days five and seven respectively (*N*=3). $CD4^+CD25^-$ T cells cultured under identical conditions without anti-CD3/CD28 stimulation did not proliferate.

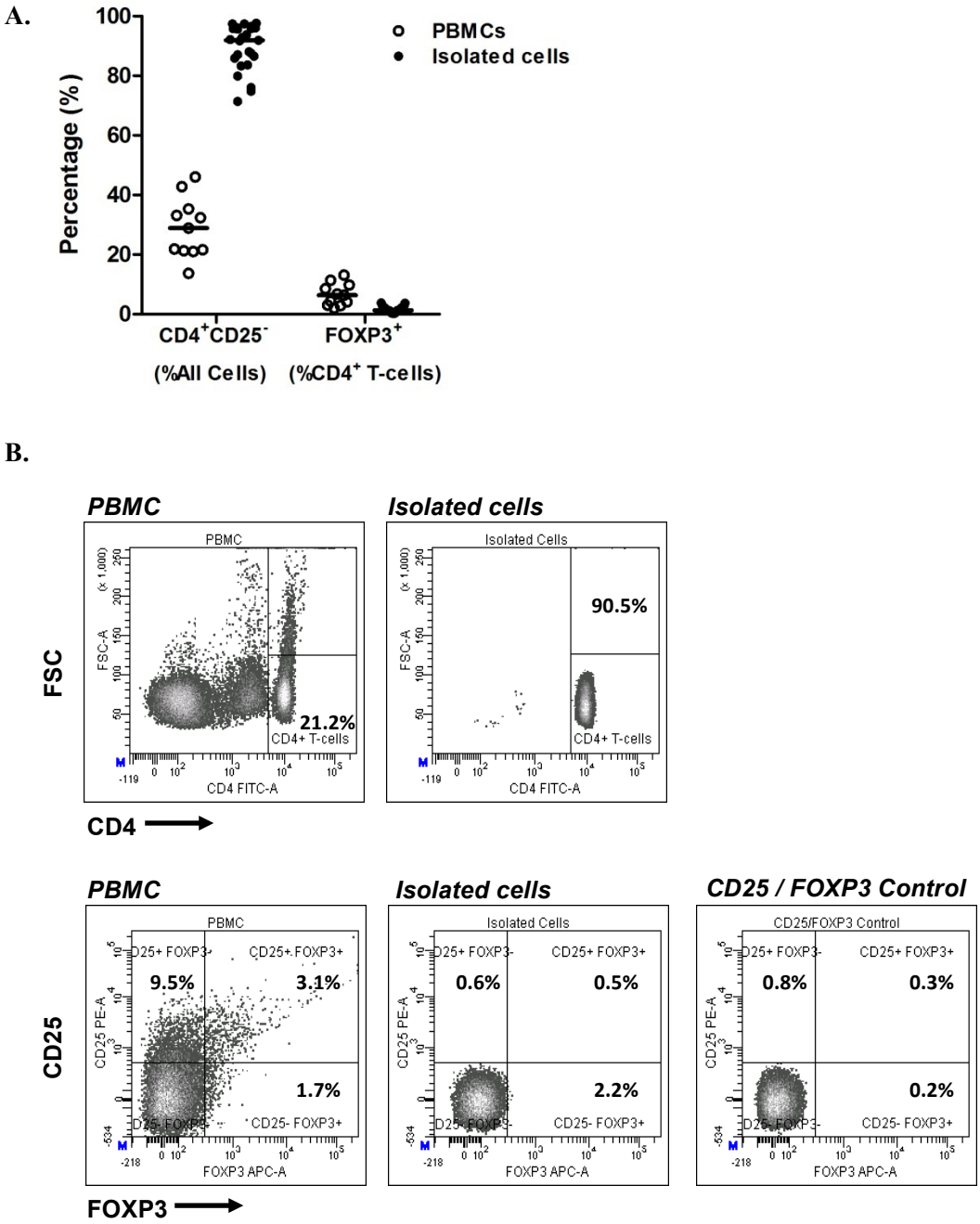


Figure 6.1: Isolation of CD4+CD25- T Cells

Human PBMC were isolated from buffy coats/leucocyte cones using Ficoll-Paque separation. $CD4^+CD25^-$ T cells were then isolated using the magnetic bead $CD4^+$ T-cell isolation kit II (Miltenyi Biotech) and CD25 MicroBeads (Miltenyi Biotech). **(A)** Proportion of $CD4^+CD25^-$ cells (% all cells) and the proportion of $FOXP3^+$ cells (%$CD4^+$ cells) in the starting PBMC (*N*=11) and isolated $CD4^+CD25^-$ cell populations (*N*=25). All isolations were performed on PBMC from different buffy coats/leucocyte cones. Horizontal bar shows the median value. **(B)** Density plots from a single isolation which is representative of 25 separate procedures. CD4 and CD25 gates were set using FMO controls. FOXP3 gate was set by using FMO + IgG APC isotype control.

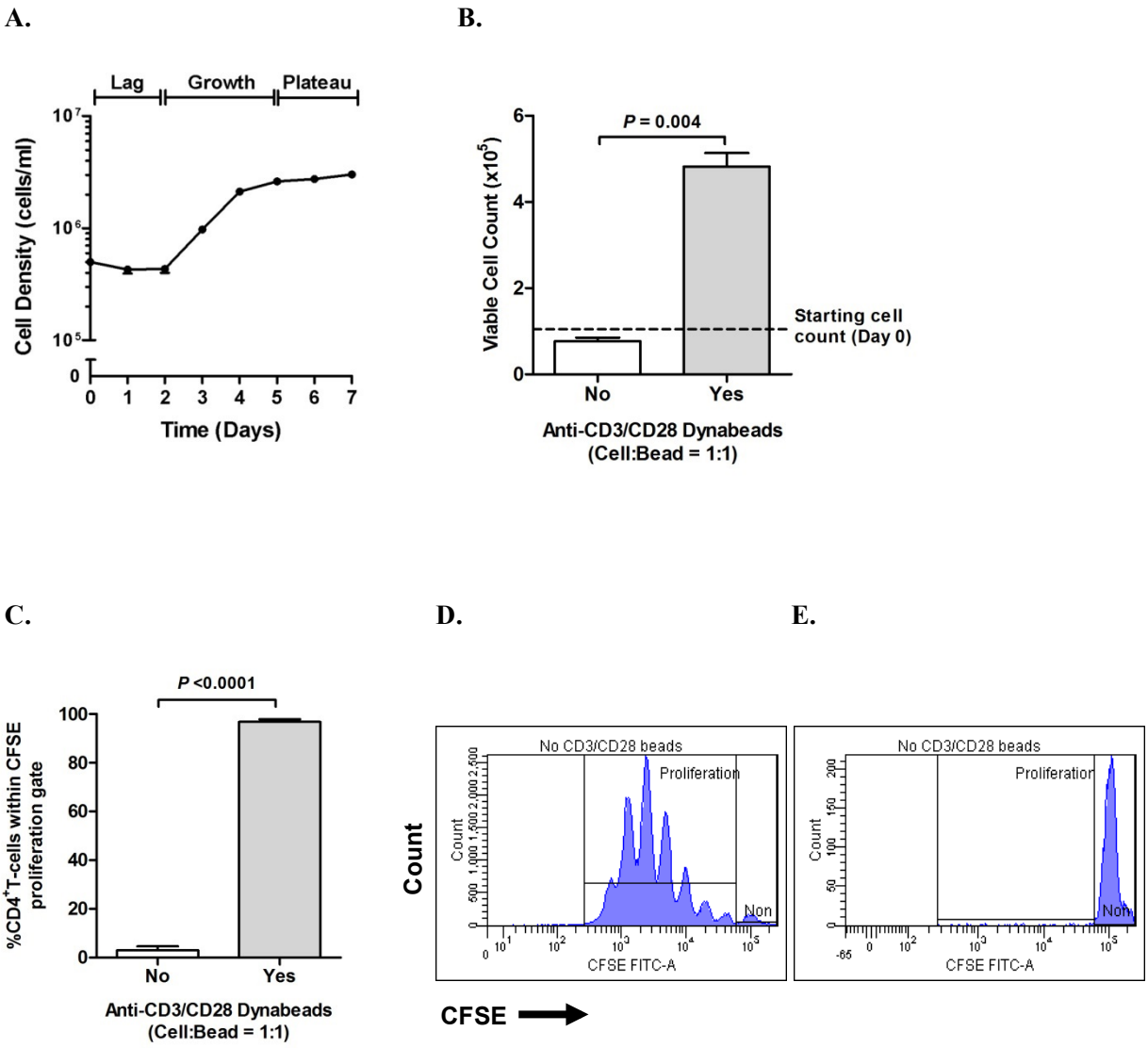


Figure 6.2: *In vitro* CD4$^+$CD25$^-$ T-Cell Proliferation

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 94% (86-97%), expressed as % all cells) were cultured in RH10 media and 30IU/ml IL2 at 0.5x10^{6}cells/ml in 96 well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for seven days. **(A)** Change in cell density over time (*N*=3); **(B)** Day five cell counts (*N*=3); **(C)** Cell division by day five as assessed by 0.5µM CFSE staining (*N*=4). **(D-E)** Flow cytometry histogram of 0.5µM CFSE staining in CD4$^+$CD25$^-$ T cells incubated with **(D)** and without **(E)** anti-CD3/CD28 Dynabeads. Figures show the mean values from *N* (specified above) separate experiments, each one performed using sorted CD4$^+$CD25$^-$ T cells from different donors. Flow cytometry plots from a single experiment which is representative of four separate experiments.

To further examine the effect of the *in vitro* culture conditions for $CD4^+CD25^-$ T cell growth, the effect of the cell-to-anti-CD3/CD28 Dynabead ratio, cell density and changing the culture media was investigated. Increasing the starting cell:anti-CD3/CD28 Dynabead ratio to 30:1 or more significantly reduced T-cell proliferation over seven days ($6.07 \pm 0.40x10^5$ v $1.95 \pm 0.14x10^5$, $P<0.0001$) (Figure 6.3A). Adjusting the seeding density had no effect on the early lag period. However, a lower starting density ($0.125x10^6$cells/ml) produced a faster rate of T-cell proliferation from day three to five, although the final plateau density reached by day five remained the same (Figure 6.3B). Passaging the cells on day five at the start of the plateau phase, allowed further proliferation over the next 48 hours, significantly increasing the total cell count produced over seven days ($7.62 \pm 0.38x10^5$ v $5.59 \pm 0.22x10^5$, $P=0.0009$) (Figure 6.3C). Similarly, culturing the cells in 48-well flat bottom plates with a larger surface area per cell, although at the same starting density, allowed greater proliferation with a significantly higher plateau density on day seven ($3.69 \pm 0.16x10^6$/ml v $2.53 \pm 0.08x10^6$/ml, $P<0.0001$) (Figure 6.3D). Increasing the frequency of changing the culture media also increased total cell proliferation over seven days (daily: $9.71 \pm 0.11x10^5$, alternate days: $6.04 \pm 0.10x10^5$, never: $4.06 \pm 0.20x10^5$) (Figure 6.3E).

In light of these findings, a standard cell culture method was used throughout this project to allow direct comparison between experiments. Isolated human $CD4^+CD25^-$ T cells were incubated at 37^oC in 96-well round-bottom plates at a seeding density $0.5x10^5$ cells/ml (200µl/well) with anti-CD3/CD28 Dynabeads at a cell-to-bead ratio of 1:1. As the plateau cell density was obtained by day five and because cell viability fell from five to seven days, cells were harvested on day five. Passaging the cells on day five was, therefore, not required. Fresh cell media was added on day three as this provided a balance between adding fresh nutrients and maintaining cell growth without excessively disrupting or losing cells. When larger cell numbers were required for analysis (cytokine staining, DNA isolation), cell cultures were performed in 48-well flat-bottomed plates. However, the results from these cultures were not directly compared or combined with the results from the 98-well cultures.

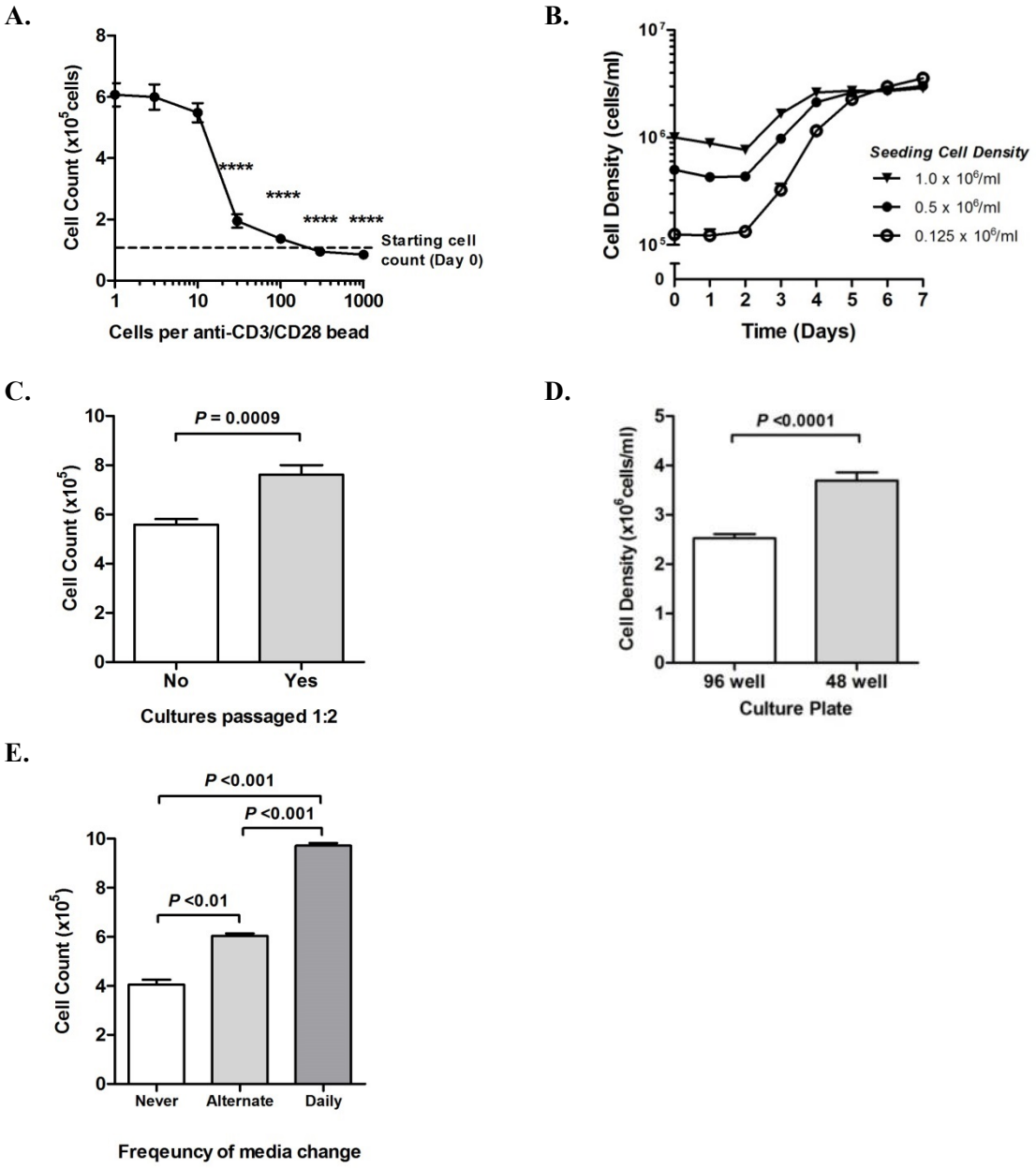


Figure 6.3: Variables Affecting *In Vitro* CD4$^+$CD25$^-$ T-Cell Proliferation

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 92% (72-98%)%), expressed as % all cells) were cultured in RH10 media and 30IU/ml IL2 at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for seven days unless otherwise stated. Culture media was changed on alternate days and viable cells counted on day seven. **(A)** Cell:Anti-CD3/CD28 Dynabead ratio (****P<0.0001 compared to baseline 1:1 ratio) (*N*=3); **(B)** Seeding cell density (*N*=3); **(C)** Cells passaged 1 in 2 on day five (*N*=6); **(D)** Culture plate size (*N*=11); **(E)** Frequency of media change (*N*=3). Figures show the mean values from *N* (specified above) separate experiments, each one performed using sorted CD4$^+$CD25$^-$ T cells from different donors.

6.3.2 CD25 AND FOXP3 EXPRESSION IN ACTIVATED $CD4^+CD25^-$ T CELLS

Having established the *in vitro* $CD4^+$ T-cell culture methods, the work presented in this section then tested the hypothesis that TCR-activation of human $CD4^+CD25^-$ T cells induces expression of CD25 and FOXP3.

After five days in culture with anti-CD3/CD28 beads, all of the $CD4^+$ T cells (98.9 ± 0.4%, *N*=5) strongly expressed CD25 with an average median fluorescence intensity (MFI) in the $CD25^+$ cells of 41275 ± 2811 (*N*=5). However, only a limited proportion of the cells expressed FOXP3 (14.3% ± 1.5%, *N*=5) with a MFI in the $FOXP3^+$ cells of 1421 ± 37 (*N*=5) (Figure 6.4 and Figure 6.5A-C). Cells cultured without anti-CD3/CD28 did not express CD25 (1.30 ± 0.55% $CD25^+$, MFI 3195 ± 1124, *N*=5) or FOXP3 (0.63 ± 0.09% $FOXP3^+$, MFI 1141 ± 93, *N*=5) (Figure 6.5A&B). As demonstrated by CFSE dilution, FOXP3 expression was not observed in the $CD4^+$ T cells that had not divided (Figure 6.6). The level of CD25 and FOXP3 expression in the $CD25^+$ and $FOXP3^+$ cells respectively was significantly greater than the levels seen in *ex vivo* $CD4^+CD25^+FOXP3^+$ T cells (CD25: 41275 ± 2811 v 3484 ± 405, $P<0.0001$; FOXP3 1421 ± 37 v 755 ± 110, $P=0.0007$) (Figure 6.5C-E). However, these $CD4^+CD25^+FOXP3^+$ T cells were unstimulated.

The expression of CD25 and FOXP3 in the *in vitro* cultured cells was also examined over time. CD25 expression occurred quickly with all the $CD4^+$ T cells expressing CD25 by day three onwards (Figure 6.7A). In contrast, the proportion of $CD4^+$ T cells expressing FOXP3 progressively increased over time with 9.1 ± 0.4%, 15.3 ± 1.9% and 19.4 ± 3.6% cells FOXP3 positive on days three, five and seven respectively. The increase from day five to seven did not reach statistical significance ($P>0.05$) (Figure 6.7B).

The results so far, therefore, confirm that in these *in vitro* cultures, all of the cultured $CD4^+$ T cells are activated, divide and express high levels of CD25 by day five in response to anti-CD3/CD28 stimulation. However, only a proportion of these cells express FOXP3.

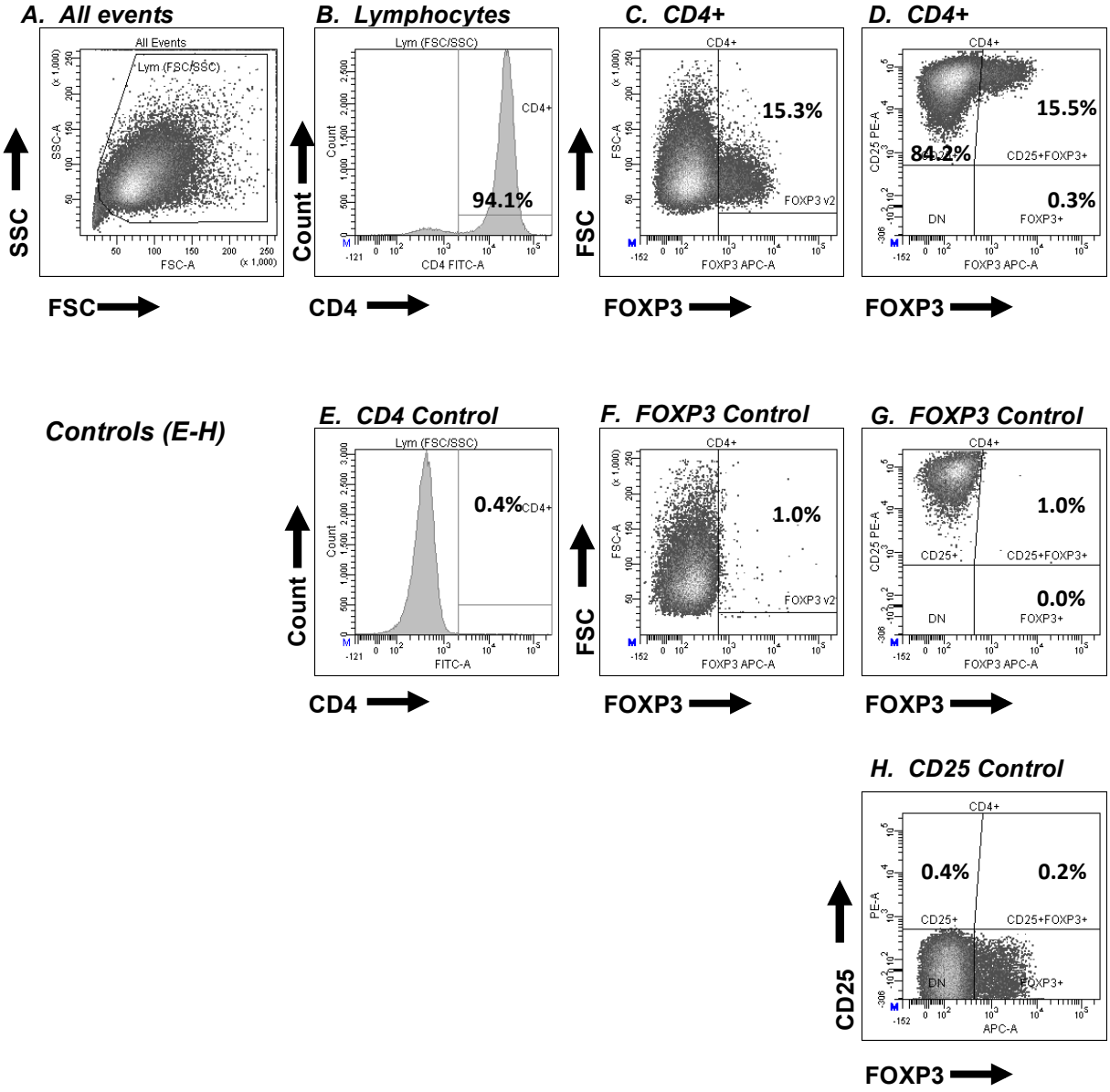


Figure 6.4: Flow Cytometry Plots of Activated CD4$^+$CD25$^-$ T Cells

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (93% purity, % all cells) were cultured in RH10 media and 30IU/ml IL2 at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Culture media was changed on day three. Flow density plots showing **(A-D)** *In vitro* cultured CD4$^+$ T cells; **(E-H)** Antibody staining controls. **(F&G)** FOXP3 control (FMO + IgG1 APC isotype control); **(H)** CD25 control (FMO). Flow cytometry plots show (x-axis/y-axis) **(A)** FSC/SSC; **(B&E)** CD4/Count; **(C&F)** FOXP3/FSC; **(D, G&H)** FOXP3/CD25. Flow plots from a single experiment which is representative of five separate experiments, each one performed using sorted CD4$^+$CD25$^-$ T cells from different donors.

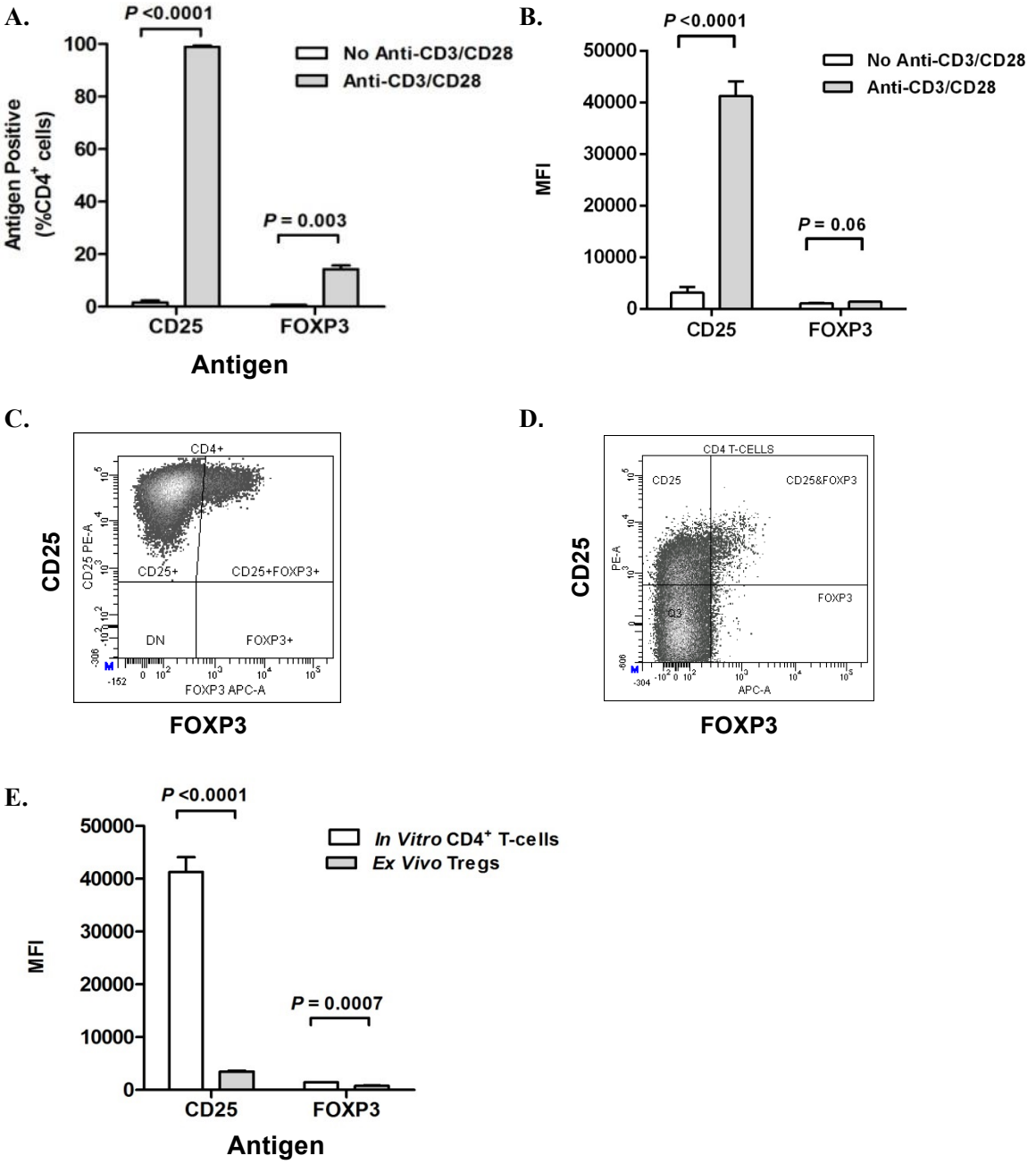


Figure 6.5: CD25 and FOXP3 Expression in Activated CD4$^+$CD25$^-$ T Cells

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 96% (87-97%), expressed as % all cells) were cultured in RH10 media and 30IU/ml IL2 at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with and without anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Culture media was changed on day three. **(A)** Proportion of the CD4$^+$ T cells expressing CD25 and FOXP3 (*N*=5); **(B)** MFI of the CD25$^+$ and FOXP3$^+$ cells (*N*=5). Flow cytometry density plots showing FOXP3 and CD25 expression in **(C)** *in vitro* cultured CD4$^+$ T cells and **(D)** *ex vivo* CD4$^+$ T cells ; **(E)** CD25 and FOXP3 MFI in the *in vitro* cultured CD4$^+$ T cells (*N*=5) and *ex vivo* CD4$^+$CD25$^+$FOXP3$^+$ T cells (*N*=8). Figures show the mean values from *N* separate experiments, each one performed using sorted CD4$^+$CD25$^-$ T cells from different donors. Flow cytometry plots are representative examples.

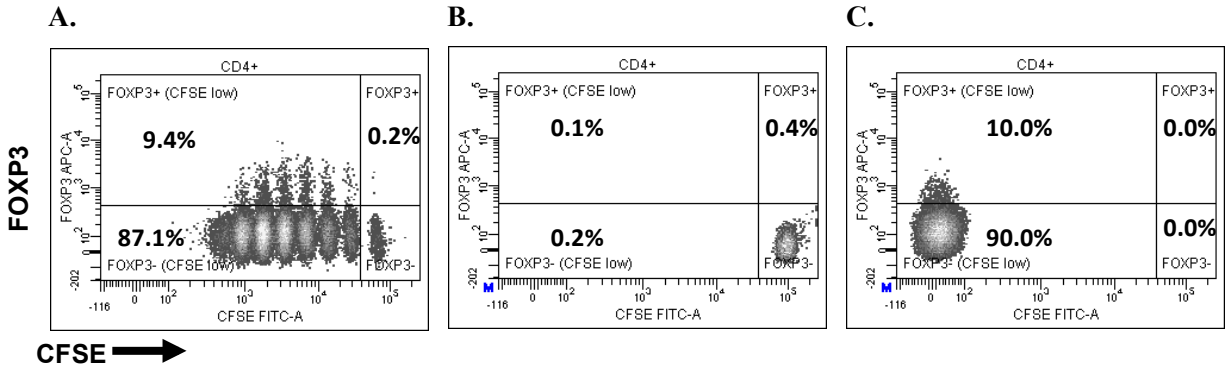


Figure 6.6: FOXP3 Expression and CFSE Staining in Activated CD4$^+$CD25$^-$ T Cells

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (91% purity, % all cells) were stained with 0.5μM CFSE and cultured in RH10 media and 30IU/ml IL2 at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with and without anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for three days. Flow density plots showing **(A)** Activated CFSE stained CD4$^+$ T cells; **(B)** No anti-CD3/CD28 beads; **(C)** No CFSE staining. Flow plots from a single experiment which is representative of four separate experiments, each one performed using sorted CD4$^+$CD25$^-$ T cells from different donors.

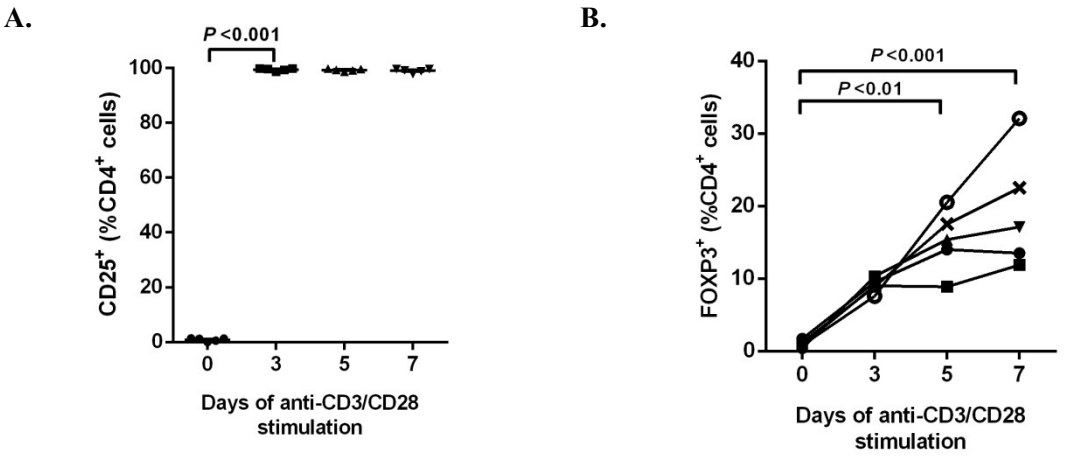


Figure 6.7: CD25 and FOXP3 Expression in Activated CD4$^+$CD25$^-$ T Cells

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 96% (71-98%, *N*=*5*) (% all cells); 1.2% (0.0-1.4%, *N*=*5*) of the sorted CD4$^+$ T cells were CD25$^+$; 0.9% (0.4-1.7%, *N*=5) were FOXP3$^+$) were cultured in RH10 media and 30IU/ml IL2 at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for seven days. Culture media was changed on day three and five. **(A)** CD25 expression over time (*N*=5); **(B)** FOXP3 expression over time (*N*=5). Each line represents an individual experiment, each one performed using sorted CD4$^+$CD25$^-$ T cells from different donors.

6.3.3 TGFB INCREASES ACTIVATION INDUCED FOXP3 EXPRESSION IN $CD4^{+}CD25^{-}$ T CELLS

It has been previously demonstrated that TCR-induced FOXP3 expression in naïve human $CD4^{+}$ T cells is TGFβ dependent (Tran *et al.,* 2007). Therefore, it was hypothesised that the addition of TGFβ to these $CD4^{+}CD25^{-}$ T-cell cultures would increase FOXP3 expression. As shown in Figures 6.8 & 6.9, the addition of TGFβ significantly increased the proportion of cell expressing FOXP3 in a concentration dependent manner with an EC_{50} of 0.2ng/ml (95% CI 0.10-0.29). Maximal response was attained by the addition of TGFβ at a concentration of 5ng/ml with 50.5 ± 1.71% of the cells expressing FOXP3 on day five (Figure 6.8A&B). The actual concentration of TGFβ in the cultures may have been higher due to background TGFβ present within the human serum (normal range 20-50ng/ml (Liem *et al.,* 1999; Li *et al.,* 2010)). TGFβ also significantly increased the level of FOXP expression (MFI) in the $FOXP3^{+}$ cells in a concentration dependent manner with a MFI of 3205 ± 896 with the addition of 5ng/ml TGFβ (Figure 6.8C&D). In contrast, the addition of TGFβ had no effect on cell proliferation (Figure 6.8E&F). Likewise, the addition of the buffer (4mM HCl/BSA) used to reconstitute the TGFβ had no effect on either FOXP3 expression or cell proliferation (Figure 6.8B/D/F).

These data are therefore in keeping with the hypothesis that TGFβ increases FOXP3 expression in TCR activated $CD4^{+}$ T cells. For all subsequent experiments, TGFβ was added at 5ng/ml.

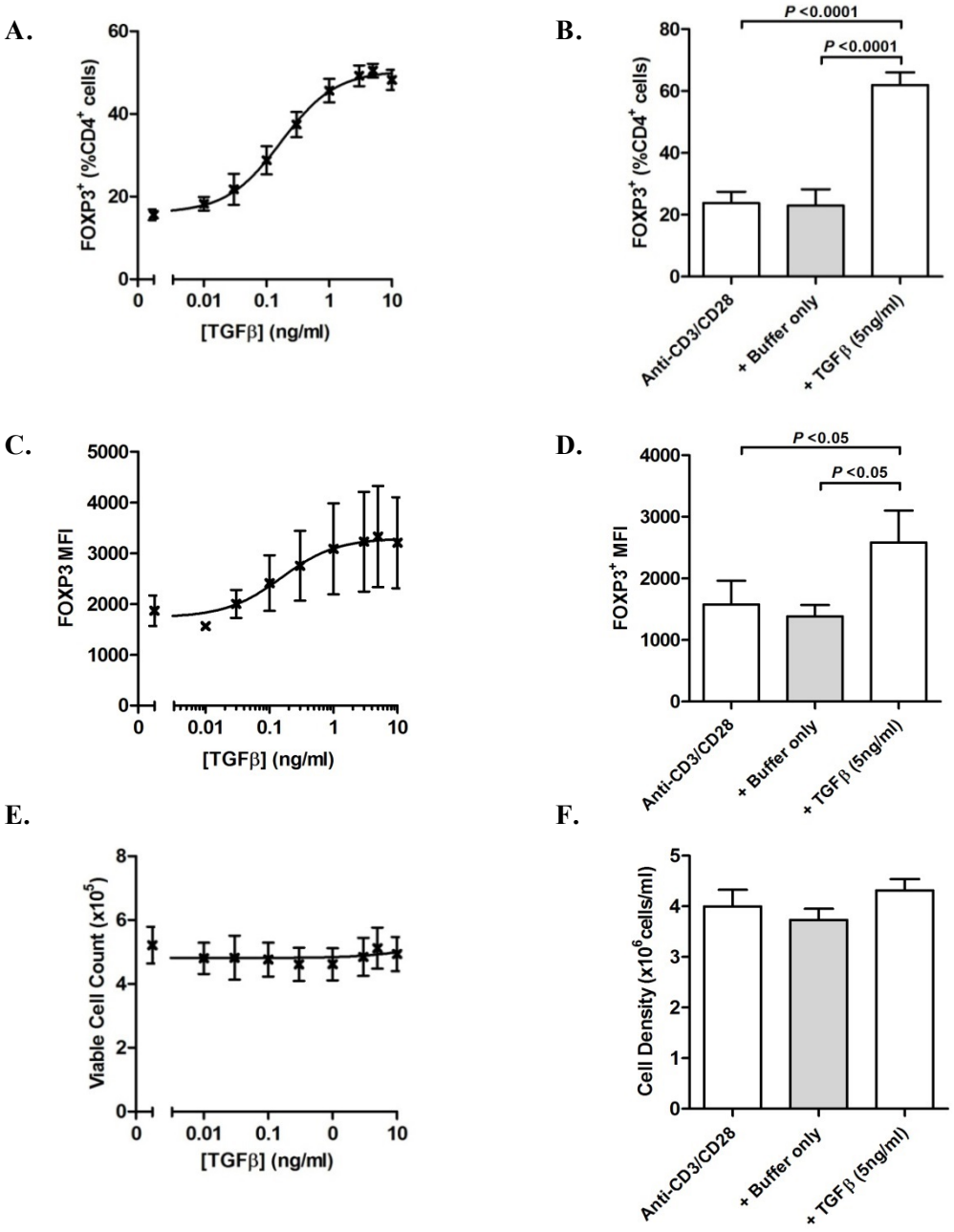


Figure 6.8: Cell Proliferation and FOXP3 Expression in CD4+CD25- T Cells Cultured with TGFβ

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 96% (84-97%), expressed as % all cells) were cultured in RH10 media, 30IU/ml IL2 and TGFβ (0-10ng/ml) at $0.5x10^6$cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Culture media was changed on day three. **(A/C/E)** Effect of increasing TGFβ concentration; **(B/D/F)** Effect of the 4mM HCl/BSA buffer used to reconstitute TGFβ. **(A&B)** Proportion of $CD4^+$ T cells expressing FOXP3 (*N*=3); **(C&D)** FOXP3 MFI in the $FOXP3^+$ cells (*N*=3); **(E&F)** $CD4^+$ T-cell counts (*N*=2). Figures show the mean values from *N* separate experiments (specified above), each performed using sorted $CD4^+CD25^-$ T cells from different donors.

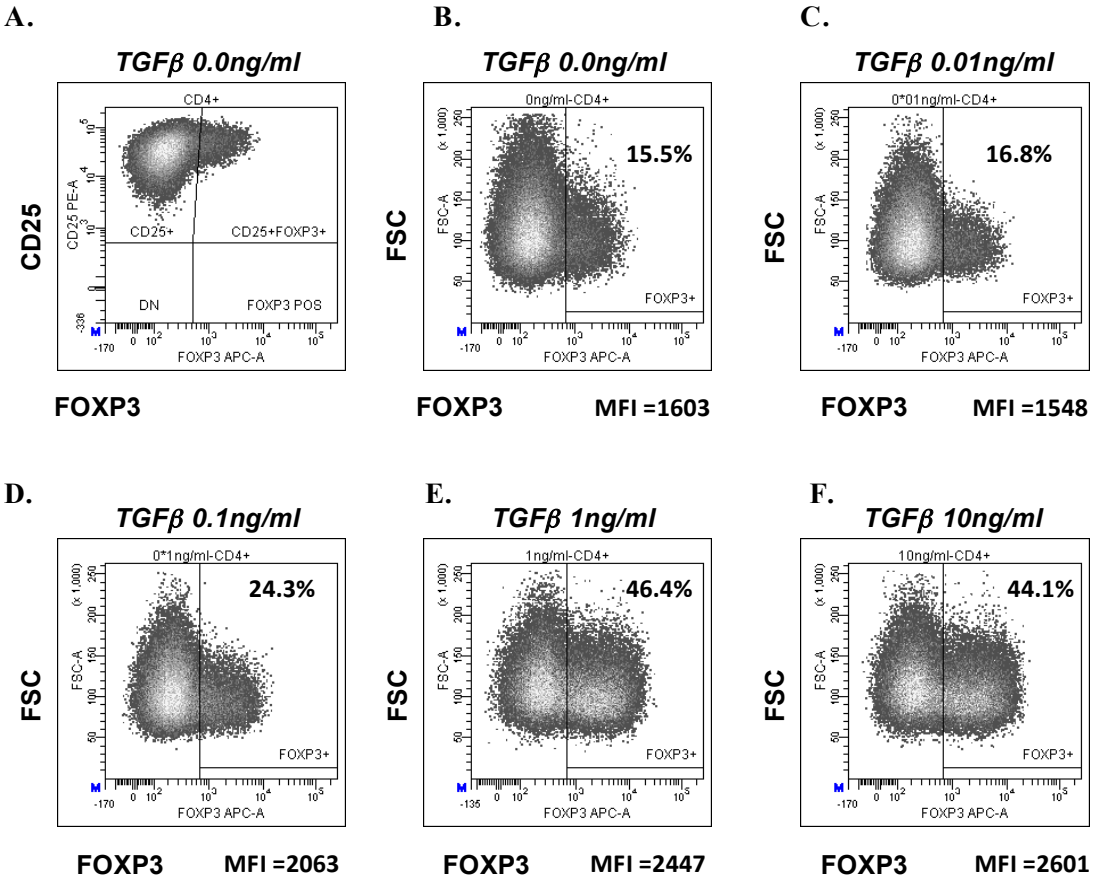


Figure 6.9: FOXP3 Expression in CD4$^+$CD25$^-$ T Cells Cultured with TGFβ

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 96%, expressed as % all cells) were cultured in RH10 media, 30IU/ml IL2 and TGFβ (0-10ng/ml) at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Culture media was changed on day three. Flow cytometry density plots show **(A)** FOXP3/CD25 expression; **(B-F)** FSC/FOXP3 expression. Plots show the proportion of cells expressing FOXP3 and the FOXP3 MFI of the CD4$^+$FOXP3$^+$ cells. Density plots from a single experiment which is representative of three separate experiments, each performed using sorted CD4$^+$CD25$^-$ T cells from different donors.

6.3.4 AZACITIDINE INCREASES ACTIVATION INDUCED FOXP3 EXPRESSION IN $CD4^+CD25^-$ T CELLS

Epigenetic regulation of the *FOXP3* gene plays a critical role in producing stable FOXP3 expression and sustaining a Treg phenotype. In particular, DNA demethylation of both the *FOXP3* promoter and a downstream regulatory element (TSDR) has been observed in nTregs and differentiates nTregs from conventional T cells (Baron *et al*., 2007). In view of these findings, it was hypothesised that addition of a DNA methyltransferase 1 inhibitor (Azacitidine) to the $CD4^+CD25^-$ T-cell cultures may produce *FOXP3* TSDR demethylation and further increase FOXP3 expression.

To test these hypotheses, Azacitidine was added to the $CD4^+CD25^-$ T-cell cultures. As Azacitidine can be toxic to the cells, the optimal concentration and duration of exposure to induce FOXP3 was first established. $CD4^+CD25^-$ T cells were cultured as before but with Azacitidine (0-10μM) for five days. Since Azacitidine is unstable in aqueous solutions with a quoted 10% loss in 2-3 hours at RT, fresh Azacitidine was added daily for the first 24, 72 or 120 hours (Figures 6.10 & 6.11) (Reynolds, 1989). Addition of Azacitidine for the first 24 hours only had little effect on the proportion of cells expressing FOXP3, although producing a modest reduction in cell proliferation at higher doses (1-10μM). Since Azacitidine requires cell division to be effective, it may be speculated that with only 24 hours exposure, the active Azacitidine was only present during the lag phase of proliferation. There was no difference between the 72 and 120 hour exposure to Azacitidine in either FOXP3 expression or cell proliferation. With both regimens, Azacitidine increased FOXP3 expression (% $CD4^+$ T cells expressing FOXP3 and the FOXP3 MFI) but reduced cell proliferation in a concentration dependent manner. Indeed, 10μM Azacitidine for 72 hours or more was toxic causing pronounced cell death with no $FOXP3^+$ cells detectable. To obtain a balance between inducing FOXP3 without markedly inhibiting cell proliferation, all subsequent experiments were performed using 1μM, added for the first 72 hours.

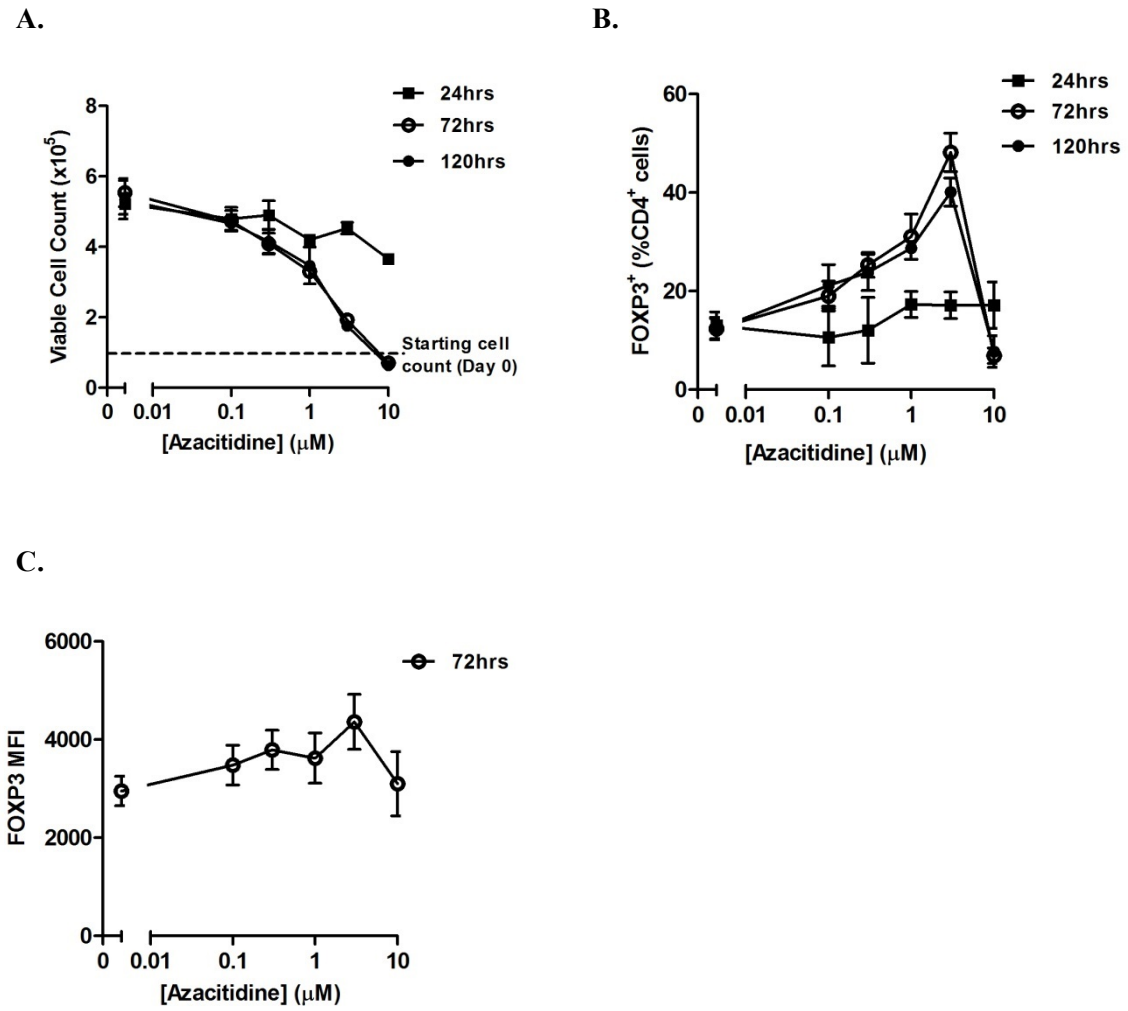


Figure 6.10: Cell Proliferation and FOXP3 Expression in $CD4^+CD25^-$ T Cells Cultured with Azacitidine

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 96% (96-98%), expressed as % all cells) were cultured in RH10 media, 30IU/ml IL2 and Azacitidine (0-10μM) at $0.5x10^6$cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 24, 72 or 120 hours. Culture media was changed on day three. **(A)** Cell counts (*N*=3); **(B)** Proportion of cells expressing FOXP3 (*N*=3); **(C)** FOXP3 MFI in the $CD4^+FOXP3^+$ cells (*N*=3). Figures show the mean values from three separate experiments, each performed using sorted $CD4^+CD25^-$ T cells from different donors.

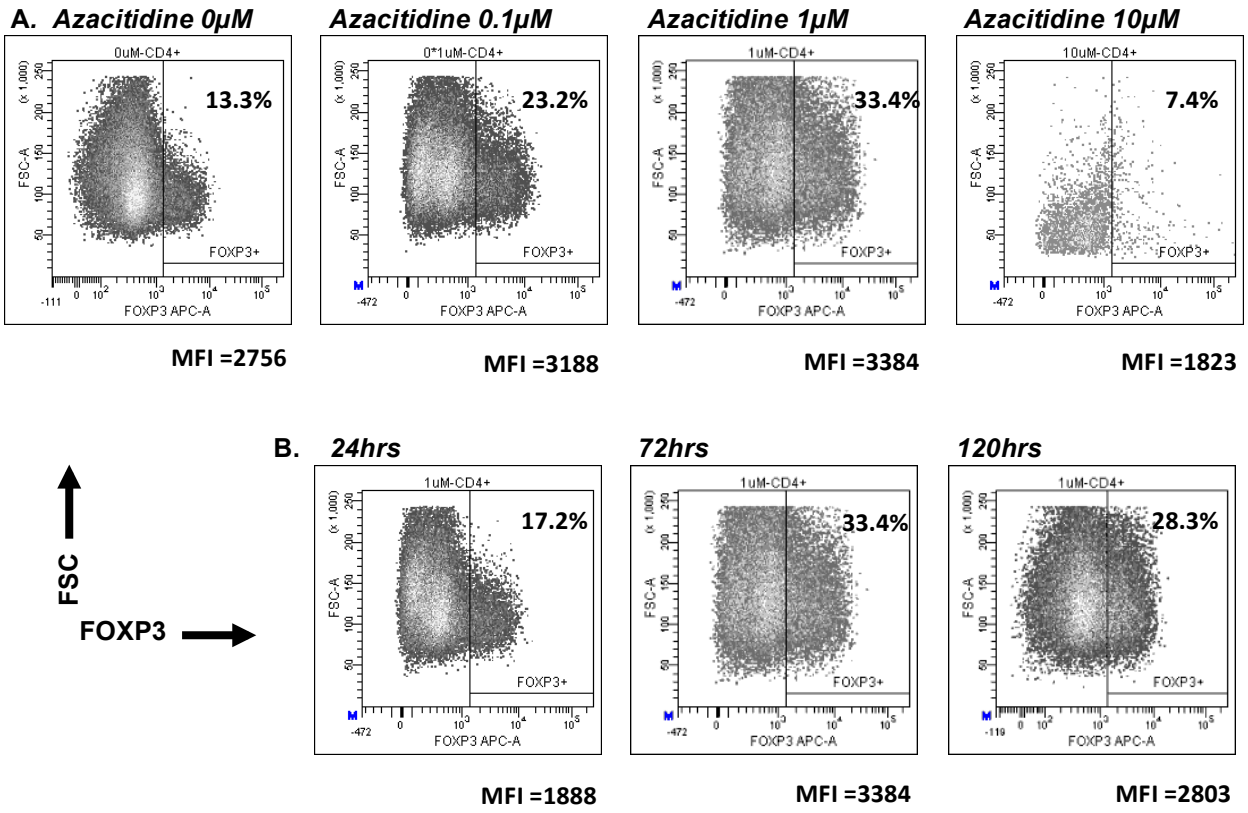


Figure 6.11: Cell Proliferation and FOXP3 Expression in $CD4^{+}CD25^{-}$ T Cells Cultured with Azacitidine

Magnetic-bead sorted $CD4^{+}CD25^{-}$ T cells (median purity 96%, expressed as % all cells) were cultured in RH10 media, 30IU/ml IL2 and Azacitidine (0-10μM) at $0.5x10^{6}$cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 24, 72 or 120 hours. Culture media was changed on day three. **(A)** Azacitidine (0-10μM) added for the first 72 hours; **(B)** Azacitidine (1μM) added daily for the first 24, 72 or 120 hours. Flow density plots show the proportion of cells expressing FOXP3 and the FOXP3 MFI of the $CD4^{+}FOXP3^{+}$ cells. Density plots from a single experiment which is representative of three separate experiments, each one performed using sorted $CD4^{+}CD25^{-}$ T cells from different donors.

Although Azacitidine increased FOXP3 expression, its action is not specific to FOXP3 alone and will inhibit DNA methylation in other genes during cell division. Therefore, to ensure that the phenotype of the cultured cells was not dramatically altered by Azacitidine, the expression of CD3, CD4 and CD25 was examined. In addition, the cells were also stained to ensure that Azacitidine did not cause expression of other lymphocyte lineage markers (CD8, CD19, CD16 and CD56) (Figure 6.12). Azacitidine significantly increased the level of CD25 expression in the $CD4^+CD25^+$ cells (58794 ± 5150 v 36815± 2609, *P*=0.008). However, 1μM Azacitidine for the first 72 hours of culture had no effect on the expression the other antigens investigated.

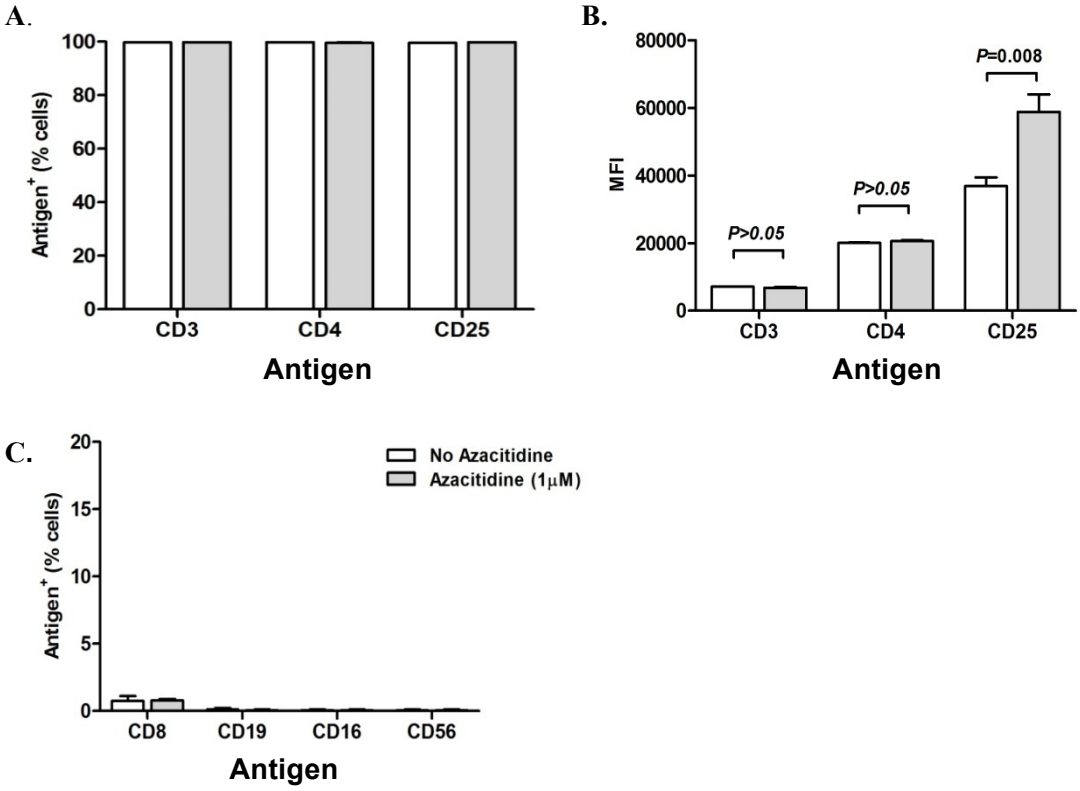


Figure 6.12: Antigens Expressed in $CD4^+CD25^-$ T Cells Cultured with Azacitidine

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 96% (96-98%), expressed as % all cells) were cultured in RH10 media, 30IU/ml IL2 and Azacitidine (0-10μM) at $0.5x10^6$cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours (*N*=3). No Azacitidine (white bars); Azacitidine (grey bars). **(A)** Proportion of cells expressing CD3, CD4 or CD25; **(B)** MFI of CD3, CD4 and CD25 in the antigen positive cells; **(C)** Proportion of cells expressing CD8, CD19, CD16 and CD56. Figures show the mean values from three separate experiments, each performed using sorted $CD4^+CD25^-$ T cells from different donors.

6.3.5 TGFB AND AZACITIDINE INCREASE ACTIVATION INDUCED FOXP3 EXPRESSION IN $CD4^+CD25^-$ T CELLS

Given the observation that the *FOXP3* TSDR and the TGFβ-sensor are separate enhancers for FOXP3 expression, this study tested the hypothesis that Azacitidine can further increase TCR/TGFβ induced FOXP3 expression (Baron *et al.,* 2007; Huehn *et al.,* 2009).

A) FOXP3 EXPRESSION AND CELL PROLIFERATION

As observed above, when 1μM Azacitidine was added to the TGFβ (5ng/ml) supplemented cell cultures, it significantly reduced the cell density after five days ($4.14 \pm 0.14x10^6$/ml v $2.68 \pm 0.16x10^6$/ml, $P<0.001$) (Figure 6.13A). In contrast, the addition of TGFβ to the Azacitidine treated cultures produced a small but significant increase in cell density after five days ($2.12 \pm 0.05x10^6$/ml v $2.68 \pm 0.16x10^6$/ml, $P<0.01$) (Figure 6.13A). Although such a response was not observed when TGFβ was added to the anti-CD3/CD28 cultures (TCR only), it should be noted both cultures had already reached the plateau cell density at the time of testing. In keeping with the hypothesis, the combination of Azacitidine and TGFβ together produced a significantly higher proportion of $CD4^+$ T cells expressing FOXP3 than using either agent alone (Figure 6.13B&D). This combination also significantly increased the level of FOXP3 expression in the $CD4^+FOXP3^+$ T cells (Figure 6.13C&D).

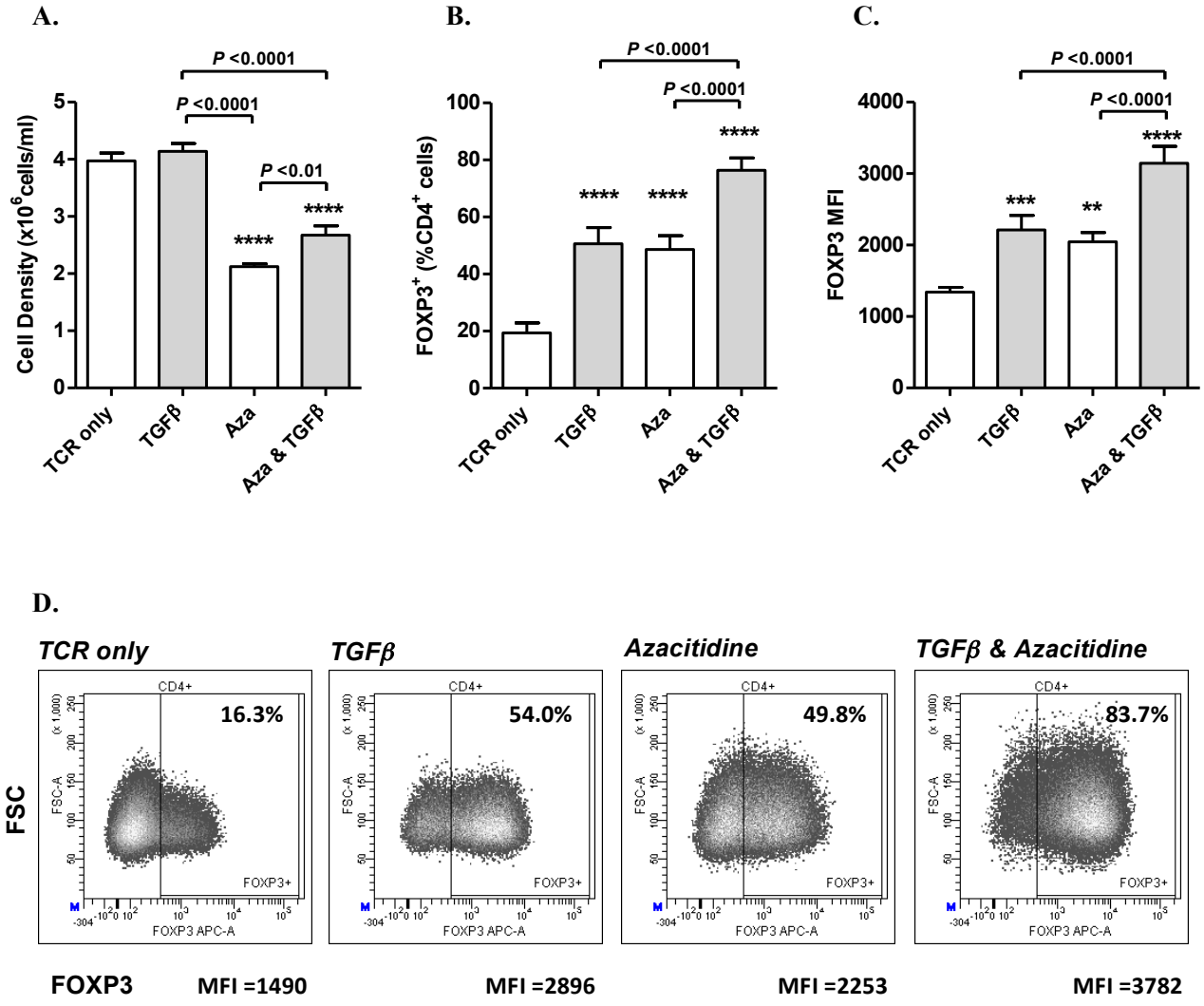


Figure 6.13: Cell Proliferation and FOXP3 Expression in CD4⁺CD25⁻ T Cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 87% (76-94%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. Figure shows (A) Cell counts (*N*=7); (B) Proportion of $CD4^+$ T cells expressing FOXP3 (*N*=6); (C) FOXP3 MFI in the $CD4^+FOXP3^+$ cells (*N*=6); (D) FSC/FOXP3 flow cytometry density plots from a single representative experiment. Figures show the mean values from *N* separate experiments (specified above), each performed using sorted $CD4^+CD25^-$ T cells from different donors.

While a significant proportion of the cultured $CD4^+$ T cells expressed both CD25 and FOXP3, it was unknown whether they also contained a demethylated *FOXP3* TSDR, as seen in nTregs. Therefore, $CD4^+CD25^-$ T cells were cultured and harvested as above, DNA extracted and *FOXP3* TSDR methylation status determined using the *FOXP3* MSRT-qPCR assay. After five days activation with anti-CD3/CD28, 23.7 ± 3.7% of the $CD4^+$ T cells expressed FOXP3. However, none of the cultured cells contained a demethylated *FOXP3* TSDR (0.05 ± 0.03%) (Figure 6.14). The addition of TGFβ significantly increased the proportion of cells expressing FOXP3 (61.9 ± 4.1%) but less than 1% of cells (0.98 ± 0.93%) were demethylated at the *FOXP3* TSDR. In contrast, Azacitidine significantly increased the proportion of cells with a demethylated *FOXP3* TSDR, when added alone or in combination with TGFβ (69.1 ± 7.2% and 91.9 ± 3.2% respectively). Interestingly, the addition of TGFβ to the Azacitidine treated cells significantly increased the percentage of cells with *FOXP3* TSDR demethylation (Figure 6.14).

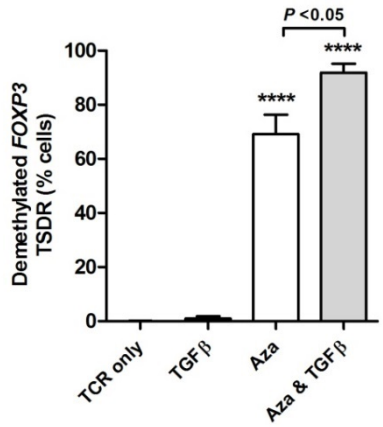


Figure 6.14: FOXP3 TSDR Methylation Status in $CD4^+CD25^-$ T Cells Cultured with or without TGFβ and Azacitidine

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 76% (75-86%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. DNA was then extracted from the cultured cells and tested for FOXP3 methylation using the FOXP3 MSRT-qPCR assay. Figure shows the mean percentage of cells with an unmethylated FOXP3 TSDR from three separate experiments, each performed using sorted $CD4^+CD25^-$ T cells from different donors. **** P<0.0001(compared to TCR only).

6.3.6 THE INDUCED CD4+FOXP3+ T CELLS PRODUCE PRO-INFLAMMATORY CYTOKINES

In the experiments so far, the majority of the activated $CD4^+$ T cells cultured with TGFβ and Azacitidine, expressed FOXP3 (76.4 ± 4.31%, *N*=6) and contained a demethylated *FOXP3* TSDR (91.9 ± 3.24%, *N*=6). Although *in vivo* these markers are present on nTregs, it was uncertain if these induced $CD4^+FOXP3^+$ cells had a nTreg phenotype and/or suppressive function (Baron *et al.,* 2007). Therefore, to test the hypothesis that the combination of TGFβ and Azacitidine can produce iTregs, the cultured cells were tested for their ability to produce pro-inflammatory cytokines and/or inhibit the proliferation of autologous $CD4^+$ T cells.

A) CYTOKINE EXPRESSION

To investigate whether the induced $CD4^+FOXP3^+$ T cells were able to produce inflammatory cytokines, the cultured cells were activated and stained for CD4, FOXP3 and the cytokines IL2, TNFα, IFNγ or IL17. In contrast to $CD4^+CD25^+FOXP3^+$ nTregs (Figure 6.15), many of the TCR induced $FOXP3^+$ cells produced IL2 and TNFα (47.0 ± 9.62% and 79.2 ± 3.76%) (Figures 6.16 & 6.17) (Levings *et al*., 2001; Stephens *et al*., 2001). A small minority of the $FOXP3^+$ cells also produced IFNγ or IL17 (7.05 ± 0.90% and 2.18 ± 0.39%). When $CD4^+CD25^-$ T cells were cultured in the presence of TGFβ, similar proportions of the induced $FOXP3^+$ cells produced Il2, TNFα, IFNγ and IL17 (49.4 ± 10.3%, 83.7 ± 4.79%, 3.35 ± 0.77% and 2.28 ± 1.15%). In contrast, when Azacitidine was added, significantly smaller proportions of the $FOXP3^+$ cells produced IL2 or TNFα (16.7 ± 2.90% and 56.7 ± 4.75%) but more produced IFNγ (21.5 ± 2.51%). In contrast to the hypothesis, a large proportion of the $CD4^+FOXP3^+$ cells from the TGFβ & Azacitidine cultures also produced IL2, TNFα or IFNγ (29.4 ± 3.08%, 78.3 ± 2.96% and 31.0 ± 1.80%). A smaller minority also produced IL17 (11.5 ± 5.67%). Therefore, in contrast to $CD4^+CD25^+FOXP3^+$ nTregs, a significant proportion of the induced $CD4^+CD25^+FOXP3^+$ T cells produced pro-inflammatory cytokines. For all culture conditions, the proportion of cells producing pro-inflammatory cytokines was not lower in the $FOXP3^+$ population compared to the $FOXP3^-$ cells. Hence, the induction FOXP3 did not appear to significantly inhibit the cells ability to produce inflammatory cytokines.

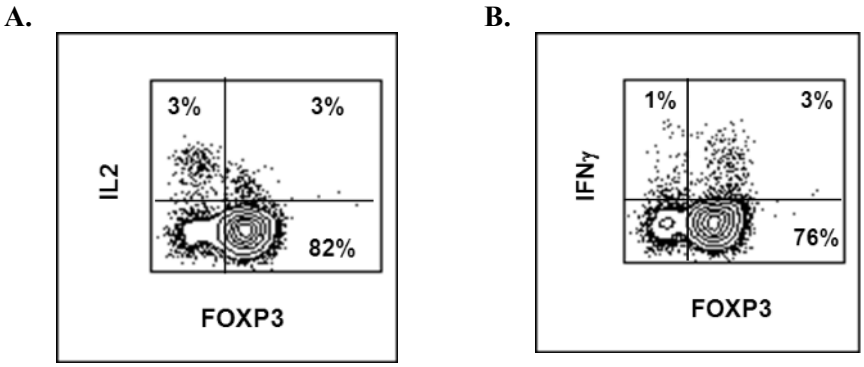


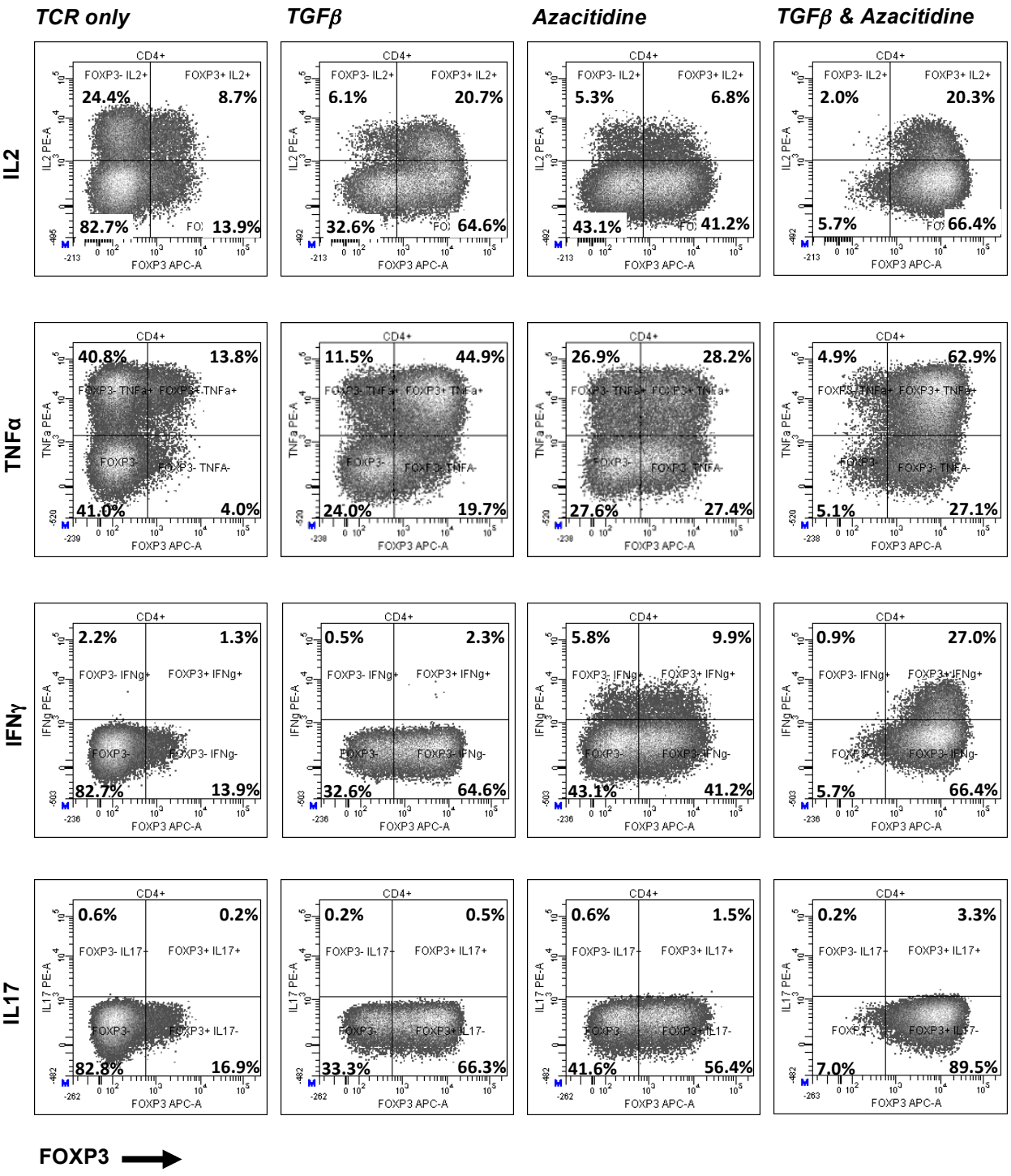
Figure 6.15: Pro-inflammatory Cytokine Production in *Ex Vivo* Isolated CD4+CD25high T cells

Ex vivo isolated $CD4^+CD25^{high}$ T cells (putative Tregs) from a healthy control donor were stimulated with 50ng/ml phorbol myristate acetate, 1μM Ionomycin and 5μg/ml Brefeldin A for four hours at 37°C. The activated cells were stained for FOXP3 and (**A**) IL2; (**B**) IFNγ. Very few of the $FOXP3^+$ cells produced IL2 or IFNγ. *Data and flow plots were kindly supplied by Dr Wei Zhang (Blood Research Laboratory, NHSBT, Oxford, UK).*

On next page

Figure 6.16: Cytokine Production in $CD4^+CD25^-$ T Cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 90% (80-97%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. The cultured cells were then harvested, re-stimulated (50ng/ml phorbol myristate acetate, 1μM Ionomycin and 5μg/ml Brefeldin A) for four hours at 37°C and stained for CD4, FOXP3 and IL2, TNFα, IFNγ or IL17. Figure shows flow cytometry density plots from a single experiment which is representative of four separate experiments each performed using sorted $CD4^+CD25^-$ T cells from different donors.


TCR only
TGFβ
Azacitidine
TGFβ & Azacitidine
IL2
TNFα
IFNγ
IL17
FOXP3
CD4+
FOXP3- IL2+
FOXP3+ IL2+
24.4%
8.7%
82.7%
13.9%
6.1%
20.7%
32.6%
64.6%
5.3%
6.8%
43.1%
41.2%
2.0%
20.3%
5.7%
66.4%
IL2 PE-A
FOXP3 APC-A
FOXP3- TNFa+
FOXP3+ TNFa+
FOXP3-
FOXP3- TNFA-
40.8%
13.8%
41.0%
4.0%
11.5%
44.9%
24.0%
19.7%
26.9%
28.2%
27.6%
27.4%
4.9%
62.9%
5.1%
27.1%
TNFa PE-A
FOXP3- IFNg+
FOXP3+ IFNg+
FOXP3- IFNg-
2.2%
1.3%
82.7%
13.9%
0.5%
2.3%
32.6%
64.6%
5.8%
9.9%
43.1%
41.2%
0.9%
27.0%
5.7%
66.4%
IFNg PE-A
FOXP3- IL17+
FOXP3+ IL17+
FOXP3+ IL17-
0.6%
0.2%
82.8%
16.9%
0.2%
0.5%
33.3%
66.3%
0.6%
1.5%
41.6%
56.4%
0.2%
3.3%
7.0%
89.5%
IL17 PE-A

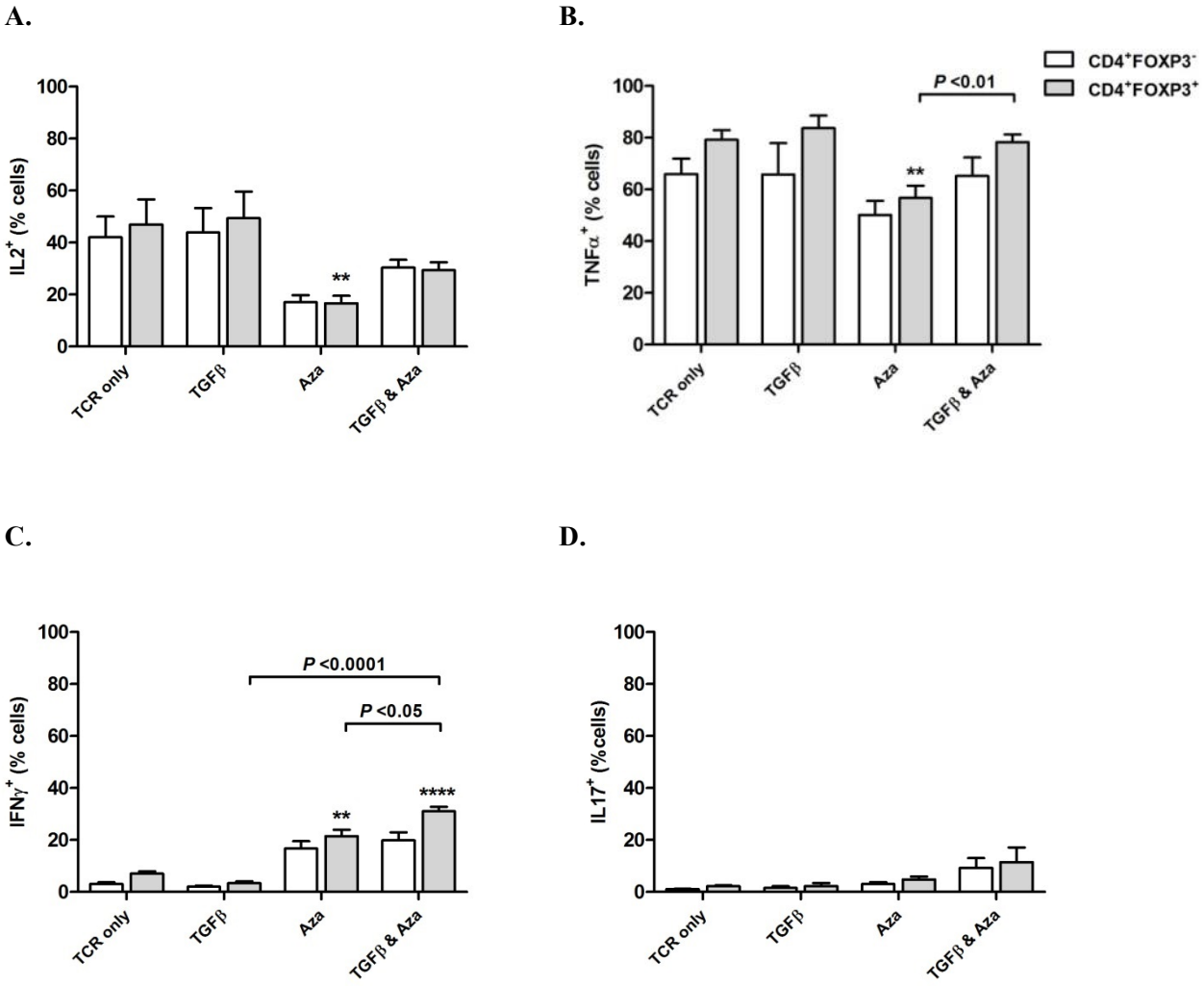


Figure 6.17: Cytokine Production in CD4+CD25- T Cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 90% (80-97%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. The cultured cells were then harvested, re-stimulated (50ng/ml phorbol myristate acetate, 1μM Ionomycin and 5μg/ml Brefeldin A) for four hours at 37°C and stained for CD4, FOXP3 and **(A)** IL2, **(B)** TNFα, **(C)** IFNγ, **(D)** IL17. Figures show the mean percentage of cytokine positive cells within the $FOXP3^-$ (white bars) and $FOXP3^+$ cells (grey bars) from four separate experiments. each performed using sorted $CD4^+CD25^-$ T cells from different donors. **P<0.01, ****P<0.0001(compared to TCR only).

B) SUPPRESSIVE FUNCTION

To test whether the *in vitro* induced $CD4^+FOXP3^+$ T cells had suppressive function, *in vitro* proliferation assays were set up as per Methods 2.3.6. In this assay, anti-CD3/CD28 driven proliferation of CFSE-labelled autologous $CD4^+CD25^-$ T cells was performed in the presence of serial dilutions of the TCR/TGFB/Azacitidine cultured $CD4^+CD25^-$ T cells. As FOXP3 is intracellular, it was not possible to stain and isolate induced $CD4^+FOXP3^+$ cells only for these functional tests. Therefore, these assays assess the suppressive function of the whole cell culture. Unfortunately, these proliferation assays had a number of problems limiting analysis (Figure 6.18).

A. The autologous $CD4^+CD25^-$ T cells failed to proliferate sufficiently. In the example shown, only 11.9% of the $CD4^+CD25^-$ T-cell population had divided one or more times (Figure 6.18A).
B. In contrast to *ex* vivo isolated $CD4^+CD25^+CD127^{-/dim}$ cells (Figure 3.26), the TCR/TGFB/Azacitidine cultured $CD4^+CD25^-$ T cells continued to proliferate. Therefore, although the proliferation assays were set up with a 50:50 ratio, after five days, the cultured T cells (non-CFSE labelled cells) accounted for 95.4%, 93.9 %, 87.5% and 85.2% of all the cells in the TCR, TGFβ, Aza and TGFβ&Aza assays respectively (Figure 6.18B-E). As such, anti-CD3/CD28 driven proliferation of the autologous $CD4^+CD25^-$ T cells may have been inhibited by consumption of nutrients and/or increasing cell density.
C. Resting the TCR/TGFB/Azacitidine cultured $CD4^+CD25^-$ T cells for one week before adding them to the functional assays was unable to overcome these issues. However, resting them for two weeks caused excessive cell death (>50%).

Despite these issues, there were two interesting observations. First, in contrast to *ex* vivo isolated $CD4^+CD25^+CD127^{-/dim}$ cells, the autologous $CD4^+CD25^-$ T cells proliferated more when the cultured T cells were added. Secondly, the proportion of proliferating CFSE-labelled $CD4^+CD25^-$ T cells was lower when TCR/TGFβ&Azacitidine cultured $CD4^+CD25^-$ T cells were added compared to the TCR alone or TCR/TGFβ cultured cells. However, due to the problems highlighted above, there were insufficient experiments to perform statistical analysis to confirm these findings.

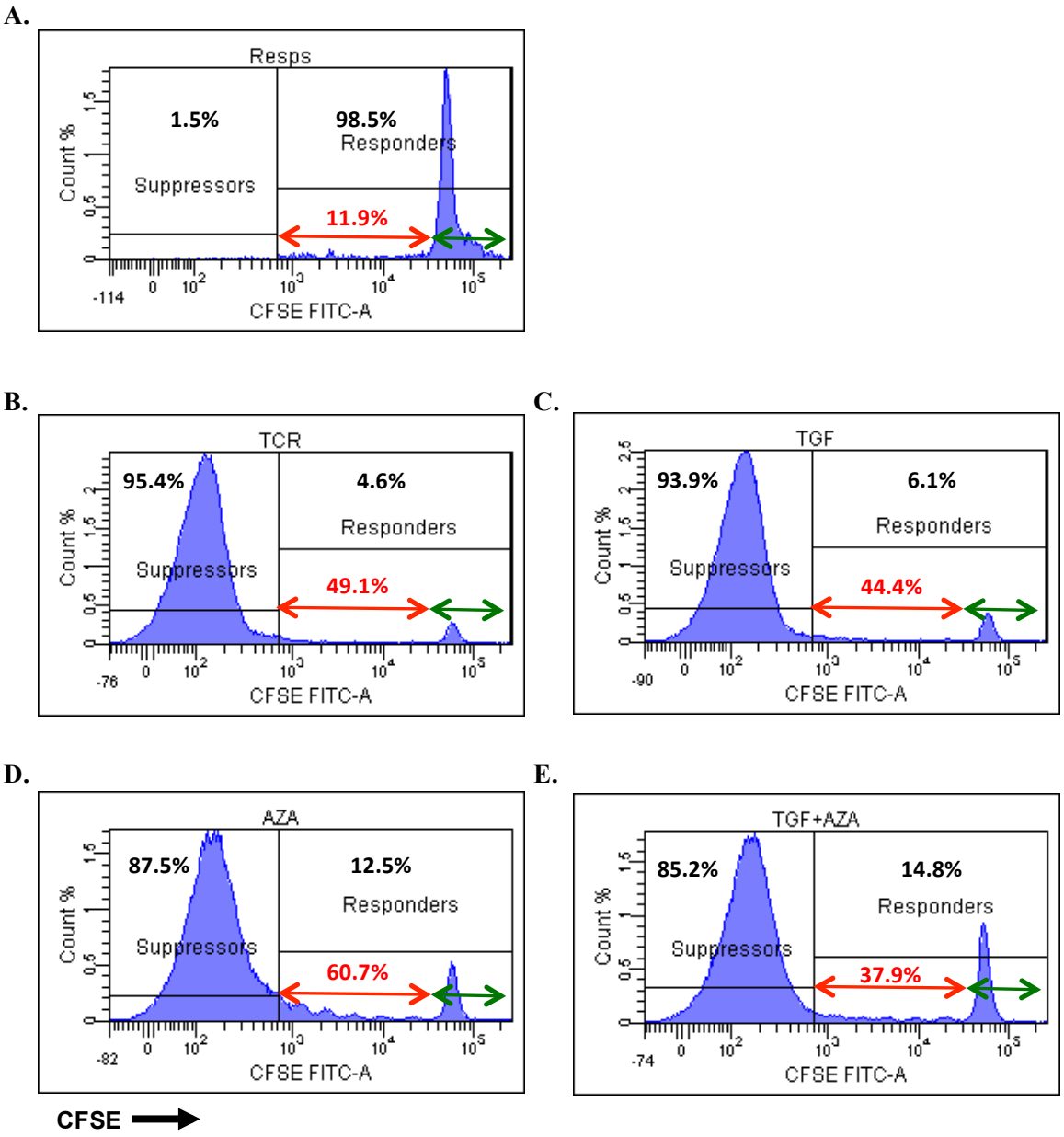


Figure 6.18: Suppressive Function of the CD4^{+}CD25^{-} T Cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted CD4^{+}CD25^{-} T cells (median purity 76% (75-86%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at 0.5x10^{6}cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. The cultured cells were then harvested and used in the *in vitro* proliferation assays. In these assays, 5x10^{4} CFSE-labelled (0.5μM) autologous CD4^{+}CD25^{-} T cells (median purity 76% (75-86%), expressed as % all cells) were activated for five days in 96-well U-bottomed plates with plate bound anti-CD3 (5μg/ml) and soluble anti-CD28 (1μg/ml) in the

presence **(B-E)** or absence **(A)** of an equal number of pre-cultured $CD4^+CD25^-$ T cells: cultured with **(B)** Anti-CD3/CD28 beads only **(C)** Anti-CD3/CD28 beads plus TGFβ (5ng/ml); **(D)** Anti-CD3/CD28 beads plus Azacitidine (1μM); **(E)** Anti-CD3/CD28 beads plus TGFβ (5ng/ml) & Azacitidine (1μM). Red arrow and % shows the proliferating autologous CFSE-labelled $CD4^+CD25^-$ T cells (% of all the CFSE-labelled $CD4^+CD25^-$ T cells). Green arrow shows the non-proliferating CFSE-labelled $CD4^+CD25^-$ T cells. Data shown are from a single experiment which is representative of four separate experiments, each performed using sorted $CD4^+CD25^-$ T cells from different donors.

6.3.7 ANTI-CD3/CD28 STIMULATION

Under these *in vitro* culture conditions, a large proportion of the induced FOXP3$^+$ cells produced IL2 and TNFα. It was speculated that this may be because the cultured CD4$^+$ T cells continued to receive anti-CD3/CD28 stimulation up to the time of analysis. As a consequence, the anti-CD3/CD28 co-stimulation could drive second messenger pathways (e.g. Calcineurin/NFAT) to up-regulate the transcription of IL2 and TNFα and prevent FOXP3 from inhibiting the production of these cytokines. There were also questions about the stability of FOXP3 expression and whether removing the initial culture conditions would lead to a loss of FOXP3. Of particular concern for Treg cellular therapies is the potential plasticity of induced FOXP3$^+$ T cells, such that when placed into a pro-inflammatory environment they may up-regulate their effector phenotype and exacerbate inflammation.

To address these questions, isolated human CD4$^+$CD25$^-$ T cells were cultured *in vitro* as before followed by removal of the anti-CD3/CD28 Dynabeads and allowed to rest for seven days in RH10 culture medium only. In all culture conditions, there was no significant difference in the overall proportion of CD4$^+$ T cells that expressed FOXP3 after seven days without anti-CD3/CD28 stimulation (Figure 6.19). Since FOXP3 is an intracellular protein, it was not possible to isolate only the FOXP3 cells on day five for further culture. Therefore, it cannot be concluded that the FOXP3$^+$ cells on day five were the same cells that were positive on day twelve. The level of FOXP3 expression in the CD4$^+$FOXP3$^+$ fell for all culture conditions after seven days rest although this did not reach significance (P>0.05) for the Azacitidine treated cells.

In contrast to the working hypothesis, even after removing the anti-CD3/CD28 stimulation for one week, almost all of the induced FOXP3$^+$ cells were still able to produce TNFα (Figure 6.20). Likewise, a large proportion of the FOXP3$^+$ cells were also able to produce IL2, although as before, this proportion was lower in those cell cultures treated with Azacitidine.

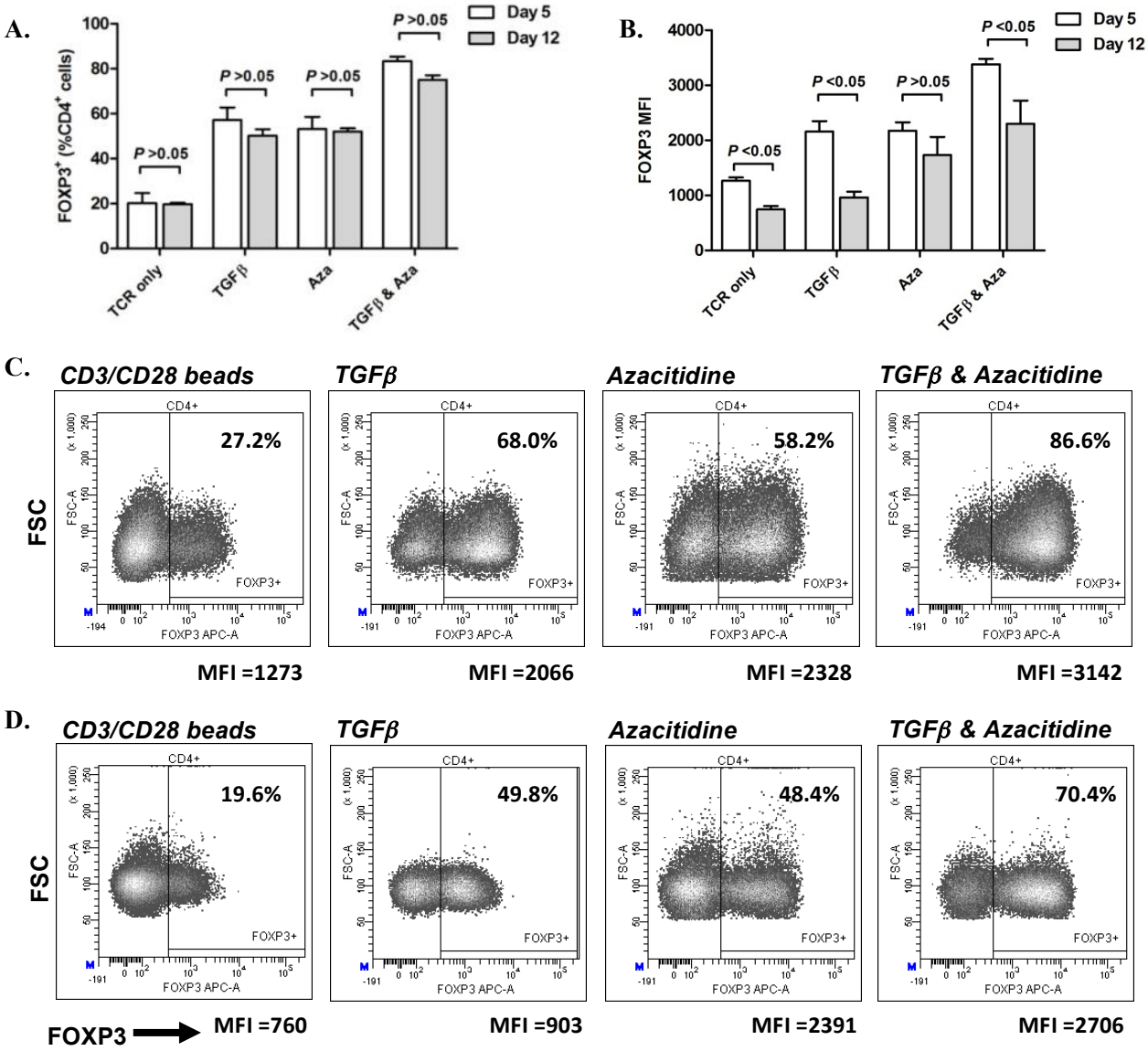


Figure 6.19: FOXP3 Expression in CD4⁺CD25⁻ T Cells Cultured with TGFβ and Azacitidine and Rested for Seven Days

Magnetic-bead sorted $CD4^+CD25^-$ T cells (median purity 90% (80-97%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. Anti-CD3/CD28 beads were then removed and the cells rested for seven days in RH10 media with 30IU/ml IL2. Figure shows **(A)** Proportion of $CD4^+$ T cells expressing FOXP3 **(B)** FOXP3 MFI in the $CD4^+FOXP3^+$ cells **(C-D)** FSC/FOXP3 flow cytometry density plots: **(C)** Day 5; **(D)** Day 12 (rested for seven days). Figures show the mean values from four experiments, each performed using sorted $CD4^+CD25^-$ T cells from different donors. Density plots are from a single representative experiment.

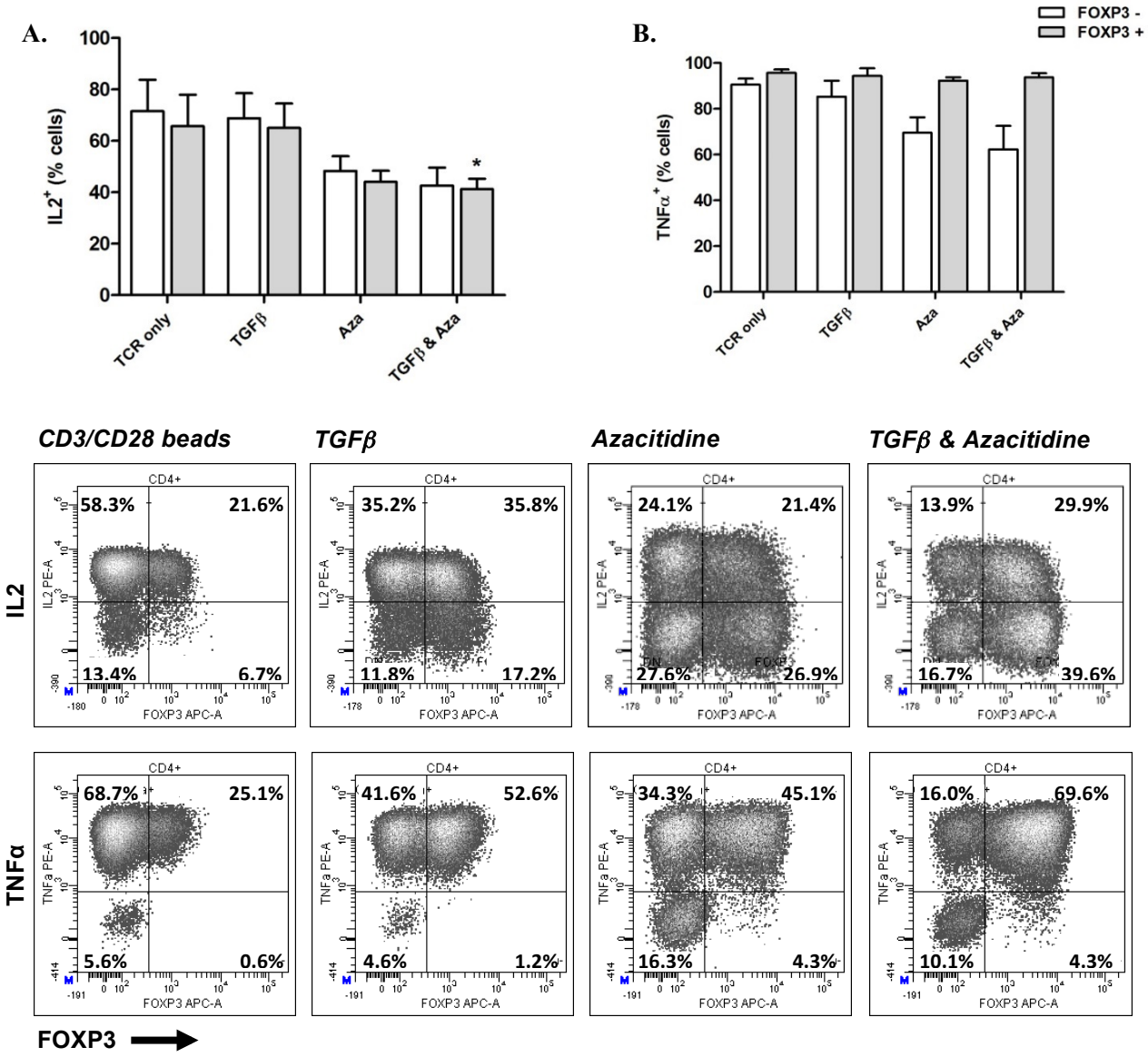


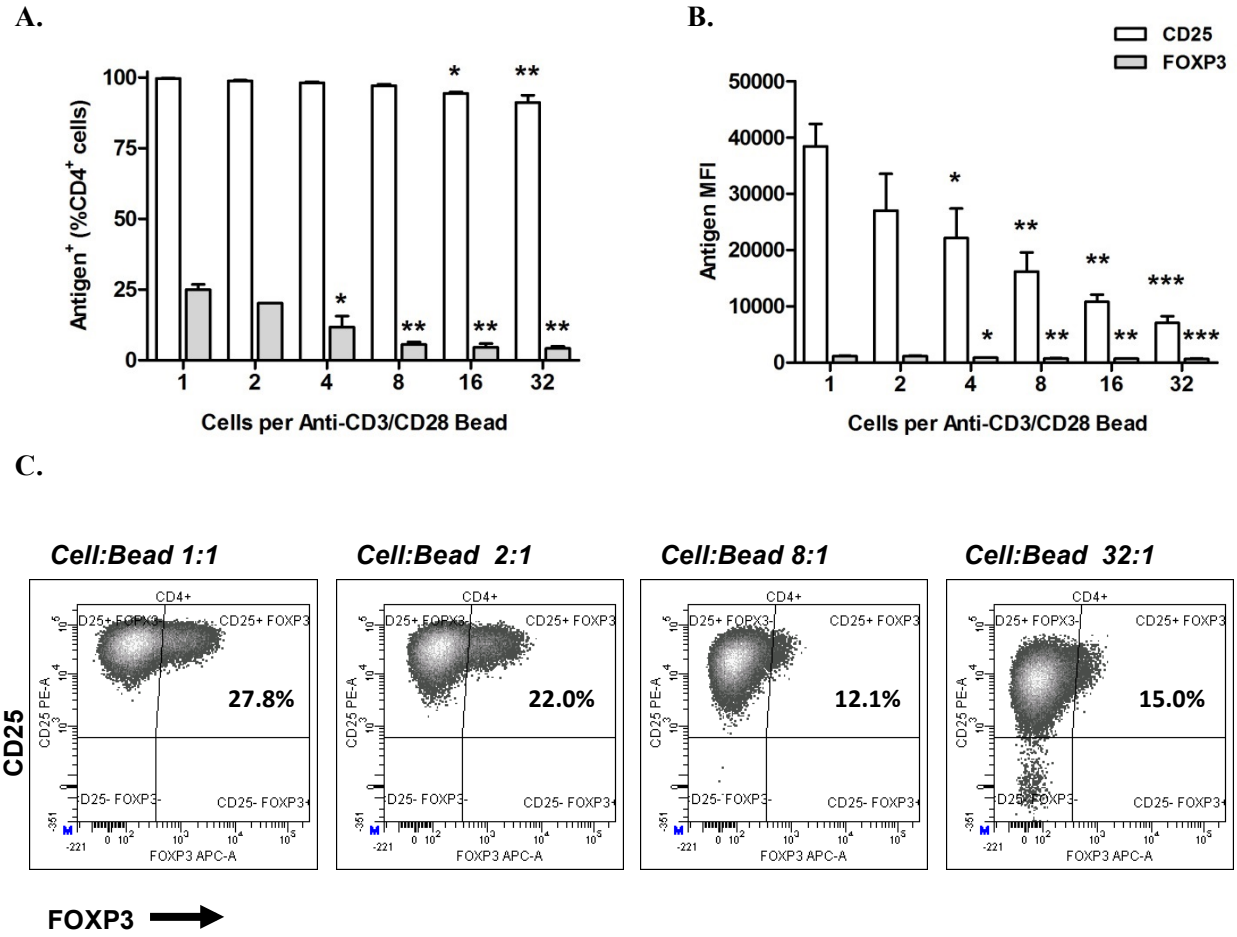
Figure 6.20: Cytokine Production from CD4$^+$CD25$^-$ T Cells Cultured with TGFβ and Azacitidine and Rested for Seven Days

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 90% (80-97%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at 0.5x10^{6}cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. Anti-CD3/CD28 beads were removed and the cells rested for seven days in RH10 media with 30IU/ml IL2. The cultured cells were harvested, re-stimulated (50ng/ml phorbol myristate acetate, 1μM Ionomycin and 5μg/ml Brefeldin A) for four hours at 37^{o}C and stained for CD4, FOXP3 and **(A)** IL2, **(B)** TNFα. Figures show the mean percentage of cytokine positive cells within the FOXP3$^-$ (white bars) and FOXP3$^+$ cells (grey bars) from four experiments. each performed using sorted CD4$^+$CD25$^-$ T cells from different donors. *P<0.05, (compared to TCR only). Density plots are from a single representative experiment.

Since removing the CD3/CD28 co-stimulation for one week did not appear to make any significant difference in the ability of the induced $FOXP3^+$ cells to produce inflammatory cytokines, it was questioned whether the initial anti-CD3/CD28 Dynabead to $CD4^+CD25^-$ T-cell ratio to was too high to induce a regulatory phenotype. It was hypothesised that excessive TCR stimulation may up-regulate many intracellular second messenger pathways, driving the production of inflammatory cytokines and overriding the ability of FOXP3 to suppress IL2 and TNFα. To test this hypothesis, the number of anti-CD3/CD28 Dynabeads used in the cultures was reduced.

After five days in culture, increasing the T-cell to anti-CD3/CD28 Dynabead ratio from 1:1 to 32:1 produced a small reduction in the proportion of $CD4^+$ T cells expressing CD25 (99.7 ± 0.1% v 91.2 ± 2.55%, $P<0.01$), although there was a more marked reduction in the level of CD25 expression in the $CD4^+CD25^+$ cells (38436 ± 3991 v 7078 ± 1203, $P<0.001$) (Figure 6.21). However, reducing the number of anti-CD3/CD28 Dynabeads reduced both the proportion of cells expressing FOXP3 (25.0 ± 1.80 4.25 ± 0.75, $P<0.01$) and the level of expression in the $CD4^+FOXP3^+$ cells (1169 ± 69.5 v 681 ± 72.0, $P<0.001$) (Figure 6.21). Even though the proportion of cells expressing FOXP3 fell, the majority of the $CD4^+FOXP3^+$ cells were still able to produce inflammatory cytokines. At cell to bead ratios of 32:1, the proportion of induced $FOXP3^+$ producing Il2 and TNFα was 57.4 ± 14.7% and 73.1 ± 13.1% respectively (Figure 6.22).

Therefore, within the limits of the cell to bead ratios tested, reducing the number of anti-CD3/CD28 Dynabeads did not appear to induce FOXP3 expression but prevent their ability to produce pro-inflammatory cytokines.



	1:1	2:1	8:1	32:1
CD25 MFI	42426	33580	19599	8280
FOXP3 MFI	1099	1072	698	609

Figure 6.21: CD25 and FOXP3 Expression in CD4$^+$CD25$^-$ T Cells Cultured with Reducing Numbers of Anti-CD3/CD28 Dynabeads

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 92% (80-94%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2. Cells were cultured at 0.5x10^{6}cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratios 1:1 to 32:1) for five days. Culture media was changed on day three. **(A)** Proportion of CD4$^+$ T cells expressing CD25 and FOXP3; **(B)** CD25 and FOXP3 MFI in the CD4$^+$CD25$^+$ and CD4$^+$FOXP3$^+$ cells respectively. All statistics show comparison to the 1:1 cell to bead ratio. **(C)** Flow cytometry density plots and the CD25 and FOXP3 MFI. Percentages show the % of CD4$^+$ T cells expressing FOXP3. Figures show the mean values from three experiments, each performed using sorted CD4$^+$CD25$^-$ T cells from different donors. Density plots are from a single representative experiment.

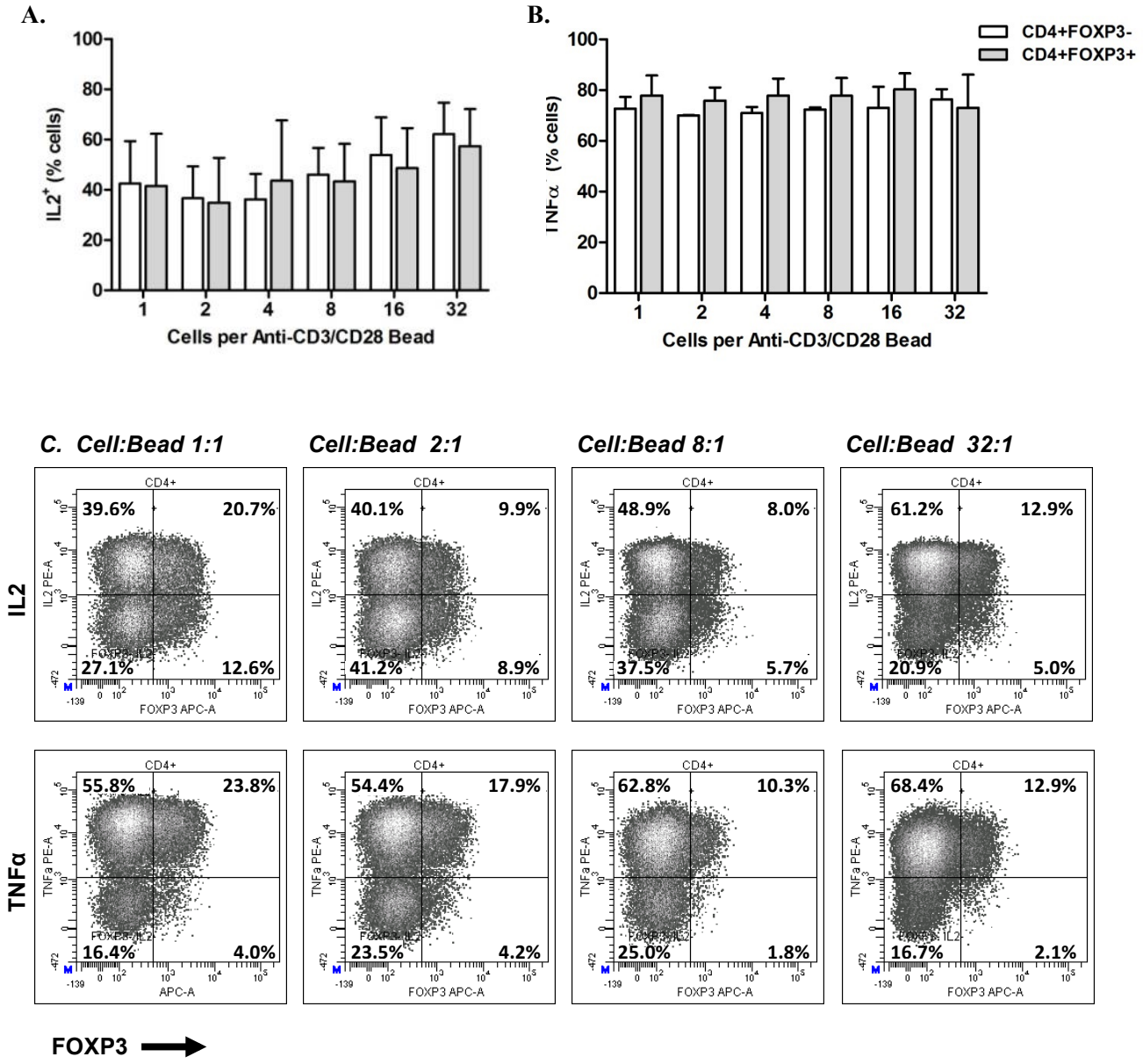


Figure 6.22: Cytokine Production in CD4$^+$CD25$^-$ T Cells Cultured with Reducing Numbers of Anti-CD3/CD28 Dynabeads

Magnetic-bead sorted CD4$^+$CD25$^-$ T cells (median purity 92% (80-94%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2. Cells were cultured at 0.5x10^{6}cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratios 1:1 to 32:1) for five days. Culture media was changed on day three. The cultured cells were harvested, re-stimulated (50ng/ml phorbol myristate acetate, 1µM Ionomycin and 5µg/ml Brefeldin A) for four hours at 37^{o}C and stained for CD4, FOXP3 and **(A)** IL2, **(B)** TNFα. Figures show the mean percentage of cytokine positive cells within the FOXP3$^-$ (white bars) and FOXP3$^+$ cells (grey bars) from three experiments. each performed using sorted CD4$^+$CD25$^-$ T cells from different donors. Density plots are from a single representative experiment.

6.3.8 INDUCING FOXP3 IN NAÏVE $CD4^+CD25^-$ T CELLS

All the *in vitro* cell culture experiments performed above used isolated human $CD4^+CD25^-$ T cells, containing a mixture of both naive and effector/memory T cells. Since the effector/memory T cells will have already been exposed to antigen *in vivo* and undergone further differentiation, it was proposed that these cells may be more resistant to the induction of FOXP3 and/or possible conversion into an iTreg. Therefore, it was hypothesised that isolation and *in vitro* culture of naïve $CD4^+CD25^-$ T cells only would produce a much greater proportion of $FOXP3^+$ cells. In addition, the induction of FOXP3 in naive $CD4^+CD25^-$ T cells may be able to inhibit and/or occur separately from the production of pro-inflammatory cytokines.

Using the methods described in 2.3.1-2.3.2, human $CD45RA^+CD4^+CD25^-$ T cells were isolated from buffy coats and leucocyte cones. Before sorting, PBMC contained 11.0-19.4% $CD45RA^+CD4^+CD25^-$ T cells (*N*=5) (% all nucleated cells). Following magnetic bead isolation, $CD45RA^+CD4^+CD25^-$ T cells accounted for 83.0% of all the nucleated cells (75.6-95.8%, *N*=8). The isolated naïve $CD4^+$ T cells contained only 1.5% $FOXP3^+$ cells (0.0-4.3%, *N*=8) (Figure 6.23).

To ensure the TGFβ and Azacitidine were still being used at appropriate concentrations when culturing naïve $CD4^+$ T cells, the TGFβ and Azacitidine concentration curves were re-tested (Figure 6.24). In keeping with before, both TGFβ and Azacitidine increased the proportion of $FOXP3^+$ cells in a concentration dependent manner. However, 10μM Azacitidine for the first 72 hours of culture remained toxic causing marked cell death.

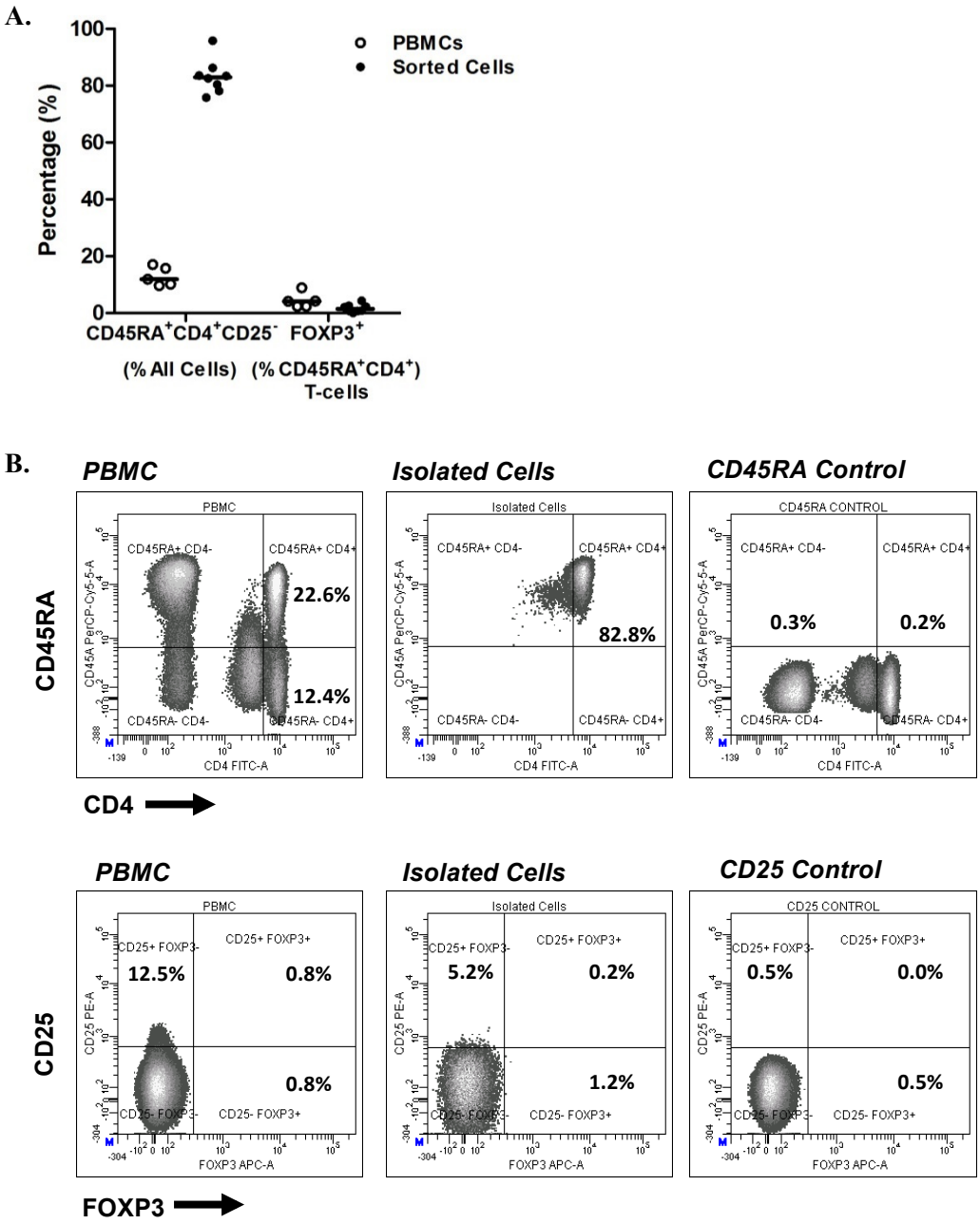


Figure 6.23: Isolation of CD45RA$^+$CD4$^+$CD25$^-$ T Cells

Human PBMC were isolated from buffy coats/leucocyte cones using Ficoll-Paque separation. CD45RA$^+$CD4$^+$CD25$^-$ T cells were then isolated using the magnetic bead Naïve CD4$^+$ T-cell isolation kit II (Miltenyi Biotech) and CD25 MicroBeads (Miltenyi Biotech). **(A)** Proportion of CD45RA$^+$CD4$^+$CD25$^-$ cells (% all cells) and the proportion of FOXP3$^+$ cells (%CD45RA$^+$CD4$^+$ cells) in the starting PBMC (*N*=5) and isolated CD4$^+$CD25$^-$ cell populations (*N*=8). All isolations were performed on PBMC from different buffy coats/leucocyte cones. Horizontal bar shows the median value. **(B)** Density plots from a single isolation which is representative of eight separate procedures. CD45RA, CD4 and CD25 gates were set using FMO controls. FOXP3 gate was set by using FMO + IgG APC isotype control.

A. **B.**

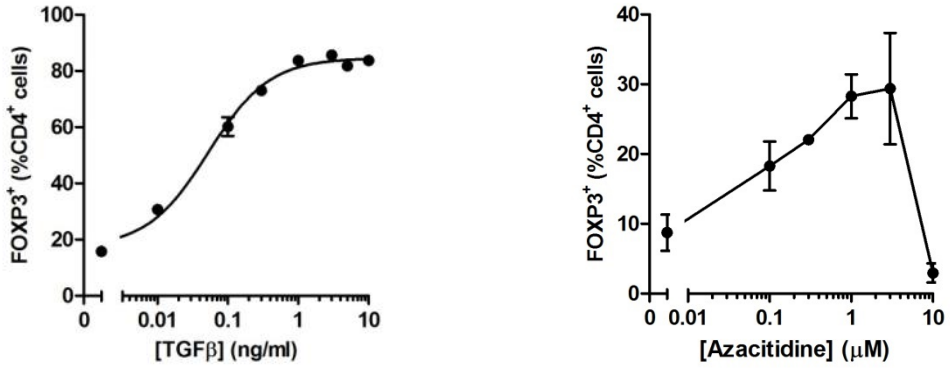


Figure 6.24: FOXP3 Expression in Naïve CD4+CD25- T Cells Cultured with TGFβ or Azacitidine

Magnetic-bead sorted CD45RA$^+$CD4$^+$CD25$^-$ T cells (purity 84%. expressed as % all cells) were cultured in RH10 media, 30IU/ml IL2 and **(A)** TGFβ (0-10ng/ml) or **(B)** Azacitidine (0-10μM, added daily the first 72 hours) at 0.5x10^{6}cells/ml in 96-well U-bottomed plates with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Culture media was changed on day three. Figures show the proportion of CD4$^+$ T cells expressing FOXP3 from a single experiment

To compare the induction of CD25 and FOXP3 expression in CD4$^+$CD25$^-$ and naïve CD4$^+$CD25$^-$T cells, both populations were isolated from the same source and cultured *in vitro* with anti-CD3/CD28 Dynabeads, TGFβ and Azacitidine for five days. Under all conditions, all the CD4$^+$ T cells (>99%) from both starting populations expressed CD25 by the fifth day of culture (Figure 6.25A). In keeping with the hypothesis, the cultured naïve CD4$^+$CD25$^-$ T cells contained a higher proportion of FOXP3$^+$ cells after five days culture (Figure 6.25B). However, this only reached statistical significance for the TGFβ treated cells (61.9 ± 7.85% v 46.2 ± 3.47%; P<0.05). Even with TGFβ plus Azacitidine, 20.0 ± 5.10 % of the cells in the naïve CD4$^+$CD25$^-$ T-cell cultures did not express FOXP3 even though all the cells expressed CD25. The level of FOXP3 expression in the CD4$^+$FOXP3$^+$ cells was not significantly different between the CD4$^+$CD25$^-$ and CD45RA$^+$CD4$^+$CD25$^-$ T-cell cultures (all conditions) (Figure 6.25C).

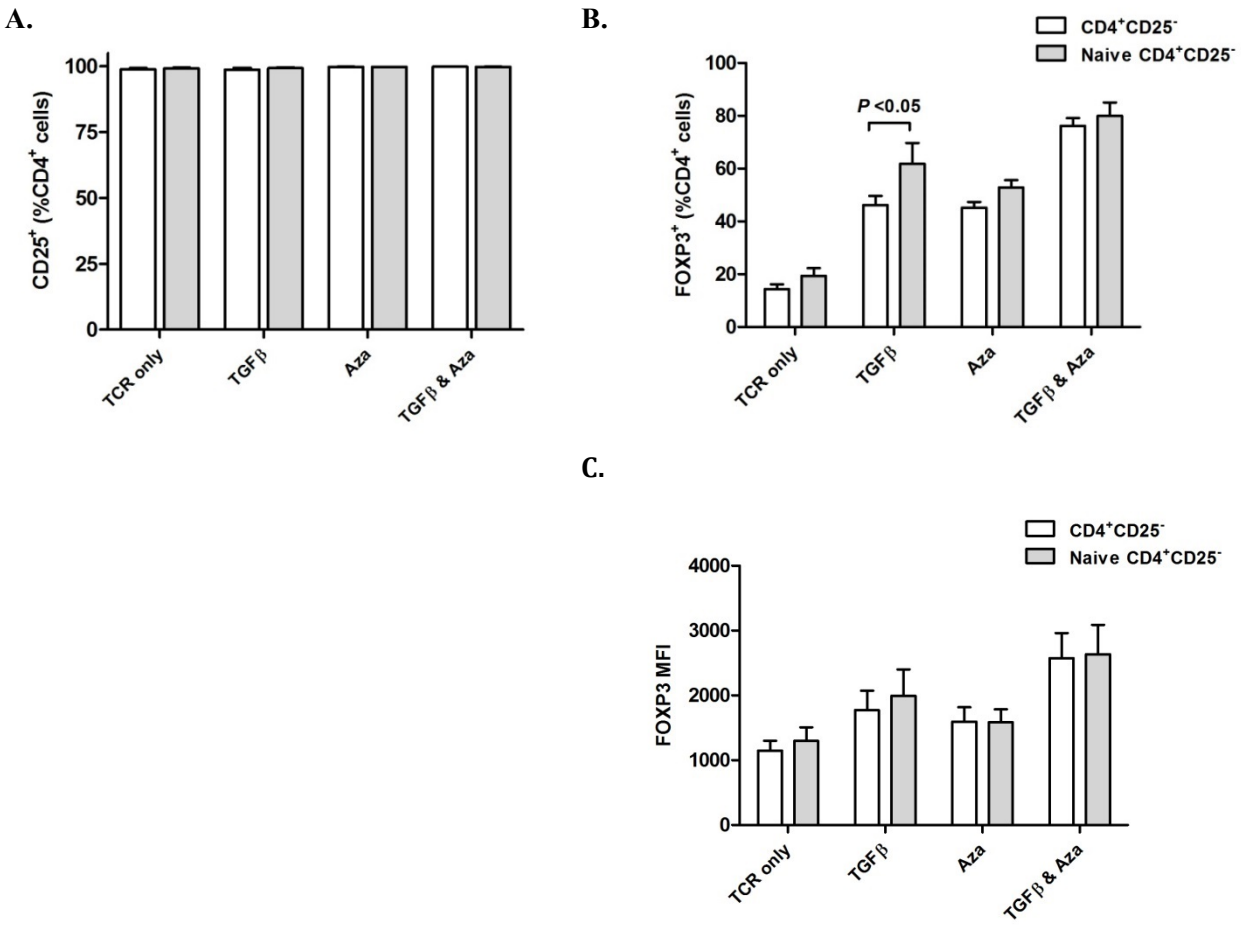


Figure 6.25: Comparison of CD25 and FOXP3 Expression in Cultured $CD4^+CD25^-$ and Naïve $CD4^+CD25^-$ T Cells

Magnetic-bead sorted $CD4^+CD25^-$ T cells (white bars) (median purity 86% (75-88%), expressed as % all cells) and $CD45RA^+CD4^+CD25^-$ T cells (grey bars) (median purity 80% (78-96%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. Figures show **(A)** Proportion of $CD4^+$ T cells expressing CD25; **(B)** Proportion of $CD4^+$ T cells expressing FOXP3; **(C)** FOXP3 MFI in the $CD4^+FOXP3^+$ cells. Figures show the mean values from four experiments, each performed using sorted cells from different donors.

On analysis of the methylation status of the *FOXP3* TSDR from the naïve $CD4^+CD25^-$T-cell cultures on day five, none of the TCR/TGFβ activated $CD4^+$ T cells contained an unmethylated *FOXP3* TSDR. In contrast, almost all the cells exposed to Azacitidine had an unmethylated F*OXP3* TSDR (96.0 ± 2.77% and 99.2 ± 0.01% for the Azacitidine and TGFβ/Azacitidine cultures respectively) (Figure 6.26).

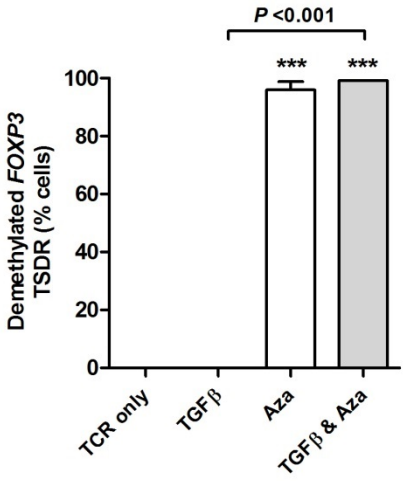


Figure 6.26: FOXP3 TSDR Methylation Status in Naïve $CD4^+CD25^-$ T cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted $CD45RA^+CD4^+CD25^-$ T cells (median purity 79% (78-80%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at $0.5x10^6$cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. DNA was then extracted from the cultured cells and tested for *FOXP3* methylation using the FOXP3 MSRT-qPCR assay. Figure shows the mean percentage of cells with an unmethylated *FOXP3* TSDR from three separate experiments, each performed using sorted $CD4^+CD25^-$ T cells from different donors. *** P<0.001(compared to TCR only).

Although culturing $CD45RA^{+}CD4^{+}CD25^{-}$T cells only, increased the proportion of cells expressing FOXP3, the majority of the induced $CD4^{+}FOXP3^{+}$ cells were still able to produce inflammatory cytokines (Figures 6.27 & 6.28). In particular, more than 75% of the $FOXP3^{+}$ cells from all the cultures were able to produce TNFα. The addition of TGFβ and/or Azacitidine did not significantly alter the proportion of $FOXP3^{+}$ that produced TNFα (84.1 ± 2.99%, 75.3 ± 1.89%, 80.0 ± 7.13%, 76.1± 6.67% for the TCR, TGFβ, Aza and TGFβ&Aza cultures respectively, $P>0.05$) (Figure 6.27B). Many of the $FOXP3^{+}$ cells were also able to produce IL2 (61.7 ± 7.42%, 54.8 ± 7.15%, 37.5 ± 4.36%, 39.7 ± 3.22% for the TCR, TGFβ, Aza and TGFβ&Aza cultures), although culturing the cells with Azacitidine produced a small but significant reduction in the proportion of $FOXP3^{+}$ that could produce IL2 ($P<0.01$) (Figure 6.27A). In contrast, culturing the cells with Azacitidine significantly increased the proportion of $FOXP3^{+}$ cells able to produce IFNγ (50.6 ± 7.83% and 42.3 ± 8.46% of the $FOXP3^{+}$ cells in the Azacitidine and TGFβ/Azacitidine cultures respectively) (Figure 6.27C). Again, inducing FOXP3 expression did not appear to inhibit the production of inflammatory cytokines since the proportion of cytokine positive cells was greater in the $FOXP3^{+}$ cells compared to the $FOXP3^{-}$ population.

These findings, suggest that even though TCR activation with TGFβ and Azacitidine can induce FOXP3 expression and demethylation of the *FOXP3* TSDR in the majority of naive $CD4^{+}$ T cells, the cells' cytokine profile is more in keeping with effector T cells than Tregs.

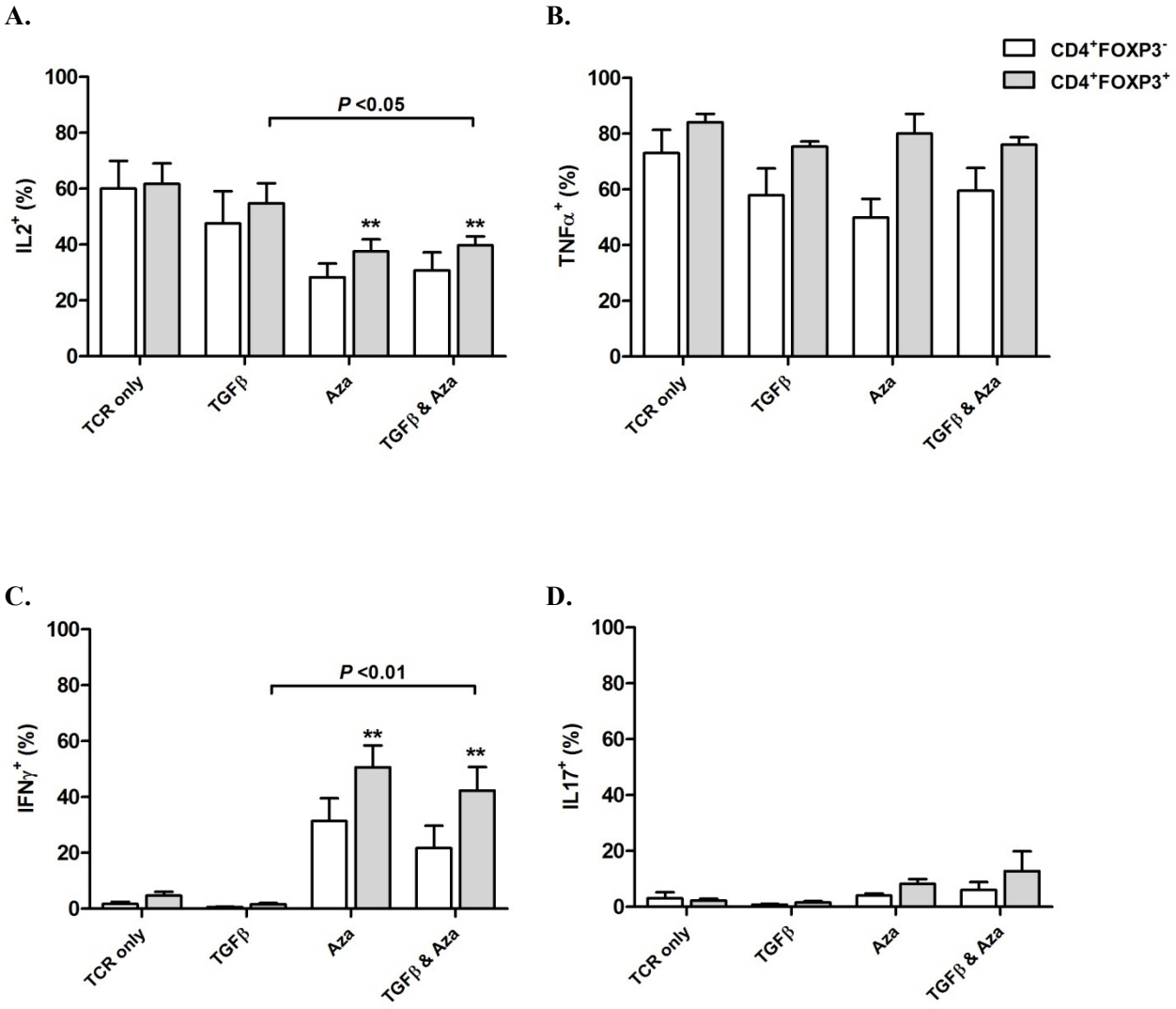


Figure 6.27: Cytokine Staining from Naïve CD4+CD25- T Cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted CD45RA$^+$CD4$^+$CD25$^-$ T cells (median purity 84% (82-86%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at 0.5x10^{6}cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell-to-bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. The cultured cells were then harvested, re-stimulated (50ng/ml phorbol myristate acetate, 1μM Ionomycin and 5μg/ml Brefeldin A) for four hours at 37^{o}C and stained for CD4, FOXP3 and **(A)** IL2, **(B)** TNFα, **(C)** IFNγ, **(D)** IL17. Figures show the mean percentage of cytokine positive cells within the FOXP3$^-$ (white bars) and FOXP3$^+$ cells (grey bars) from three experiments, each one performed using sorted CD45RA$^+$CD4$^+$CD25$^-$ T cells from different donors. **P<0.01 (compared to TCR only).

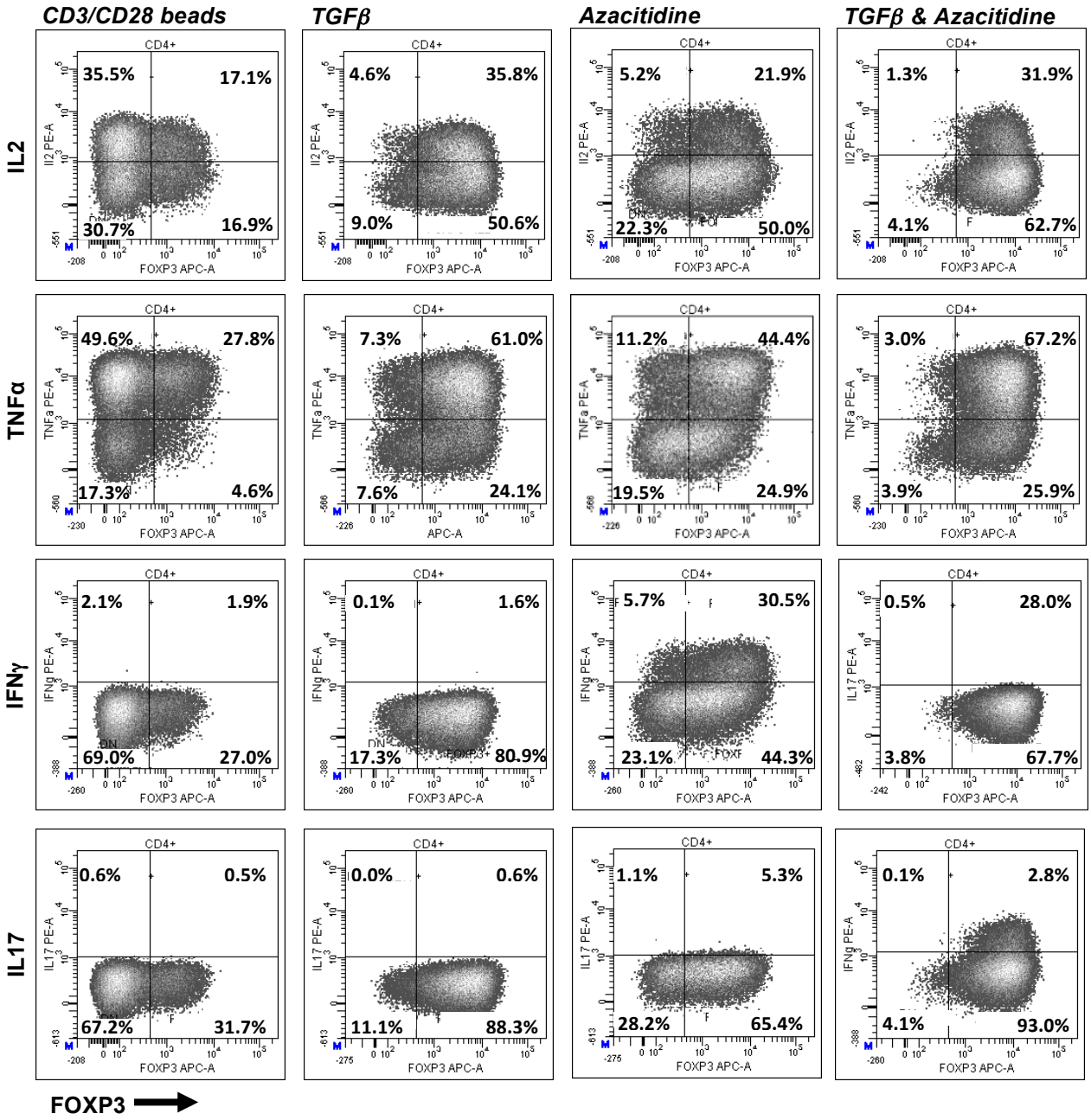


Figure 6.28: Cytokine Production from Naïve CD4⁺CD25⁻ T Cells Cultured with TGFβ and Azacitidine

Magnetic-bead sorted CD45RA$^+$CD4$^+$CD25$^-$ T cells (median purity 84% (82-86%), expressed as % all cells) were cultured in RH10 media with 30IU/ml IL2 ± TGFβ (5ng/ml) and/or Azacitidine (1μM). Cells were cultured at 0.5x10^{6}cells/ml in 48-well plates and activated with anti-CD3/CD28 Dynabeads (cell to bead ratio 1:1) for five days. Azacitidine was added daily the first 72 hours. Culture media was changed on day three. The cultured cells were then harvested, re-stimulated (50ng/ml phorbol myristate acetate, 1μM Ionomycin and 5μg/ml Brefeldin A) for four hours at 37^{o}C and stained for CD4, FOXP3 and IL2, TNFα, IFNγ or IL17. Figure shows flow cytometry density plots from a single experiment which is representative of three separate experiments, each one performed using sorted CD45RA$^+$CD4$^+$CD25$^-$ T cells from different donors.

6.4 CONCLUSIONS

In mice, induced Foxp3 expression in $CD4^+$ T cells confers regulatory phenotype and function (Chen *et al.,* 2003b; Zheng *et al.,* 2004). In humans, however, FOXP3 may be expressed in activated conventional $CD4^+$ T cells (Allan *et al.,* 2007; Tran *et al.,* 2007). The initial work in this chapter tested the hypotheses that FOXP3 can be induced in activated human $CD4^+$ T cells and that this is enhanced by the addition of TGFβ. It then tested the hypothesis that Azacitidine inhibits *FOXP3* TSDR methylation, enhances TCR/TGFβ induced FOXP3 expression and induces a regulatory T-cell phenotype in $CD4^+$ T cells.

In the initial experiments, *in vitro* cell culture methods were established to activate isolated human $CD4^+CD25^-$ T cells using anti-CD3/CD28 beads. The main conclusions from these preliminary experiments were:

- Anti-CD3/CD28 Dynabeads induce $CD4^+$ T-cell activation and proliferation.
- $CD4^+$ T-cell proliferation showed an initial lag phase of 48 hours followed by 72 hours of growth. The maximum cell density was achieved after five days under these culture conditions.
- $CD4^+$ T-cell proliferation was dependent on the $CD4^+$ T-cell to anti-CD3/CD28 Dynabead ratio, $CD4^+$ T-cell density and availability of fresh media.

With these *in vitro* culture methods, this project then tested the hypotheses that CD25 and FOXP3 can be induced in $CD4^+$ T cells in response to anti-CD3/CD28 stimulation and that FOXP3 expression is enhanced by TGFβ. In these experiments:

- All CD3/CD28 activated $CD4^+$ T cells expressed high levels of CD25 although only a limited proportion of these cells expressed FOXP3.
- The proportion of $CD4^+$ T cells expressing FOXP3 and the level of FOXP3 expression within the $CD4^+FOXP3^+$ cells was increased by TGFβ in a concentration dependent manner.

- In contrast to $CD4^+CD25^+FOXP3^+$ nTregs, high proportions of the induced $FOXP3^+$ cells were able to produce IL2 and TNFα and did not contain an unmethylated *FOXP3* TSDR. Furthermore, the proportion of $FOXP3^+$ cells producing pro-inflammatory cytokines was not significantly different from the proportion of $FOXP3^-$ cells producing these cytokines.
- In conclusion, TCR/TGFβ induced FOXP3 expression in $CD4^+$ T cells does not significantly inhibit the production of IL2 or TNFα and does not confer these cells with a Treg phenotype.

Since demethylation of the *FOXP3* promoter and TSDR enhancer are thought to be necessary and specific for stable FOXP3 expression in $CD4^+CD25^+FOXP3^+$ nTregs (Huehn *et al.,* 2009), this project tested the hypothesis that Azacitidine, can enhance TCR/TGFβ induced FOXP3 expression, demethylate the *FOXP3* TSDR and induce a Treg phenotype in $CD4^+$ T cells. In these experiments:

- Azacitidine reduced $CD4^+$ T-cell proliferation in a concentration dependent manner but was toxic to cells at concentrations of 10μM.
- Azacitidine enhanced TCR/TGFβ induced FOXP3 expression in $CD4^+$ T cells in a concentration dependent manner. Azacitidine also enhanced activation induced CD25 expression but did not affect the expression of CD3, CD4, CD8, CD19, CD16 or CD56.
- Azacitidine inhibited methylation of the *FOXP3* TSDR in proliferating $CD4^+$ T cells.
- Although most of TCR/TGFβ/Azacitidine cultured cells expressed CD25 and FOXP3 and contained an unmethylated *FOXP3* TSDR enhancer, the majority of these cells were still able to produce IL2 and TNFα. Therefore, TCR/TGFβ/Azacitidine induced FOXP3 expression and TSDR demethylation in $CD4^+$ T cells does not confer these cells with a Treg phenotype.

Following these observations, it was hypothesised that the CD3/CD28 stimulation used in these cultures may up-regulate the expression of IL2 and TNFα and prevent induced FOXP3 from inhibiting the transcription of inflammatory cytokines. Therefore, the TCR stimulation was modified by increasing the T-cell to anti-CD3/CD28 Dynabead ratio. In summary:

- Reducing the number of anti-CD3/CD28 Dynabeads reduced $CD4^+$ T-cell proliferation, CD25 expression and FOXP3 expression. However, this did not significantly reduce the proportion of $FOXP3^+$ cells producing IL2 and TNFα.
- In contrast to the hypothesis, reducing the number of anti-CD3/CD28 Dynabeads was not able to maintain FOXP3 expression while preventing the production of IL2 and TNFα.

Finally, it was proposed that effector/memory T cells contained within the isolated $CD4^+CD25^-$ T cells may be resistant to the induction of FOXP3 and/or conversion to a Treg phenotype. Therefore, it was hypothesised that *in vitro* culture of naïve $CD4^+CD25^-$ T cells only with TCR/TGFβ/Azacitidine may be able to induce FOXP3 expression and TSDR demethylation without producing inflammatory cytokines. The experiments were therefore repeated with naïve $CD4^+$ T cells only. In these experiments:

- All of the cultured naïve $CD4^+$ T cells expressed CD25.
- A greater proportion of the cultured naïve $CD4^+$ T cells expressed FOXP3 in response to TCR/TGFβ than the original $CD4^+$ T-cell population. However, the level of FOXP3 expression in the $CD4^+FOXP3^+$ cells was not significantly different.
- All the cells cultured with Azacitidine contained an unmethylated *FOXP3* TSDR.
- In contrast to the hypothesis, the $CD4^+FOXP3^+$ cells were still able to produce TNFα.

In conclusion, in these *in vitro* cultures, FOXP3 can be expressed in TCR activated $CD4^+$ T cells and is further enhanced by TGFβ and/or Azacitidine. However, FOXP3 expression and/or demethylation of the *FOXP3* TSDR are not sufficient to induce a Treg phenotype.

Chapter 7

Discussion

7. DISCUSSION

Over the last 20 years, there have been considerable advances in our understanding and ability to perform allogeneic haematopoietic stem cell transplantation (alloHSCT). Use of peripheral blood stem cell harvests (PBSCH), reduced-intensity conditioning (RIC) and improved supportive care have reduced transplant related mortality (TRM) and increased the number of successful transplants performed each year (Bacigalupo *et al.,* 2004; Valcárcel *et al.,* 2005; Gratwohl *et al.,* 2010). It is also becoming a viable treatment option for more patients as the treatment indications increase, the recipients' age range broadens and HLA-typing and donor registries improve. However, the challenge continues to be how to regulate the host's new immune system, preventing excessive responses such as graft-versus-host disease (GvHD) but allowing the beneficial graft-versus-tumour (GvT) response and preventing infections or relapse.

Following the discovery of regulatory T cells (Tregs) and their functional ability to suppress T-cell activation and proliferation, interest developed in their role and use in GvHD (Sakaguchi *et al.,* 1995; Shevach, 2001; Hoffmann *et al.,* 2002). This was heightened when it was demonstrated in mouse models that $CD4^{+}CD25^{+}$ Tregs could prevent GvHD while maintaining a GvT response (Edinger *et al.,* 2003). Subsequently, many human studies tested the hypothesis that Tregs were low in GvHD (Zorn *et al.,* 2005; Rezvani *et al.,* 2006; Rieger *et al.,* 2006; Magenau *et al.,* 2010). However, the results were variable, probably reflecting the different methods and varying criteria used to define Tregs. Furthermore, despite this interest, relatively little is known about the effect of Treg numbers in human HSC grafts and the potential implications for engraftment, immune reconstitution, GvHD and other transplant outcomes. Similarly, the rate, pattern and factors effecting Treg reconstitution have not been clearly defined and whether this affects the development of GvHD.

The overall aims of this study were to develop accurate methods to quantify putative Tregs (phenotypically defined in this thesis as $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells); to perform a

prospective clinical study of $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell reconstitution and GvHD post-alloHSCT; and to test the hypotheses that low $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in the HSC graft or early post-transplant are associated with increased risk of GvHD, NRM and inferior overall survival. Confirming these hypotheses would support the use of Treg cellular therapies in alloHSCT. This is important since progression from murine studies to human trials has been disappointing due to difficulties of GMP-grade Treg isolation and high costs. In addition, since Treg isolation and expansion remain challenging, this study also tested the hypotheses that, in the presence of TGFβ and Azacitidine, FOXP3 can be stably expressed in conventional $CD4^{+}$ T cells and investigated whether this approach could produce viable Tregs for use in clinical studies.

7.1 QUANTIFICATION OF PUTATIVE TREGS

In this project, two new in-house assays were developed, optimised and tested to quantitatively measure putative Tregs and lymphocyte populations from clinical samples and *in vitro* cell cultures. The first used multi-colour flow cytometry, producing a quantitative $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell assay which was reliable, reproducible, had acceptable variability and good specificity and sensitivity. The lymphocyte assay also showed very strong correlation with a more expensive commercially available kit. Using these methods, the relative proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) was shown to be dependent on both sample anti-coagulant and storage time. The second assay used the principle of methylation-specific real-time quantitative PCR (MSRT-qPCR) using the recently described $CD4^+CD25^+FOXP3^+$ Treg and T-cell specific unmethylated DNA loci (Baron *et al.,* 2007; Sehouli *et al.,* 2011). This new technique showed good specificity for putative Tregs and T cells and showed strong correlation with the flow cytometry methods.

Multi-colour flow cytometry allows the quantification of putative Tregs and other cell populations through the simultaneous detection of multiple antigens. To maximise the specificity of the this assay, the multi-colour panel was designed to simultaneously detect CD3, CD4, CD8, CD25, FOXP3 and CD127. Although some studies use $CD4^+CD25^{high}$ only, this approach has limited specificity since CD25 is also expressed in activated human T cells and there is no universal definition of what constitutes $CD25^{high}$ (Wallace and Beverley, 1990; Clark *et al.,* 2004; Stanzani *et al.,* 2004). Similarly, using only CD4 and FSC to identify $CD4^+$ T cells could include $CD4^+$ monocytes, although most have higher FSC and lower CD4 expression compared to $CD4^+$ T cells. Many publications use $CD4^+CD25^+FOXP3^+$ (Meignin *et al.,* 2005; Rezvani *et al*., 2006; Wolf *et al.,* 2007). However, CD25 and FOXP3 can be expressed in activated human T cells and may not be specific to Tregs alone, particularly in the setting of acute inflammation (Allan *et al.,* 2007; Wang *et*

al., 2007). Therefore, to improve specificity and minimise T-cell contamination, CD127 was also included as it inversely correlates with FOXP3 expression but is expressed in the majority of effector and memory T cells (Liu *et al.,* 2006; Seddiki *et al.,* 2006b). Hence, Tregs were defined as $CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}CD127^{dim/-}$. While this combination improves the specificity of the assay compared to other studies, CD127 is also down-regulated in T cells upon activation and this panel could, therefore, still include some recently activated T cells (Liu *et al.,* 2006). Conversely, since designing this assay, it has been demonstrated that CD127 can be expressed during *in vivo* and *in vitro* activation of $CD4^{+}FOXP3^{+}$ Tregs (Simonetta *et al.,* 2010). Therefore, it is possible that activated Tregs could have been excluded from analysis. Simultaneous detection of additional antigens e.g. GARP, was not possible since spillover of the emission spectra from seven fluorochromes on this flow cytometer caused unacceptable loss of sensitivity, particularly for weakly expressed antigens such as FOXP3.

The ability and sensitivity of the multi-colour 'Treg' assay to accurately quantify its target population was dependent upon: (A) The antibody/fluorochrome combinations so as to minimise spectral overlap and prevent spreading of negative populations into weakly positive cells; (B) Optimal antibody concentrations to ensure saturation staining while minimising non-specific background; (C) Use of appropriate controls for multi-colour staining including fluorescence-minus-one controls and known negative cell populations (Roederer *et al.,* 2001; Maecker *et al.,* 2004; Maecker and Trotter 2006). As shown during testing, by careful selection and design, the combination of all six antibody-fluorochromes used in this assay did not significantly impair the ability to detect any individual antigen or cell type. The reliability and reproducibility of the assay is also important, especially when measuring clinical samples over a prolonged period. In particular, there were concerns that isolation of PBMC would introduce unacceptable variability. PBMC isolation, however, was necessary as the low T-cell numbers in the lymphopenic post-transplant blood samples meant there were insufficient $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells for reliable analysis by staining and lysing whole blood only. Despite this, the variance in the staining was small with the coefficients of variation less than 5%. Only the variation in FOXP3 staining was

higher with a CV of approximately 10%, probably reflecting the low proportions of $FOXP3^+$ cells and the inherent variability of cell permeabilization and staining intracellular antigens. However, this was acceptable as clinical differences in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts are probably greater than 10% given the reported wide variation in $CD4^+CD25^+FOXP3^+$ Treg and $CD4^+$ T-cell counts post-alloHSCT (Matthews *et al.,* 2009; Magenau *et al.,* 2010). Notably though, this project demonstrated that delaying sample processing for 24 hours or using lithium heparin anti-coagulant significantly reduced the relative proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). It is unknown if these findings were due to preferential loss of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells, altered expression of CD25/FOXP3/CD127 or because sample processing or antibody binding was affected. To ensure consistency, blood samples were always collected in EDTA and analysed on the day of collection. Where specified, most studies investigating human Tregs in alloHSCT analyse fresh blood samples, although some used frozen isolated PBMC (Meignin *et al.,* 2005; Magenau *et al.,* 2010, Matsuoka *et al.,* 2010). The sample anti-coagulant used is rarely specified but includes both EDTA and heparin (Meignin *et al.,* 2005; Matsuoka *et al.,* 2010, Pastore *et al.,* 2012). Although not specific to Tregs, previous studies have also shown that storage of blood in EDTA and heparin can cause changes to $CD4^+$ T-cell numbers and phenotype (Thornthwaite *et al.,* 1984; Miller and Levy, 1989). It is possible, therefore, that variation in the assays, sample collection and processing may account for some of the variation in the results between different clinical Treg studies.

Using the same principles, a second flow cytometry assay was created to quantify T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), B cells ($CD19^+$) and NK cells ($CD3^-CD56^+$). In this assay, there were sufficient cells to stain and lyse whole blood, thus avoiding possible errors due to PBMC isolation. Absolute counts were determined by multiplying each cell type (% lymphocytes) by the total lymphocyte count obtained from an automated FBC analyser. Although this two-stage method is commonly used to measure lymphocyte counts in many published studies,

potential limitations and possible sources of error were detected (Meignin *et al.,* 2005; Matthews *et al.,* 2009; Magenau *et al.,* 2010). First, using FSS/SSC to identify the lymphocyte population incorporated some non-lymphocytes, while excluding some lymphocytes. In retrospect, this could have been reduced by adding the CLA (CD45) to the panel and using FSC/SSC/CD45 to identify the lymphocytes (Whitby *et al.,* 2002). However, the addition of a seventh fluorochrome would have caused more spectral overlap. Second, it was observed that using a stain-lyse-wash method caused some cells to acquire higher FSC and become $CD16^{+}$, possibly due to clumping with monocytes or neutrophils. Using a stain-lyse-no-wash method may have prevented this issue. Due to these observations, the lymphocyte flow cytometry data were analysed using the calculated lymphosum ($CD3^{+}$&$CD19^{+}$&$CD3^{-}CD56^{+}$) (Schenker *et al.,* 1993). Using this modification, the results showed excellent correlation with a commercial one-stage stain-lyse-no-wash T-cell assay (CD45/CD3/CD4/CD8 Multitest with Trucount tubes (BD Biosciences)). Therefore, this in-house assay produced accurate and reliable results for use in this project. However, if this study was repeated, the six-colour CD45/CD3/CD4/CD8/CD19/CD16&CD56 Multitest with Trucount tubes (BD Biosciences) would be used as it is avoids these potential issues with FSC/SSC gating and cell clumping.

Following the discovery that unmethylated regions of DNA within the *FOXP3* and *CD3* genes can discriminate $CD4^{+}CD25^{high}FOXP3^{+}$ Tregs and $CD3^{+}$ cells respectively from all other cells, this project also developed a MSRT-qPCR assay to quantify putative Tregs (Baron *et al.,* 2007; Sehouli *et al.,* 2011). This assay was developed because it has been proposed that this method can discriminate $FOXP3^{+}$ natural Tregs (nTregs) from $FOXP3^{+}$ activated T cells, permitting a more accurate quantitation of nTregs in an inflammatory environment. In keeping with the findings of Baron and colleagues (2007), only sorted $CD4^{+}CD25^{high}CD127^{-/dim}$ T cells (88% $FOXP3^{+}$) contained high proportions of cells with unmethylated *FOXP3* TSDR. Low levels of methylated *FOXP3* TSDR were detected in the $CD4^{+}CD25^{high}CD127^{-/dim}$ T-cell samples while low levels of

unmethylated *FOXP3* TSDR were detected in the 'non-Treg' samples. However, as magnetic-bead sorted cells are not entirely pure, this could be due to contaminating 'non-Tregs' and $FOXP3^+$ nTregs respectively. To confirm the specificity of unmethylated *FOXP3* TSDR for nTregs, flow-sorted cell populations with higher purity would be required. Likewise, only sorted $CD3^+$ populations contained high proportions of the unmethylated *CD3* locus and the presence of low numbers of methylated and unmethylated *CD3* in the $CD3^+$ and $CD3^-$ samples respectively could be due to contamination in the sorted cell populations. Importantly though, for both cell types, the MSRT-qPCR assay showed strong correlation with the flow cytometry results for healthy control samples (ρ=0.88, P<0.0001 and ρ=0.85, P<0.0001 respectively).

In light of these findings, it is proposed that this method could be used to quantify $CD3^+$ cells and putative Tregs in future large, multi-centre studies in alloHSCT. The advantage of this technique is that DNA can be easily extracted from small clinical samples and stored for batch processing/transportation to a central laboratory. Samples do not need to be analysed fresh and MSRT-qPCR is cheaper than using multiple fluorochrome-conjugated monoclonal antibodies. As proposed by Baron and colleagues (2007) and supported by the *in vitro* T-cell culture experiments in this project, it can also differentiate $FOXP3^+$ nTregs from $FOXP3^+$ activated T cells. For such projects, two important factors should be considered. First, the accuracy of the assay is dependent on the efficiency of bisulphite conversion since non-converted unmethylated DNA will be incorrectly detected as methylated. In this study, the bisulphite conversion efficiency was consistently greater than 99.5%. Second, in this project, the *FOXP3*, *CD3* and *GAPDH* plasmid standards were developed separately. However, to improve accuracy and reliability of the $FOXP3^+$ nTreg to $CD3^+$ cell ratios, all three methylated or unmethylated standards should be synthesised and inserted into a single plasmid, as per Sehouli and colleagues (2011). For consistency and reproducibility, sufficient quantities of the plasmid dilutions should also be produced for the entire study to avoid variation in the reference standards.

7.2 $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS IN HSC GRAFTS

In this project, a prospective, observational clinical study of 101 alloHSCT was performed, analysing the cellular composition of HSC grafts and investigating whether low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses were associated with higher rates of GvHD and other transplant related events. In contrast to the initial hypothesis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were not associated with increased acute or chronic GvHD. However, for the first time, this project demonstrated that low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft is independently associated with poor engraftment, increased non-relapse mortality (NRM) and inferior overall survival. Low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell doses and/or low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the grafts were not, however, associated with an increased incidence of CMV reactivation or disease relapse.

In this study of 101 allogeneic HSC grafts, the median $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell, $CD3^+$ cell and $CD34^+$ cell doses transplanted were $4.60x10^6$/kg (0.20-20.6), $274x10^6$/kg (21.0-801) and $6.10x10^6$/kg (0.00-19.8) respectively. While the $CD3^+$ and $CD34^+$ cell doses were consistent with previous reported values from GCSF stimulated PBSCH, the median putative Treg count was noticeably lower (Pabst *et al.,* 2007; Wolf *et al.,* 2007; Pastore *et al.,* 2012). Likewise, the median proportion of putative Tregs of 2.94% $CD4^+$ T cells (0.81-8.56%) was also lower than previously described (Pabst *et al.,* 2007; Wolf *et al.,* 2007). Although this project contained four BM harvests, this would not account for this difference since BM harvests contain higher $CD25^{hi}$ Treg frequencies (%$CD4^+$ T cells) than GSCF stimulated PBSCH (Blache *et al.,* 2010). A more likely explanation was the use of a different antibody clone to stain for FOXP3. While the other studies of allogeneic HSC grafts used antibody PCH101 (eBiosciences), this project used 3G3 (Miltenyi-Biotech), due to previous laboratory experience and reports that PCH101 may be associated with

higher non-specific binding. However, as subsequently shown by Law and colleagues (2009), clone 3G3 stains significantly fewer cells than PCH101. Tighter CD25/FOXP3 gating and the additional use of $CD127^{dim/low}$ may have also contributed to the lower values.

The cumulative incidence of grade II-IV acute GvHD was 28.7% (20.0-37.9%) at 100 days post-transplant, increasing to 40.2% (29.9-50.3%) at one year using the 2005 NIH Consensus Conference criteria. The incidence of chronic GvHD was 74.7% (61.9-83.4%) and 42.0% (30.1-53.3%) at one year using the Shulman and NIH Consensus criteria respectively. These rates were comparable to this centre's historical data and previous published results (Medd *et al.,* 2011). Although broadly in keeping with other published studies, direct comparison is difficult due to the heterogeneity of the study population and difference in the patient/transplant characteristics (Malladi *et al.,* 2009; Moon *et al.,* 2010; Jagasia *et al.,* 2012). Using the NIH Consensus criteria increased the incidence of acute GvHD and reduced chronic GvHD due to the re-classification of patients with GvHD beyond 100 days from chronic to late acute GvHD. 11 patients re-classified with late acute GvHD subsequently went on to develop chronic GvHD and, therefore, 41% (*N*=21) of the patients with cGvHD by the Shulman criteria did not have cGvHD by the NIH Consensus criteria. A higher proportion of our patients (61%) were reclassified as late aGvHD compared to the 15-48% previously reported in retrospective studies (Vigorito *et al.,* 2009; Arai *et al.,* 2011). This may be due to the higher proportion of RIC transplants within this project and the withdrawal of immunosuppression at three months, delaying the onset of acute GvHD to beyond 100 days post-transplant. Alternatively, this could reflect the complexity of the NIH Consensus Classification which was difficult to use prospectively in the busy out-patient clinic. Similarly, obtaining histological biopsies to confirm cGvHD was not always feasible. As a result, it is possible that GvHD beyond 100 days was more likely to be classified as late aGvHD rather than cGvHD meeting the NIH Consensus criteria i.e. cGvHD could have been under diagnosed. For future projects, further training of the NIH Consensus criteria would be required, using a simplified GvHD data collection form that can be completed quickly and accurately by clinical staff.

In contrast to this study's initial hypothesis, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the HSC grafts (absolute counts and $\%CD4^+$ T cells) were not associated with an increased risk of acute or chronic GvHD. This contrasts to the early mouse data which demonstrated that depletion of $CD4^+CD25^+$ regulatory cells from the grafts rapidly increased the development of GvHD (Taylor *et al.,* 2002). Conversely, GvHD was reduced in these models by the addition of isolated $CD4^+CD25^+$ Tregs at the time of transplantation (Hoffmann *et al.,* 2002). However, as recently noted by Rosenzwajg and colleagues (2011), these effects were observed following complete $CD4^+CD25^+$ Treg depletion or when using high $CD4^+CD25^+$ Treg to $CD4^+$ T-cell ratios (1:1). It is possible, therefore, that the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell to $CD4^+$ T-cell ratios in the grafts within this study (1:12 to 1:123) were too low to demonstrate a difference in clinical GvHD between the low and high 'Treg' groups. It is also important to consider that the $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell to $CD4^+$ T-cell ratios in the graft may not correlate with the equivalent values *in vivo* in the recipient post-infusion. In particular, most of the transplants in this study (84%) used a RIC protocol in which host haematopoietic cells are still present at the time of transplantation. The majority of the transplants (62%) also used Alemtuzumab conditioning which causes profound T-cell depletion. Although given five to six days before the graft, due to its long half-life, it will also deplete T cells within the graft and it remains unknown whether Alemtuzumab alters the infused donor $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell to $CD4^+$ T-cell ratio (Chakraverty *et al.,* 2010). Due to the low lymphocyte counts on day zero, it was not possible to measure these ratios during or shortly after the infusion of the grafts. The heterogeneity of the study population may also create too much variability in the data to be able demonstrate an effect of graft $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells on the incidence of GvHD.

In keeping with our results, a recent study of 49 HLA-matched sibling allogeneic transplants found that the $CD4^+FOXP3^+$ Treg content in BM grafts did not predict the occurrence of aGvHD (Rosenzwajg *et al.,* 2011). However, in contrast, Pastore and colleagues (2012) recently demonstrated that the $CD3^+$ to $CD4^+CD25^{high}FOXP3^+$ Treg ratio in PBSCH from 65 alloHSCT was

linked to the onset of aGvHD. This study used MAC only and none of the transplants used Alemtuzumab although transplants using unrelated donors did receive ATG. This group analysed their data using CD3 to $CD4^{+}CD25^{high}FOXP3^{+}$ Treg ratios and used the median value to separate the two groups. However, using this approach to reanalyse our data still showed no difference in the incidence of grade II-IV aGvHD (26.4% (14.9-39.3) v 31.3% (18.6-44.6), *P*=0.63 at 100 days (Consensus); 36.2 % (22.4-50.1) v 45.1% (29.3-59.8), *P*=0.48 at one year (NIH)). Likewise, there are two publications in which graft $CD4^{+}FOXP3^{+}$ Treg doses below the median were significantly associated with an increased risk of grade II-IV aGvHD (Pabst *et al.,* 2007; Wolf *et al.,* 2007). Although our project used a lower value to separate the two groups, this did not account for the difference in our findings as re-analysing our data using their approach still showed no significant difference in grade II-IV aGvHD (34.8% (21.6-48.3) v 22.6% (12.0-35.2), *P*=0.17 at 100 days (Consensus); 42.5% (27.8-56.4) v 37.6% (23.3-51.9), *P*=0.40 at one year (NIH)). Importantly, Wolf and colleagues (2007) only observed a significant difference within their MAC transplants. Subgroup analysis of the effect of graft $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells in MAC transplants only was not feasible within this project due to the low numbers performed in Oxford during the study period (*N*=16). The reasons why a significant difference in aGvHD has only been observed following MAC is unknown but it has been speculated that high $CD4^{+}FOXP3^{+}$ Treg doses in the graft may be able to suppress early activation of T cells and/or more effectively repopulate secondary lymphoid organs following MAC only (Wolf *et al.,* 2007; Pastore *et al.,* 2012). In view of these findings, when designing human clinical studies to establish whether adding *ex vivo* isolated Tregs can prevent GvHD, it may be necessary to use MAC transplants only.

The incidence of CMV reactivation was 35.8% (26.1-45.7%) at one year and the incidence of disease relapse was 21.6% (13.1-31.5%) and 27.6% (6.10-47.6%) at one and two years respectively. While Tregs in the graft could suppress T-cell responses to CMV and tumour cells, it has been hypothesised that $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs promote immune reconstitution and allow the development of a T-cell repertoire against CMV and the underlying disease (Nguyen *et al.,* 2008). In this study, neither the $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell dose nor the relative proportion of

$CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the HSC grafts were significantly associated with CMV reactivation. In keeping with these findings, an association between graft $CD4^+FOXP3^+$ Tregs and CMV was not observed by Wolf and colleagues (2007). However, high $CD3^+$ to $CD4^+CD25^{high}FOXP3^+$ Treg ratios in PBSCH were associated with higher rates of CMV reactivation (Pastore *et al.,* 2012). This study was limited to myeloablative transplants only, again suggesting that Tregs within the graft are more important when administered into a lymphopenic environment. Neither this study nor others have shown any association between graft $CD4^+FOXP3^+$ Tregs and disease relapse (Wolf *et al.,* 2007; Pastore *et al.,* 2012). This is in keeping with mouse data which showed the addition of $CD4^+CD25^+$ cells did not increase relapse and supports the hypothesis that Tregs do not inhibit the GvT response.

Although low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the graft were not significantly associated with GvHD, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) were independently associated with increased NRM and inferior OS. Even though previous studies have suggested that low $CD4^+FOXP3^+$ Tregs in the graft are associated with inferior survival, this was observed when analysing $CD4^+FOXP3^+$ Treg doses in MAC transplants only and, importantly, did not remain significant in multivariate analysis (Wolf *et al.,* 2007). Other variables independently associated with NRM and inferior OS were low doses of $CD34^+$ cells, low doses of $CD3^-CD56^+$ cells and previous recipient exposure to CMV. Given that low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) and absolute $CD3^-CD56^+$ cell doses in the graft were associated with NRM but not GvHD or CMV reactivation, it was hypothesised that these variables may be associated with higher rates of infection. Supporting this hypothesis, low $CD3^-CD56^+$ cells post-transplant have been associated with higher TRM, possibly due to infectious complications (Bühlmann *et al.,* 2011). However, it has also been suggested that NK-cell recovery is not influenced by the number of NK cells in the graft (Bosch *et al.,* 2012). Although this project did not directly record the rates of infection or antibiotic use, the mortality from infectious complications was available. While taking the low number of events into consideration, in multivariate regression, both the relative proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T

cells) and absolute $CD3^-CD56^+$ doses in the grafts were independently associated with increased infectious mortality. As NK cells are an important part of the innate immune response and recover quickly post-transplant, it is speculated that low $CD3^-CD56^+$ cells in the graft increases the recipient's susceptibility to early infection due to reduced innate immunity. However, why low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft are associated with increased infectious mortality is less clear. In adoptive immunotherapy experiments with $CD4^+CD25^+$ Tregs in haploidentical transplants, NK-cell reconstitution was faster and with an enhanced NK-cell repertoire compared to historical controls (Di Ianni *et al.,* 2011). T cells with specificity to opportunistic pathogens, e.g. Aspergillus *fumigatus*, also emerged significantly earlier. Similarly, in mouse GvHD models, adoptive transfer of $CD4^+CD25^+$ Tregs accelerated lymphoid immune reconstitution with a diverse TCR repertoire and protection against viral infections (Winstead *et al.,* 2010). Hence, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the graft may impair immune reconstitution. However, in this project, it was evident that most of the infectious mortality occurred early post-transplant (median 22 days), at a time when bacterial and/or fungal infections are generally more prevalent due to profound neutropenia. Therefore, this study investigated whether low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were associated with delayed engraftment. On multivariate analysis, low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the HSC graft was independently associated with inferior neutrophil engraftment by day 30. This effect was independent of low $CD34^+$ cell dose which was also associated with poor engraftment and has been previously observed in autologous and allogeneic HSCT (Kamel *et al.,* 2005; Specchia *et al.,* 2006; Pastore *et al.,* 2008). These findings are in keeping with the mouse data that shows donor $CD4^+CD25^+$ T cells can promote early and sustained engraftment of allogeneic HSC (Hanash and Levy, 2005). While the exact mechanisms are unknown, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells may inhibit graft rejection through the inhibition of residual host T cells. Fujisaki and colleagues (2011) demonstrated in mouse models using high resolution *in vivo* imaging that allogeneic HSC co-localised with $Foxp3^+$ Tregs on the endosteal surface of the bone, potentially forming an immune privileged site for engraftment. Depletion of

Foxp3^{+} Tregs resulted in loss of the allogeneic HSC. Taking all these findings together, it may be hypothesised that the addition of Tregs around the time of the HSC graft infusion can promote early HSC engraftment, decreasing the duration of the neutropenic period and reducing the risk of severe infectious complications. They may also reduce NRM and improve OS by promoting immune reconstitution, NK-cell recovery and T-cell diversity. Further pre-clinical and clinical studies investigating the effect of Tregs in the graft on HSC engraftment will be required.

7.3 $CD4^+CD25^+FOXP3^+CD127^{-/DIM}$ T CELLS AND IMMUNE RECONSTITUTION

Treg reconstitution post-alloHSCT may regulate the developing immune system, promoting balanced immune reconstitution and the development of a diverse T-cell repertoire (Nguyen *et al.,* 2008). However, the pattern and factors affecting Treg recovery post-alloHSCT have not been clearly defined in humans. This project described the kinetics of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell and immune reconstitution and presents new data that demonstrates that Alemtuzumab conditioning may significantly alter $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell recovery. In particular, Alemtuzumab may increase the proportion of peripheral blood $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) compared to healthy controls and non-Alemtuzumab transplants. CMV reactivation and/or treatment may also reduce post-alloHSCT $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. In contrast to the original hypothesis, low peripheral $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the first few months post-transplant were not significantly associated with an increased risk of developing GvHD, CMV reactivation, disease relapse, non-relapse mortality or overall survival. A fall in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts from one to three months, however, was associated with a significantly higher risk of NRM and inferior OS.

Studying haemopoietic and immune reconstitution post-alloHSCT in humans is difficult due to the large diversity of clinical factors that can affect blood counts before, during and after transplantation. Very large studies would be required to determine all the variables, how they interact and the implications of these findings. Nevertheless, this study of 101 transplants allows exploratory analysis of post-alloHSCT $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell reconstitution and whether this is associated with development of transplant related events. While the heterogeneity of the study population could limit the findings, it does allow multivariate testing of which factors

affect $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell reconstitution from a typical clinical setting and in a patient cohort that is reflective of many UK transplant centres.

For the study population as a whole, granulocyte and monocyte counts recovered quickly post-transplant with the median counts being within the normal range by one month. The majority of these transplants used RIC (84%) with GSCF stimulated PBSCH (97%) which are both known to have faster recovery than myeloablative conditioning and BM harvests respectively (Gahrton *et al.,* 2007). As previously described, a second nadir in the neutrophil and monocyte counts was also observed after the first month post-transplant before gradually increasing again around six months (Storek, 2008). Lymphocyte recovery was much slower, with counts gradually increasing over the first year and not reaching the normal range until nine months post-transplant. Since Alemtuzumab causes profound lymphopenia and has been detected at sufficient levels to induce antibody-mediated cellular cytotoxicity two months post-transplant, lymphocyte recovery in patients conditioned with and without Alemtuzumab were analysed separately (Chakraverty *et al.,* 2010). In keeping with previous reports, Alemtuzumab conditioning was associated with significantly lower lymphocyte counts early post-alloHSCT (Dodero *et al.,* 2005; Schmidt-Hieber *et al.,* 2010). BM harvests and RIC were also independently associated with low lymphocyte counts at one month post-transplant. While BM grafts are associated with slower engraftment and immune recovery, RIC transplants have generally been associated with faster recovery of $CD3^+$ cell and total lymphocytes, at least during the early months post-transplant (Jiménez *et al.,* 2005; Gahrton *et al.,* 2007; Jiménez *et al.,* 2007). It remains unclear why the RIC transplants had lower lymphocyte counts at one month in this study and further analysis would be required.

In the T-replete transplants (no Alemtuzumab), $CD3^-CD56^+$, $CD3^+$ and $CD3^+CD8^+$ cell counts recovered rapidly. However, in keeping with the findings of others, $CD3^+CD4^+$ cell recovery was more delayed with low counts until nine months post-transplant (Imamura *et al.,* 2003; Bühlmann *et al.,* 2011). T-cell reconstitution post-transplant is through a combination of peripheral expansion of donor T cells and generation of new naïve T cells from the thymus. However, thymic function is

often reduced due to age, the effects of previous chemotherapy and GvHD (Jiménez *et al.,* 2006; Castermans *et al.,* 2011). Peripheral expansion of memory/effector $CD8^+$ T cells is more effective than $CD4^+$ T cells although the biological explanation for this remains uncertain (Bosch *et al.,* 2012). $CD19^+$ counts remained significantly suppressed until nine months post-transplant, in keeping with previous reports that B-cell reconstitution is not completely restored until 1-2 years post-transplant (Storek *et al.,* 2001; Bosch *et al.,* 2012).

In the T-deplete transplants (Alemtuzumab), $CD3^-CD56^+$ cells still recovered rapidly. However, in keeping with previous reports, reconstitution of all $CD3^+$ populations was significantly delayed with $CD3^+CD8^+$ and total $CD3^+$ cell counts not reaching normal levels until six months post-transplant (D'Sa *et al.,* 2003; Dodero *et al.,* 2005). As with the T-replete transplants, the $CD3^+CD8^+$ cells recovered before $CD3^+CD4^+$ cells. However, in contrast to the T-replete setting and other published studies, we observed faster $CD19^+$ recovery using Alemtuzumab with median counts reaching the normal range by three months (D'Sa *et al.,* 2003; Matthews *et al.,* 2009). Importantly, rapid B-cell recovery has been observed in renal transplantation using Alemtuzumab, to levels exceeding baseline and is associated with a shift towards naïve B cells (Heidt *et al.,* 2012). Therefore, a similar response may be seen post-alloHSCT. Further investigation of B-cell subtypes will be required.

Reconstitution of Tregs in human alloHSCT has not been extensively studied. In this project, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell numbers recovered rapidly in the non-Alemtuzumab alloHSCT, being within the normal range from one month post-transplant. In keeping with $CD3^+CD4^+$ cells, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts in Alemtuzumab conditioned transplants have a more delayed recovery, increasing over the first six months. However, of particular interest, the relative proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (% of $CD4^+$ T cells) post-transplant exceeded the upper limit of the normal range. In the T-replete setting, proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) was above the normal range from one month onwards. In the T-deplete setting, the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T

cells) increased over the first six months and exceeded the levels seen in the T-replete transplants from three to six months. This increase could be explained by several factors. First, early $CD4^+$ T-cell reconstitution post-alloHSCT predominantly occurs via homeostatic expansion driven by profound lymphopenia and cytokines such as IL7 and IL2 (Mackall *et al.,* 1997; Fry *et al.,* 2001). Healthy donor $CD4^+CD25^+CD127^{-/dim}$ T-cell populations contain more proliferating cells than conventional T-cell populations and during homeostatic expansion post-alloHSCT this difference is exacerbated further (Matsuoka *et al.,* 2010). Therefore, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell proliferation due to $CD4^+$ lymphopenia may be enhanced above that of the conventional T cells, increasing $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell to $CD4^+$ T-cell ratios (Matsuoka *et al.,* 2010). Second, increased $CD4^+CD25^+FOXP^+/CD4^+CD25^-FOXP^-$ ratios have also been observed following immune depletion with Alemtuzumab in renal transplants (Bloom *et al.,* 2008). This increase was not fully explained by homeostatic expansion, increased thymic output or Alemtuzumab-sparing of $CD4^+CD25^+FOXP^+$ Tregs. Instead, Bloom and colleagues (2008) proposed that $CD4^+CD25^+FOXP^+$ Tregs may have been generated from conversion of $CD4^+CD25^-$ T cells. In keeping with this hypothesis, *in vitro* stimulation of PBMC with Alemtuzumab increased the number of $CD4^+CD25^+FOXP3^+$ cells with suppressive function (Bloom *et al.,* 2008). Alemtuzumab also increased $CD4^+CD25^+$ Treg percentages in *in vitro* mixed lymphocyte reactions through induction of immunoregulation in naïve T cells and reportedly induced $CD4^+CD25^+$ regulatory T cells in anti-CD3 stimulated $CD4^+CD25^-$ T cells, although without Foxp3 expression (Watanabe *et al.,* 2006; Levitsky *et al.,* 2012).

In light of these findings, this study also investigated which other transplant related variables may affect $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell reconstitution post-alloHSCT. Analysing the median time to $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell recovery was not possible because $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were only measured at monthly intervals and this would not provide sufficient sensitivity. Therefore, exploratory univariate and multivariate analysis assessed

which pre-, peri- and post-transplant variables significantly affected $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute counts and $\%CD4^+$ T cells) at one, three and six month post-alloHSCT. In keeping with the findings above, Alemtuzumab was independently associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at one and three months. Although initially associated the lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells), Alemtuzumab was significantly associated with higher proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (% of $CD4^+$ T cells) at three months post-transplant.

Low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells post-alloHSCT were also observed in CMV reactivation. It is well known that CMV reactivation post-alloHSCT is significantly increased by poor recovery of CMV-specific $CD8^+$ T cells (Gratama *et al.,* 2001; Lamba *et al.,* 2005). Furthermore, the recovery of CMV-specific $CD8^+$ T cells post-alloHSCT correlates with peripheral blood $CD4^+CD25^{high}FOXP3^+$ Treg counts (Pastore *et al.,* 2011). CMV reactivation post-alloHSCT could, therefore, be associated with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. Alternatively, it may be hypothesised that CMV reactivation is associated with low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) because of the up-regulation of the $CD4^+$ T-cell responses to eradicate the CMV virus while down-regulating the immunosuppressive $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. It should also be considered that the low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells may not be due to the CMV infection but as a consequence of the CMV therapy. Although exact details for each patient were not available, it is standard practice in Oxford to treat CMV reactivation with Val/ganciclovir. The effect of these agents on $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells and/or FOXP3 expression is currently unknown and would be interesting to investigate further.

Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) at one month were also observed with RIC and PBSCH. It may be speculated that the profound lymphopenia associated with MAC and the delayed engraftment seen with MAC and BM harvests may increase the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells ($\%CD4^+$ T cells) though increased homeostatic

expansion as previously discussed above in relation to Alemtuzumab. Low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at three months were also seen with active GvHD at the time the blood samples were collected and notably, this effect was independent of concurrent corticosteroid therapy and Alemtuzumab conditioning. Low peripheral blood Tregs have been previously associated with GvHD and conversely, GvHD may impair $CD4^+$ T-cell and Treg recovery by inhibition of thymic output (Clave *et al.*, 2009; Magenau *et al.,* 2010). Similarly, significantly lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts and lower proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at six months were observed with corticosteroid therapy. While corticosteroids are known to be toxic to lymphocytes, their effect on Tregs and FOXP3 expression remains unclear. Although some studies show that glucocorticoids can selectively up-regulate FOXP3, others have shown no effect on $FOXP3^+$ Treg number or function (Moraes-Fontes *et al.,* 2009; Prado *et al.,* 2011; Stary *et al.,* 2011). While this analysis is exploratory in nature and further prospective studies will be needed to confirm these findings, these results provide useful insights into which transplant variables are associated with post-alloHSCT $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells. In particular, further investigations into the relationship between Alemtuzumab or CMV reactivation and putative Tregs should be explored.

Following the work in mouse GvHD models, it has been proposed that human Tregs are reduced in GvHD. However, while some human studies have observed low Tregs (absolute counts and relative proportions), other studies have not (Meignin *et al.,* 2005; Rezvani *et al.,* 2006; Magenau *et al.,* 2010). In this project, although $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were significantly lower in blood samples from patients with grade I-IV aGvHD, there was no significant difference in the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells). However, in this project, the GvHD and non-GvHD groups were not evenly matched for other confounding variables. First, a significantly higher proportion of the aGvHD samples were collected from patients that had received Alemtuzumab conditioning. Second, the majority of the GvHD samples were collected

from patients that had already commenced corticosteroid therapy. Both these factors could have reduced the total lymphocyte, $CD3^+$ cell and $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts in the GvHD samples. Importantly, in the main published studies demonstrating low Tregs in GvHD, none used T-cell depleting antibodies (Rezvani *et al.,* 2006; Magenau *et al.,* 2010). Similarly, blood samples were collected at the onset of GvHD symptoms before starting corticosteroid therapy. In this setting, the frequency of $CD4^+CD25^{hi}FOXP3^+$ Tregs (% total lymphocytes) decreased with increasing grades of GvHD, predicted response to treatment and were independent predictors of NRM and survival (Magenau *et al.,* 2010). Unfortunately, collecting blood samples using a similar strategy was not possible within this project. Subgroup analysis of the Alemtuzumab and non-Alemtuzumab transplants separately, excluding samples from patients' already on steroids, did not show any difference in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells between the GvHD and non-GvHD groups. However, $CD4^+CD25^+FOXP3^-$ T-cell counts (activated conventional T cells) were significantly higher in the aGvHD samples (Alemtuzumab-conditioned transplants only), suggesting there may be a relative imbalance of pro-inflammatory T cells to regulatory T cells in this setting. Of cautionary note though, excluding samples could significantly bias the results and care must be taken when reviewing these findings. For example, those patients with more severe GvHD, requiring urgent corticosteroid therapy, are more likely to have been excluded than those with mild GvHD whose $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts may not significantly differ from the non-GvHD samples. Similar confounding factors were also seen when analysing the cGvHD data. Although in keeping with the study hypothesis, the significantly lower proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) in the cGvHD samples could have been due to lower proportion of these samples being from patients conditioned with Alemtuzumab. As demonstrated above, the Alemtuzumab transplants had higher proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) than the non-Alemtuzumab from three months onwards. As with aGvHD, the two groups were also not evenly matched for corticosteroid therapy with a significantly higher proportion of the cGvHD samples collected from patients on corticosteroids. Subgroup analysis of the Alemtuzumab and non-Alemtuzumab transplants

separately again showed no difference in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells between the cGvHD and non-cGvHD samples. However, as with acute GvHD, $CD4^+CD25^+FOXP3^-$ T-cell counts (activated conventional T cells) were significantly higher in the cGvHD samples (Alemtuzumab-conditioned transplants only), again suggesting there may be a relative imbalance of pro-inflammatory T cells to regulatory T cells in GvHD.

Although reduced peripheral blood $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were not observed in the setting of GvHD, significantly lower $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells were seen with CMV reactivation. As CMV reactivation is more common with Alemtuzumab conditioning, it was not surprising that the CMV positive group had a significantly greater proportion of samples collected from Alemtuzumab treated patients (Ljungman *et al.,* 2006). To ensure the difference in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells was not only due to this confounding variable, the results were also analysed in the Alemtuzumab and non-Alemtuzumab transplants separately. In both subgroups, $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells remained lower in the CMV positive samples, although the proportion of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) did not reach significance in the non-Alemtuzumab transplants. These findings are in keeping with the work of Pastore and colleagues (2011) which also demonstrated significantly lower $CD4^+CD25^{hi}FOXP3^+$ Treg counts in patients with CMV infection. As such, it was proposed that higher $CD4^+CD25^{hi}FOXP3^+$ Tregs may prevent GvHD, allowing immune reconstitution and recovery of CMV-specific $CD8^+$ T cells (Pastore *et al.,* 2011). As discussed before, in this project, the difference in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells could have been due to the CMV therapy and further studies will be required, collecting blood samples prior to commencing treatment for CMV.

Although low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the HSC grafts were not associated with increased rates of GvHD, it was hypothesised that $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells in the PB early post-transplant may be a better predictor of GvHD and transplant related events. However, at one and three months post-transplant, low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (counts and

%$CD4^+$ T cells) in the peripheral blood were not associated with an increased risk of aGvHD, CMV, relapse or NRM. Low proportions of $CD25^+FOXP3^+CD127^{-/dim}$ cells (%$CD4^+$ T cells) at three months were significantly associated with higher rates of cGvHD but this did not remain significant in multivariate analysis. Contrary to the study hypothesis, transplants with low $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts at one month showed a non-significant trend towards improved overall survival. If real, this observation is rather difficult to explain, particularly as there was no significant difference observed between the groups in relation to mortality from either relapse, GvHD, sepsis or other causes. It is only when all causes are combined that a non-significant trend was observed.

To obtain a better assessment of $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell recovery within each patient, this study also investigated the change in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells over time. Although the number of transplants analysed using this approach was more limited (*N*=66), the majority of these (71%) showed an increase in $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T cells (absolute and %$CD4^+$ T cells) from one to three months. Falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts/proportions were not associated with a significant difference in late aGvHD, cGvHD, CMV reactivation or relapse. However, falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts were associated with a significant increase in NRM and inferior OS. This observation has not been previously reported. Falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts over time could be indicative of a general failure of the graft. However, this observation was not seen when analysing the data using total $CD4^+$ T-cell, $CD4^+CD25^-FOXP3^-$ T-cell, $CD4^+CD25^+FOXP3^-$ T-cell counts or total lymphocytes. Therefore, it is hypothesised that falling $CD4^+CD25^+FOXP3^+CD127^{-/dim}$ T-cell counts could be an indication of failing regulation of T-cell immunity and in turn, may be associated with higher mortality due to the combination of infection, GvHD and relapse. Larger prospective clinical studies are needed to confirm this observation and test this hypothesis.

Finally, during the course of this study, it was apparent that the proportion of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) from the post-transplant samples showed very wide variation, ranging from 0.29% to 48.1% (normal range 2.70-4.30%). Since CD25 and FOXP3 are both up-regulated following T-cell activation, there were concerns whether all the $CD4^{+}CD25^{+}FOXP3^{+}$ cells are really Tregs, particularly in the setting of acute inflammation. Therefore, a cohort of the post-transplant samples was analysed using both the flow cytometry assay and the MSRT-qPCR as detection of unmethylated *FOXP3* TDSR should only detect $FOXP3^{+}$ nTregs (Baron *et al.,* 2007; Wieczorek *et al.,* 2009). Although the MSRT-qPCR assay was unable to detect unmethylated *FOXP3* TSDR in some of the samples, the two methods still showed good correlation when measuring T cells and putative Tregs. Importantly, there was no significant difference in the proportion of putative Tregs or the putative Treg to T-cell ratios between the two methods when analysing the post-alloHSCT samples, even when samples collected at the time of acute inflammation (GvHD). These findings suggest that either significant numbers of activated $FOXP3^{+}$ cells were not present in the post-alloHSCT samples or that the in-house 'Treg' flow cytometry assay did not measure significant numbers of activated $FOXP3^{+}$ T cells. Either way, these data suggest that the flow cytometry results from the post-transplant samples were an accurate measurement of putative Tregs in the peripheral blood. Importantly, it also demonstrates that high proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) (20-40%) can be observed post-alloHSCT. Further examination of these samples and/or transplants could provide crucial understanding of what factors promote *in vivo* Treg expansion and whether this could be used to increase Tregs in all alloHSCT patients. It is also proposed that the MSRT-qPCR assay should be used to analyse the stored HSC graft samples from this study to determine whether the clinical findings seen earlier remain when using this method. If so, this would further strengthen this study's clinical findings and we would then propose to analyse frozen HSC graft samples from other UK transplant centres and/or use this assay in national prospective Treg clinical studies.

In mice, Foxp3 expression in naïve $CD4^+$ T cells induces Treg phenotype and function (iTregs) (Chen *et al.,* 2003a; Liang *et al.,* 2005; Lan *et al.,* 2012a). However, in humans, FOXP3 can be expressed in activated $CD4^+$ T cells without conferring suppressive function and it remains uncertain whether these cells can become iTregs (Allan *et al.,* 2007; Tran *et al.,* 2007). In this project, using *in vitro* cell cultures, we confirmed that FOXP3 can be induced in a proportion of TCR activated naïve and conventional $CD4^+$ T cells and that FOXP3 expression is enhanced by TGFβ. However, in contrast to $CD4^+CD25^{high}$ Tregs, the majority of these $CD4^+FOXP3^+$ T cells produced TNFα and did not possess an unmethylated *FOXP3* TSDR. This project then demonstrated that the DNA hypomethylating agent, Azacitidine, can inhibit *FOXP3* TSDR methylation and enhance TCR induced CD25 and FOXP3 expression. However, most of these cells still produced TNFα, suggesting that FOXP3 expression and demethylation of the *FOXP3* TSDR are not sufficient to induce a Treg phenotype.

There is widespread interest in using Treg therapies in the setting of alloHSCT, solid organ transplantation and autoimmune disease (Leventhal *et al.,* 2012; Rosenblum *et al.,* 2012; Tang and Lee, 2012). However, producing GMP-grade human Treg-rich products with sufficient Treg numbers and purity has proved difficult. In the absence of Treg-specific surface markers, isolation strategies rely on magnetic bead cell sorting using positive selection with anti-CD25 and depletion with anti-CD8/CD19/CD127, generating cellular products with 40-90% $FOXP3^+$ Tregs (Hoffmann *et al.,* 2006a; Wichlan *et al.,* 2006; Peters *et al.,* 2008). However, the contaminating effector T cells within these products could exacerbate inflammation. Similarly, *ex vivo* expansion of these products can be accompanied by absolute and relative expansion of conventional T and B cells and down regulation of FOXP3 expression, reducing $CD4^+CD25^+FOXP3^+$ Treg purity and overall suppressive function (Peters *et al.,* 2008; Hoffmann *et al.,* 2009). While isolation only strategies

may be sufficient for Treg therapies in lymphopenic recipients, it is likely that *ex vivo* expansion will be required to produce sufficient $FOXP3^+$ Tregs for use in lymphocyte-replete settings (Tang and Lee, 2012). The high cost of performing large scale Treg isolation and expansion is also limiting further progress. In particular, the development of GMP-compliant flow cytometry cell sorters to improve isolated Treg purity is an option to only few centres due to the high expense and need to comply with stringent GMP regulations. Given these limitations and difficulties, there remains interest in whether human $CD4^+CD25^+FOXP3^+$ Tregs can be induced from $CD4^+CD25^-$ T cells. In mice, Foxp3 expression can be induced in naïve $CD4^+$ T cells through TCR stimulation and TGFβ and these iTregs have a similar phenotype and function to $CD4^+CD25^+FOXP3^+$ nTregs (Chen *et al.,* 2003b; Zheng *et al.,* 2004; Lan *et al.,* 2012a; Lan *et al.,* 2012b). Therefore, the observation that FOXP3 can also be induced in human conventional $CD4^+$ T cells renewed interest as to whether these $FOXP3^+$ cells are activated $FOXP3^+$ T cells or functional $FOXP3^+$ iTregs (Allan *et al.,* 2007, Tran *et al.,* 2007).

In this study, *in vitro* cell cultures were established using isolated human conventional and naïve $CD4^+CD25^-$ T cells and anti-CD3/CD28 stimulation. Although under these conditions, all the conventional and naïve $CD4^+$ T cells were activated, divided and expressed high levels of CD25, only a limited proportion of these cells (15-20%) expressed FOXP3. This is in keeping with previous studies using anti-CD3/CD28 stimulation of human conventional and naïve $CD4^+CD25^-$ T cells (Allan *et al.,* 2007; Baron *et al.,* 2007; Nagar *et al.,* 2008). Therefore, FOXP3 is not a universal marker of T-cell activation. In humans, TCR stimulation probably induces FOXP3 expression through up-regulation of the transcription factors NFAT and AP1 and binding to the *FOXP3* promoter (Mantel *et al.,* 2006; Sauer *et al.,* 2008). However, since only a limited proportion of the activated cells expressed FOXP3, additional regulatory factors are required and/or only certain $CD4^+$ T-cell subtypes can express FOXP3.

One factor that appears necessary for TCR-induced FOXP3 expression in human $CD4^+$ T cells is TGFβ (Tran *et al.,* 2007). It is proposed that TGFβ enhances FOXP3 expression by binding to its

cell surface receptor, up-regulating SMAD3, which interacts with NFAT at an enhancer region downstream of the *FOXP3* promoter (Tone *et al.,* 2008). Tran and colleagues (2007) demonstrated addition of TGFβ could enhance *in vitro* TCR-induced FOXP3 expression in naïve $CD4^+$ T cells. Furthermore, addition of neutralising anti-TGFβ antibodies significantly reduced the proportion of cells expressing FOXP3. In keeping with these results, addition of TGFβ to our cultures significantly increased both the proportion of activated $CD4^+$ T cells expressing FOXP3 and the level of FOXP3 expression in the $CD4^+FOXP3^+$ cells in a concentration dependent manner. However, even at the maximum TGFβ response, 40-50% of the activated conventional $CD4^+$ T cells did not express FOXP3 by day five. In part, this could have been because the isolated $CD4^+CD25^-$ T-cell populations contained both naïve and effector/memory T cells. It has been previously demonstrated that memory T cells are more resistant to TGFβ-induced FOXP3 expression (Tran *et al.,* 2007). In keeping with these previous studies, when culturing isolated $CD45RA^+CD4^+$ T cells only with TGFβ, approximately 70-80% of the cells expressed FOXP3 by day five (Baron *et al.,* 2007; Tran *et al.,* 2007). However, this raises the interesting question as to why the remaining 20-30% of the cells did not express FOXP3. Of note, all of the cells had been activated, expressed CD25 and had divided according to CFSE staining. As the starting cell populations contained 75.6-95.8% $CD45RA^+CD4^+$ T cells, it is possible that a proportion of these $CD4^+FOXP3^-$ cells could have been from contaminating effector T cells. However, it is unlikely that this would account for all the $FOXP3^-$ cells since $CD45RA^-$ events only represented 1-2% of the isolated $CD4^+$ T cells. It has been speculated that perhaps not all $CD45RA^+$ are truly naïve (Tran *et al.,* 2007). Alternatively, some authors have reported that TCR-activated FOXP3 expression is transient (Wang *et al.,* 2007). However, it is unlikely that some of the cells would have already lost their expression of FOXP3 since the anti-CD3/CD28 beads were still present and because the majority of cells still remained $FOXP3^+$ seven days after removing the TCR-stimulation. Isolation and further analysis of the $CD4^+CD25^+FOXP3^-$ cells on day five may provide further information as to why these cells did not express FOXP3.

Whether *in vitro* TCR-induced $FOXP3^+$ human $CD4^+$ T cells have suppressive function remains an area of considerable debate. While some studies have demonstrated suppressive function others have not and even show production of effector cytokines (Allan *et al.,* 2007; Tran *et al.,* 2007; Wang *et al.,* 2007; Nagar *et al.,* 2008). In this project, significant proportion of the anti-CD3/CD28/TGFβ induced $CD4^+FOXP3^+$ cells produced IL2 (40%) and TNFα (80%). Furthermore, this was not significantly different from the $CD4^+FOXP3^-$ cells. Therefore, the induced FOXP3 did not appear to significantly inhibit the cells' ability to produce IL2/TNFα and the cytokine profile of these cells was more in keeping with effector T cells than $CD4^+CD25^+FOXP3^+$ nTregs. In keeping with other studies, this work demonstrates that anti-CD3/CD28/TGFβ can induce FOXP3 expression in $CD4^+$ T cells but this does not inhibit proliferation or cytokine production (Allan *et al.,* 2007; Tran *et al.,* 2007; Nagar *et al.,* 2008).

Since DNA demethylation of the *FOXP3* promoter and TSDR enhancer is present in $CD4^+CD25^+FOXP3^+$ nTregs and is thought to be required for stable FOXP3 expression, it was hypothesised that the addition of the Azacitidine, may enhance anti-CD3/CD28/TGFβ-induced FOXP3 expression and may produce $CD4^+CD25^+FOXP3^+$ iTregs (Baron *et al.,* 2007; Huehn *et al.,* 2009). In keeping with this hypothesis, Azacitidine increased the proportion of conventional and naïve $CD4^+$ T cells expressing FOXP3 in a concentration dependent manner, although it caused marked cell death when given at 10μM for 72 hours. However, as seen with TGFβ, only a proportion of the cultured cells expressed FOXP3. These findings are consistent with previous reports that Azacitidine can increase activation-dependent FOXP3 expression in human conventional $CD4^+CD25^-$ T cells and mouse naïve $CD4^+CD25^-FOXP3^-$ T cells (Nagar *et al.,* 2008; Polansky *et al.,* 2008). Furthermore, Goodyear and colleagues (2012) have recently shown that administration of Azacitidine post-alloHSCT increases $CD4^+CD25^+CD127^{lo}FOXP3^+$ Treg numbers within the first three months post-transplant. However, as Azacitidine is not specific to *FOXP3*, it will also inhibit methylation of other genes during cell division. Although Azacitidine enhanced the

level of CD25 expression, it did not alter the expression of CD3, CD4, CD8, CD19, CD16 and CD56 and therefore did not alter the fundamental phenotype of these cells. Importantly, by using FOXP3-GFP reporter cells, Polansky and colleagues (2008) showed that TCR/Azacitidine-induced $Foxp3^+$ cells from mice had a demethylated TSDR while the $Foxp3^-$ cells in the same cultures did not. Furthermore, the methylation status of a control CpG rich region outside the Foxp3 locus showed no difference in methylation between the $Foxp3^+$ and $Foxp3^-$ cells. Hence, Azacitidine caused restricted demethylation in naïve $CD4^+CD25^-Foxp3^-$ T cells.

Importantly, Azacitidine also increased the proportion of conventional and naïve $CD4^+$ T cells expressing FOXP3 in response to anti-CD3/CD28/TGFβ stimulation. This suggests that either Azacitidine is able to induce FOXP3 in $CD4^+$ T cells in which anti-CD3/CD28/TGFβ stimulation could not and/or that it enhances the sensitivity of these cells to anti-CD3/CD28/TGFβ stimulation. It is proposed that TGFβ enhances anti-CD3/CD28-induced FOXP3 expression through the interaction of SMAD3 and NFAT with a *FOXP3* enhancer (TGFβ sensor) which is methylation independent (Tone *et al.,* 2008; Huehn *et al.,* 2009). In contrast, Azacitidine enhances FOXP3 expression by inhibiting methylation the *FOXP3* promoter and TSDR enhancer in dividing cells and promotes binding of the transcription factors, NFAT1/AP1 and CREB/ATF/STAT5 respectively (Polansky *et al.,* 2008; Lal *et al.,* 2009). Azacitidine, therefore, acts independently of the TGFβ-sensitive *FOXP3* enhancer and TGFβ does not cause demethylation of the TSDR enhancer. In keeping with this hypothesis, none of the CD3/CD28/TGFβ cultured cells had a demethylated *FOXP3* TSDR although many expressed FOXP3. In contrast, large proportions of the CD3/CD28/TGFβ/Azacitidine cultured cells contained a demethylated *FOXP3* TSDR and expressed FOXP3. The higher proportion of Azacitidine cultured naïve $CD4^+$ T cells with a demethylated *FOXP3* TSDR compared to $FOXP3^+$ cells could indicate that demethylation alone is not sufficient to induce FOXP3 expression. However, this could also reflect the different sensitivity of the two assays or the variability in FOXP3 expression between the different experiments. To clarify this, it would be necessary to separate the $FOXP3^+$ and $FOXP3^-$ cells at the end of culture and assess their TSDR methylation status.

While anti-CD3/CD28/TGFβ/Azacitidine induced FOXP3 expression and inhibited methylation of the *FOXP3* TSDR in a large proportion of conventional and naïve $CD4^+CD25^-$ T cells, it was unknown if these cells would have an iTreg phenotype or suppressive function. Within this study, it was not possible to isolate the induced $FOXP3^+$ for testing in functional assays since FOXP3 staining requires permeabilization of the cells. Likewise, testing the function of the whole population in an *in vitro* suppression assay was not reliable due to the continued proliferation of the cultured cells. However, it was possible to test the ability of these cells to produce pro-inflammatory cytokines, as these are not produced by $CD4^+CD25^+FOXP3^+$ nTregs (Levings *et al*., 2001; Stephens *et al.,* 2001). In contrast to $CD4^+CD25^+FOXP3^+$ nTregs, a large proportion of the CD3/CD28/TGFβ/Azacitidine-induced $FOXP3^+$ cells produced inflammatory cytokines, especially TNFα (80%). Furthermore, Azacitidine significantly increased the proportion of induced $FOXP3^+$ cells that were able to secrete IFNγ. These observations are consistent with the findings of Nagar and colleagues (2008) and together suggest that, in this *in vitro* setting, induced FOXP3 expression and demethylation of the *FOXP3* TSDR are not sufficient to induce a regulatory phenotype. Furthermore, these cytokine profiles suggest these cells they are more in keeping with $FOXP3^+$ effector T cells rather than $CD4^+CD25^+FOXP3^+$ iTregs.

In T cells, one of the main second messenger pathways through which TCR-stimulation increases TNFα production is the TCR/Calcineurin/NFAT pathway (Tsai *et al.,* 1996; Lawrence *et al.*. 2011). The transcription factors NFAT and ATF2/Jun co-operate to bind to multiple NFAT binding sites and cyclic AMP-response elements respectively in the *TNFα* promoter (Tsai *et al.,* 1996). TCR/Calcineurin/NFAT has also been implicated in TCR-induced FOXP3 expression through the binding of NFAT and AP1 to the *FOXP3* promoter (Mantel *et al*., 2006; Sauer *et al.,* 2008). Therefore, in these *in vitro* cultures, TCR-stimulation of the $CD4^+$ T cells may up-regulate both FOXP3 and TNFα using similar second messenger pathways. However, in contrast to $CD4^+CD25^+FOXP3^+$ nTregs, the induced FOXP3 does not appear to inhibit the expression of the

inflammatory cytokines. Whether FOXP3 can be induced in human conventional/naïve $CD4^+$ T cells without inducing TNFα or other pro-inflammatory cytokines remains uncertain. Of note, a minority (20%) of the *in vitro* cultured conventional/naïve $CD4^+$ T cells were $FOXP3^+$ $TNF\alpha^-$ but it remains unclear what differentiates the development of these cells from the rest.

In this study, *in vitro* TCR stimulation was produced using anti-CD3/CD28 Dynabeads. However, it is possible that the strength and duration of TCR stimulation may affect FOXP3 expression and/or the possible development of $CD4^+CD25^+FOXP3^+$ iTregs. In mice, sub-optimal T-cell stimulation with low-dose anti-CD3 or sub-optimal agonist peptide induced Foxp3 expression in $CD4^+CD25^-$ T cells and was more effective than higher TCR stimulation (Oliveira *et al.,* 2011). Molinero and colleagues (2011) demonstrated that high dose TCR stimulation of naïve T cells induces NF-KB activity and expression of effector cytokines, particularly TNFα and IFNγ, which inhibit Foxp3. In contrast, TCR/TGFβ/IL2 induction of Foxp3 to produce iTregs from peripheral T cells was independent of NF-KB. At high TCR stimulation, CD28 further inhibited Foxp3 and iTreg differentiation, although it was required at lower TCR doses (Molinero *et al.,* 2011). Therefore, although the anti-CD3/CD28/TGFβ/Azacitidine used in this study was able to induce FOXP3, it may be hypothesised that using lower dose TCR stimulation can still induce FOXP3 without inducing the NF-KB pathway and/or TNFα and IFNγ. Although this was not observed by simply reducing the cell-to-bead ratio, further experiments would be required to investigate the optimal dose, duration and timing of TCR stimulation, with and without CD28 co-stimulation, and whether NF-KB is activated. The strength of TCR signalling may also be important for the activation of the PI3K/Akt/mTOR pathway which inhibits the induction of Foxp3 (Haxhinasto *et al.,* 2008; Sauer *et al.,* 2008; Merkenschlager and van Boehmer, 2010). Inhibition of PI3K, Akt or mTOR promotes FOXP3 expression in mouse and human T cells and enables preferential expansion of human $CD4^+CD25^+FOXP3^+$ Tregs in *ex vivo* cultures (Sauer *et al.,* 2008; Battaglia *et al.,* 2012). In mice, the activity of the PI3K/Akt/mTOR pathway has been associated with the dose of the antigenic stimuli with low TCR doses producing weak Akt/mTOR activity and expansion of $FOXP3^+$ cells (Sauer *et al.,* 2008). Furthermore, lower doses of TCR/co-stimulatory signals together with the

mTOR inhibitor Rapamycin or inhibitors of MEK/ERK signalling, increased TCR/TGFβ dependent induced Foxp3 expression (Haxhinasto *et al.,* 2008; Sauer *et al.,* 2008). Retinoic acid has also been shown to overcome the inhibition of Foxp3 expression observed with higher TCR/co-stimulatory signals, possibly via expansion and phosphorylation of SMAD3 and inhibition of effector cytokines (Benson *et al.,* 2007). Therefore, it is proposed that addition of Rapamycin and/or retinoic acid to the *in vitro* CD3/CD28/TGFβ/Azacitidine cultures of human $CD4^+$ T cells may be able to induce strong FOXP3 expression with a demethylated TSDR but without stimulating significant expression of pro-inflammatory cytokines. Further experiments will be required to test these hypotheses.

7.5 CONCLUSIONS

The delicate balance between immune activation and suppression is critical to the success of clinical alloHSCT. Expanding Tregs post-transplant, either *in vivo* or through *ex vivo* isolation and expansion, remains a promising new therapy to regulate this developing donor-derived immune system without losing the important GvT response. However, the effect of Treg therapies on clinical transplantation and which factors affect Treg reconstitution post-transplant remain unknown due to the paucity of human clinical studies.

In this project, two different methods were developed to measure putative Tregs in clinical samples, potentially allowing large scale multi-centre studies of putative Tregs in alloHSCT to be performed. Using these techniques, this project demonstrated novel clinical findings that low proportions of $CD25^{+}FOXP3^{+}CD127^{-/dim}$ cells (%$CD4^{+}$ T cells) with the HSC graft are independently associated with poor engraftment, increased risk of severe infection and NRM and inferior overall survival. In keeping with the pre-clinical work of Fujisaki and colleagues (2011), it is proposed that $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells may protect the engrafting HSC within the bone marrow niche. Post-transplant, $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell reconstitution (absolute counts and %$CD4^{+}$ T cells) were significantly affected by Alemtuzumab conditioning and CMV reactivation and falling $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T-cell counts over the first three months were associated with higher NRM. Monitoring $CD4^{+}CD25^{+}FOXP3^{+}CD127^{-/dim}$ T cells post-transplant may, therefore, identify those patients with worse outcomes. Further prospective clinical studies will be required to confirm these observations and test these hypotheses. Finally, it has been proposed that inducing FOXP3 expression in conventional $CD4^{+}$ T cells may confer these cells with suppressive function and generate $CD4^{+}CD25^{+}FOXP3^{+}$ iTregs for therapeutic use (Wang *et al.,* 2007). However, in this study, *in vitro* induction of FOXP3 and TSDR demethylation using anti-CD3/CD28/TGFβ/Azacitidine was not sufficient to confer these cells with Treg characteristics. Although further investigation will be required to examine the effect of variable TCR stimulation and/or use of Rapamycin/Retinoic acid, it is proposed that current focus for generating Tregs for human clinical trials should be directed towards improving *ex vivo* Treg isolation and expansion.

Chapter 8

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8. REFERENCES

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Appendix

9. APPENDIX

This appendix shows the DNA sequence for the three genetic loci tested in the MSRT-qPCR assay (*FOXP3, CD3, GAPDH*) and the sequence of the PCR primers and probes ((Bisulphite Specific (BS), Methylation Specific (MS), Probes (Pr), unmethylated (TpG), methylated (CpG)).

The location (e!Ensembl release 58, July 2012, http://www.ensembl.org) and genomic DNA sequence for each loci is shown with the CpG dinucleotides highlighted in yellow.

The bisulphite treated DNA sequence for both the unmethylated (Tregs, T cells, All cells) and methylated versions (non-Tregs, non-T cells) are also shown with the position of the BS primers (blue), BSMS primers (red) and BSMS probes (underlined) indicated.

FOXP3 TSDR

Amplicon	Specificity	Forward	Reverse
FOXP3 **336bp**	BS	TGTTTGGGGGTAGAGGATTT	TATCACCCCACCTAAACCAA
	BSMS (TpG)	GTTTTTGATTTGTTTAGATTTTTTTGTT	CCTCTTCTCTTCCTCCATAATATCA
	BSMS Pr (TpG)	ATGGTGGTTGGATGTGTTGGGTT	
	BSMS (CpG)	GTTTTCGATTTGTTTAGATTTTTTCGTT	CCTCTTCTCTTCCTCCGTAATATCG
	BSMS Pr (CpG)	ATGGCGGTCGGATGCGTC	

Genomic DNA (GRCh37:X:49117052-49117387:+1)

5:TGTCTGGGGGTAGAGGACCTAGAGGGCCGGCTGGGCAGCCGGCTTCCTGCACTGTCTG
TTGGGACGTCCCTTTCTGACTGGGTTTCTCAGAAGCTGAATGGGGGATGTTTCTGGGACAC
AGATTATGTTTTCATATCGGGGTCTGCATCTGGGCCCTGTTGTCACAGCCCCCGACTTGCC
CAGATTTTTCCGCCATTGACGTCATGGCGGCCGGATGCGCCGGGCTTCATCGACACCACGG
AGGAAGAGAAGAGGGCAGATACCCCACCCCACAGGTTTCGTTCCGAGAACTGGCTGCCCTG
TCCTGCAGCAGGCTTGGCCCAGGTGGGGTGACA:3

Bisulphite Treated DNA from Tregs (TpG specific)

5:TGTTTGGGGGTAGAGGATTTAGAGGGTTGGGTTGGGTAGTTGGTTTTTTGTATTGTTTG
TTGGGATGTTTTTTTTTGATTGGGTTTTTTAGAAGTTGAATGGGGGATGTTTTTGGGATAT
AGATTATGTTTTTTATATTGGGGTTTGTATTTGGGTTTTGTTGTTATAGTTTTTGATTTGTT
TAGATTTTTTTGTTATTGATGTTATGGTGGTTGGATGTGTTGGGTTTTATTGATATTATGG
AGGAAGAGAAGAGGGTAGATATTTTATTTTATAGGTTTTGTTTTGAGAATTGGTTGTTTTG
TTTTGTAGTAGGTTTGGTTTAGGTGGGGTGATA:3

Bisulphite Treated DNA from Non-Tregs (CpG specific)

5:TGTTTGGGGGTAGAGGATTTAGAGGGTCGGGTTGGGTAGTCGGTTTTTTGTATTGTTTG
TTGGGACGTTTTTTTTTGATTGGGTTTTTTAGAAGTTGAATGGGGGATGTTTTTGGGATAT
AGATTATGTTTTTTATATCGGGGTTTGTATTTGGGTTTTGTTGTTATAGTTTTCGATTTGTT
TAGATTTTTTCGTTATTGACGTTATGGCGGTCGGATGCGTCGGGTTTTATCGATATTACGG
AGGAAGAGAAGAGGGTAGATATTTTATTTTATAGGTTTCGTTTCGAGAATTGGTTGTTTTG
TTTTGTAGTAGGTTTGGTTTAGGTGGGGTGATA:3

CD3

Amplicon	Specificity	Forward	Reverse
CD3 **233bp**	BS	GGTAGAGAATATGGAAAAGGTGGT	CCCAACCTCCCACTAACAAA
	BSMS (TpG)	TTTAAATATTGTTATATTTTGA	AAAAATCTAACTACTACAACTTAC
	BSMS Pr (TpG)	GAGAAGTTGTTGGTTTTATAGTGTAAG	
	BSMS (CpG)	TTAAATATTGTTATATTTCGA	AAATCTAACTACTACGACTTAC
	BSMS Pr (CpG)	TCGTCGGTTTTATAGCGT	

Genomic DNA (GRCh37:11:118213561-118213793:+1)

5:GGCAGAGAATATGGAAAAGGTGGCCTGAACTTTAGCGTCCCTATTGACAATGCAACCAC
ATTTACACACAACCTAAACACTGCCACATCTCGAAGCCCCTTGAGAGAAGCCGTCGGCCCC
ATAGCGCAAGCCGTAGCAGCTAGATTTCTCATGGAGGCTGATCTTTCTCAGGACCCTTCAC
TAGGCAGCCAGGGACACCAGATCTAGCAGCTTCTTGTCAGTGGGAGGTTGGG:3

Bisulphite Treated DNA from T cells (TpG specific)

5:GGTAGAGAATATGGAAAAGGTGGTTTGAATTTTAGTGTTTTTATTGATAATGTAATTAT
ATTTATATATAATTTAAATATTGTTATATTTTGAAGTTTTTTGAGAGAAGTTGTTGGTTTT
ATAGTGTAAGTTGTAGTAGTTAGATTTTTTATGGAGGTTGATTTTTTTTAGGATTTTTTAT
TAGGTAGTTAGGGATATTAGATTTAGTAGTTTTTTGTTAGTGGGAGGTTGGG:3

Bisulphite Treated DNA from Non-T cells (CpG specific)

5:GGTAGAGAATATGGAAAAGGTGGTTTGAATTTTAGCGTTTTTATTGATAATGTAATTAT
ATTTATATATAATTTAAATATTGTTATATTTCGAAGTTTTTTGAGAGAAGTCGTCGGTTTT
ATAGCGTAAGTCGTAGTAGTTAGATTTTTTATGGAGGTTGATTTTTTTTAGGATTTTTTAT
TAGGTAGTTAGGGATATTAGATTTAGTAGTTTTTTGTTAGTGGGAGGTTGGG:3

GAPDH

Amplicon	Specificity	Forward	Reverse
GAPDH	BS	TGGGGGTAGGGTAGTTG	AAACCCACTTCTTTAATTTACC
538bp	BSMS (TpG)	GGTTTTTGGTATTGTAGGTTTT	CCAATTACAACATAACAACCA
	BSMS Pr (TpG)	TGTTTGGATGTTGTGTTTGTGGTAGAGTG	

Genomic DNA (GRCh37:12:6644119-6644656:+1)

5:TGGGGGCAGGGTAGCTGTTCCCCGCAAGGAGAGCTCAAGGTCAGCGCTCGGACCTGGCG
GAGCCCCGCACCCAGGCTGTGGCGCCCTGTGCAGCTCCGCCCTTGCGGCGCCATCTGCCCG
GAGCCTCCTTCCCCTAGTCCCCAGAAACAGGAGGTCCCTACTCCCGCCCGAGATCCCGACC
CGGACCCCTAGGTGGGGGACGCTTTCTTTCCTTTCGCGCTCTGCGGGGTCACGTGTCGCAG
AGGAGCCCCTCCCCCACGGCCTCCGGCACCGCAGGCCCCGGGATGCTAGTGCGCAGCGGGT
GCATCCCTGTCCGGATGCTGCGCCTGCGGTAGAGCGGCCGCCATGTTGCAACCGGGAAGGA
AATGAATGGGCAGCCGTTAGGAAAGCCTGCCGGTGACTAACCCTGCGCTCCTGCCTCGATG
GGTGGAGTCGCGTGTGGCGGGGAAGTCAGGTGGAGCGAGGCTAGCTGGCCCGATTTCTCCT
CCGGGTGATGCTTTTCCTAGATTATTCTCTGGTAAATCAAAGAAGTGGGTTT:3

Bisulphite Treated DNA from all cells (TpG specific)

5:TGGGGGTAGGGTAGTTGTTTTTTGTAAGGAGAGTTTAAGGTTAGTGTTTGGATTTGGTG
GAGTTTTGTATTTAGGTTGTGGTGTTTTGTGTAGTTTTGTTTTTGTGGTGTTATTTGTTTG
GAGTTTTTTTTTTTTAGTTTTTAGAAATAGGAGGTTTTTATTTTTGTTTGAGATTTTGATT
TGGATTTTTAGGTGGGGGATGTTTTTTTTTTTTTTGTGTTTTGTGGGGTTATGTGTTGTAG
AGGAGTTTTTTTTTTATGGTTTTTGGTATTGTAGGTTTTGGGATGTTAGTGTGTAGTGGGT
GTATTTTTGTTTGGATGTTGTGTTTGTGGTAGAGTGGTTGTTATGTTGTAATTGGGAAGGA
AATGAATGGGTAGTTGTTAGGAAAGTTTGTTGGTGATTAATTTTGTGTTTTTGTTTTGATG
GGTGGAGTTGTGTGTGGTGGGGAAGTTAGGTGGAGTGAGGTTAGTTGGTTTGATTTTTTTT
TTGGGTGATGTTTTTTTTAGATTATTTTTTGGTAAATTAAAGAAGTGGGTTT:3