Selective Allodepletion to improve Anti viral and Anti leukaemic Responses after Haploidentical Transplantation

By

Sujith Samarasinghe

Institute of Child Health
University College London

A Thesis Submitted for the degree of Doctor of Philosophy

Declaration

I, Sujith Samarasinghe, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical SCT, but infection and leukaemic relapse remain problematic. To develop a rational approach to refining allodepletion, we characterized the expression of surface markers and cytokines on proliferating alloreactive T-cells flow cytometrically. CD25 was expressed on 83 % of CFSE-dim alloreactive T-cells, confirming this as an excellent target for allodepletion. 70 % of the alloreactive CD25^{-ve} population expressed CD71, identifying this as a novel marker to target alloreactive T-cells that persist after CD25 depletion. We compared residual alloreactivity to host or 3rd party after CD25 *vs* combined CD25/71 immunomagnetic depletion in 8 HLA-mismatched donor-recipient pairs. In 1° MLRs, residual responses to host were undetectable after CD25/71 depletion. In 2° MLRs, CD25/71 depletion resulted in significantly lower residual proliferative response to host than CD25 depletion (median 4.8% of the response of unmanipulated PBMC *vs* 9.9%, $p < 0.01$). Likewise, the median residual reactivity to host in IFN-γ ELISPOT assays was significantly lower after combined CD25/71 than CD25 allodepletion (14.1 % *vs* 54.6%, $p < 0.05$). Third party responses after CD25/71 allodepletion were equivalent to unmanipulated PBMCs in both assays. In pentamer and IFN-γ ELISPOT assays, anti-viral responses to CMV, EBV and adenovirus were preserved after combined CD25/71 allodepletion. Finally, we showed that CD25/71 allodepleted T-cells can be redirected to recognize and secrete IFN-γ and granzyme B in response to CD19 cell lines and primary ALL blasts through lentiviral transfer of a chimeric αCD19ζ TCR. This strategy may facilitate immunotherapy with larger doses of allodepleted T-cells after haplo-SCT, enhancing graft versus leukaemia and anti-viral effects.

TABLE OF CONTENTS

TABLE OF FIGURES
Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Appendix

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Abbreviations

α	Alpha
adeno	adenovirus
ADTs	allodepleted donor T cells
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
β	Beta
BM	Bone Marrow
BMT	Bone Marrow Transplant
Bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
cPPT	Central polypurine Tract
cpm	counts per minute
CR	Complete Remission
CTL	Cytotoxic T cell
DC	Dendritic cells
DLI	Donor Lymphocyte Infusion
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus
Env	envelope
FACs	Flow Cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Gag	Group Specific Antigens
γ	gamma
GFP	green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Good Manufacturing Practice
GVHD	Graft versus host disease
GVL	Graft Versus Leukaemia
Haplo	haploidentical
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplant
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
Kb	kilobases
LB	Luria-Bertani
LCL	Lymphoblastoid cell lines
LTR	Long terminal repeat
M	Molar
m	milli (10^{-3})
ml	millilitre

μ	micro (10^{-6})
MHC	Major Histocompatibility Complex
mHag	Minor Histocompatibility Complex
MFI	Mean fluorescent Intensity
MLR	Mixed Lymphocyte Reaction
MLV	Moloney murine leukaemia virus
MOI	Multiplicity of Infection
MSD	Matched sibling donor
MRD	Matched related donor
MUD	matched unrelated donor
NK	Natural Killer
ψ	Packaging signal
PBMC	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBSC	Peripheral blood stem cells
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEI	polyethylenimine
Pe-CY5	Phycoerythrin-Cy5
PFA	Paraformaldehyde
Pol	RNA Dependent DNA polymerase
PTLD	Post Transplant Lymphoproliferative Disease
PR	Partial Remission
PR1	Proteinase 1
R:S	Responder:Stimulator ratio
RIC	Reduced Intensity Transplant
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
SCID	Severe Combined Immunodeficiency Disease
SCT	Stem cell transplant
SFFV	Spleen focus forming virus
SIN	Self Inactivating
TAE	Tris-acetate-EDTA
TCR	T cell receptor
TNF	Tumour necrosis factor
Treg	T regulatory cell
Tween 20	Polyethylene-sorbitan monolaurate
u	units
VSV-G	Vesicular stomatitis virus glycoprotein
WPRE	Woodchuck hepatitis virus post transcriptional regulatory element
WT	Wild Type

CHAPTER 1

Introduction

INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) involves the intravenous infusion of autologous or allogeneic stem cells collected from bone marrow, peripheral blood, or umbilical cord blood to re-establish haematopoietic function in patients with damaged or defective bone marrow or immune systems. HSCT is a potential curative therapy for haematological malignancies by enabling both dose escalation in chemoradiotherapy and through an immunologically graft versus leukaemia (GVL) effect, mediated by donor T-cells. However, alloreactive donor T-cells can also cause a graft versus host disease (GVHD), which is a major complication post allogeneic transplantation.

Haploidentical Transplantation

The lack of fully HLA- matched donors is a major limitation to the applicability of HSCT. The ideal donor, a HLA-matched sibling donor is only available for 30 % of patients. For patients lacking a matched sibling donor, HSCT can be performed from an unrelated adult donor or cord blood. In contrast, the probability of finding a matched unrelated donor ranges from 10 % in poorly represented ethnic groups to 60 % in Caucasians.[1] However, almost all patients have a HLA-mismatched family haploidentical donor, generally a parent or sibling. Haploidentical donors are matched with the recipient for 1 HLA haplotype and mismatched for the other. One potential advantage of using haploidentical donors [2] is that the donor could be chosen depending upon HLA type, CMV status, or other features such as NK cell alloreactivity. Another advantage is equal availability of donors for all ethnic and racial groups in contrast to matched unrelated donors. Haploidentical transplantation is now performed for patients with a variety of haematological malignancies, non haematological malignancies, immunodeficiencies and inborn errors of metabolism.

Although encouraging data have been obtained, this approach is only being explored at centres with expertise in this area. Due to the high precursor frequency of alloreactive T cells in the haploidentical setting, rigorous T cell depletion is needed to prevent GVHD. The Perugia group pioneered progress in this field by developing the strategy of infusing mega dose,

CD34- selected stem cells (>10 x 10^6/kg) which were T cell depleted by virtue of positive CD34 immunomagnetic selection using a Clinimacs device (Miltenyi Biotec).[3] This approach resulted in over 4 log T cell depletion. Typically a dose of 10^4/kg residual T cells is infused with the graft. While this vigorous T-cell depletion is necessary to avoid GVHD, this leads to delayed immune reconstitution, predisposing to a high risk of viral infections and leukaemic relapse.

One of the largest series of haploidentical transplants in the paediatric area has been done at The University of Tuebingen.[4] Using CD34 positively selected grafts, they demonstrated in 63 patients, engraftment in 98 % and grade 2 GVHD in 7 %. Overall survival after 4 years was 40 %, with the major causes of death being relapse (30 %) and viral infections (14 %). Haploidentical transplantation has now become a well-established method and should be considered in all patients who need stem cell transplantation but lack a matched or single allelic mismatched donor. This method makes motivated donors available for almost all paediatric patients, since parents can donate stem cells for their children. Stable survival rates have been obtained and the problems of engraftment and GVHD have largely been resolved. However, the profound delay in T-cell reconstitution predisposes to high morbidity/mortality from relapse and viral infections.

Poor immune reconstitution post haploidentical transplantation is the key factor contributing to the high transplant-related mortality associated with this form of HSCT and preventing broader use of this approach. In one series of 20 children who were prospectively followed up after receiving CD34 + selected haploidentical transplants, NK cells were the first lymphoid cells to emerge during the first 4 weeks post transplantation.[5] Reconstitution of the T- and B-cell occurred much later. For the first 6 months after haploidentical transplantation, patients are profoundly T-lymphopoenic, with a few circulating memory T-cells with a limited diversity, derived from graft-contaminating T-cells that have expanded in the antigenic milieu of the host. T-cell counts reached > 100/µl after a median of 72 days, with CD8 cells recovering faster than CD4 cells. A second wave of T-cell regeneration derived from donor stem cells, was found to start after 6 months resulting in T-cells that expressed a naive phenotype (CD45RA$^+$). This increase in the number of naive T cells encompassed both CD8$^+$ and CD4$^+$ fractions and had a more diverse TCR repertoire. The mean time to normal T-cell numbers was 450 days. Immune reconstitution in adult recipients of haploidentical grafts is even slower

where CD4$^+$ counts remained below 100 and 200/µl for 10 and 16 months respectively, reflecting reduced thymic function compared to children.[6]

This profound delay in T-cell reconstitution predisposes to an increased risk of morbidity and mortality from viral infections. An updated analysis of viral morbidity and mortality in paediatric haploidentical recipients showed that until day 180 post stem cell transplant (SCT) the cumulative incidence of all lethal viral infections (adenovirus *n = 5*, cytomegalovirus *n = 3*, herpes simplex virus *n = 1*) was 16 % for the whole cohort of patients.[7] Adenovirus is a particularly important pathogen with a cumulative incidence of adenovirus-associated mortality was 8.5 %. Similarly, an update from the Perugia group showed that the transplant related mortality (TRM) for haploidentical transplants was 47 % in a 100 high risk leukaemic patients who were in complete remission, and 63 % in those who were transplanted in relapse.[8] 70 % of deaths were due to infection. The probability of relapse depended on the status of remission at the time of transplant, with most relapses occurring in patients not in remission at the time of transplant. The probability of relapse for AML patients in complete remission (CR1) was 18 % and for ALL it was 22 %. For patients not in remission, the figures for AML were 34 %, and ALL 90 %. Thus, improving immune reconstitution in haploidentical transplantation is vitally important to resolve the issue of virus and relapse associated mortality.

In order to overcome the slow immune reconstitution after haplo-SCT, and transfer NK and other accessory cells with the graft, a number of groups have investigated negatively selecting haploidentical grafts with CD3 immunomagnetic beads (typically in combination with a B cell depletion), rather than positively selecting for CD34, using a reduced intensity conditioning (RIC) regime. The resulting grafts typically contain rather more T-cells than a CD34 selected graft (median 14 x 10^4/kg) and several logs more NK cells (35 x 10^6/kg after CD3/19 depletion compared to 0.003 x 10^6/kg after CD34 selected grafts) .[9,10] Engraftment occurred in 91 % of patients, with more rapid T-cell reconstitution (median CD3 count 350/µl at 3 months) compared to previously reported with CD34 selected grafts. However, 36 % of patients developed significant acute GVHD (grade 2 or greater), thus suggesting that the enhanced T-cell reconstitution was in fact due to the higher T-cell dose that was infused with the graft. Furthermore, it was not clear if the improved T-cell reconstitution is simply due to expansion of alloreactive T-cells in patients who have developed GVHD. In an adult cohort using this regime, T-cell reconstitution remained slow (median CD3 count was 227 cells/µl on day

+100), there was a high incidence of severe acute GVHD (48 %), with relapse and infection accounting for a majority of deaths (overall survival 31 % after 241 days).[9] An update of this group's experience with CD3/19 depleted RICs in 29 children/adolescents demonstrated that significant acute GVHD remained problematic (17 %) as did relapse (34 %), and deaths from adenoviraemia (7 %).[11] There has been no formal assessment of anti-viral immune responses and T-cell repertoire using this approach. Thus immune reconstitution after CD3/19 depleted haploidentical HSCTs needs further study.

In summary, strategies using mega doses of CD34-selected stem cells have shown that haplo-SCT is feasible, but is compromised but the profound delay in T-cell reconstitution. Newer regimes using RIC, with combined CD3/19 depletion have emerged, leading to somewhat quicker T-cell reconstitution, but they have been complicated with significant rates of GVHD. What is needed is a means of selectively enhancing desirable anti-viral and anti-leukaemic T-cell responses after haploidentical-SCT without causing GVHD. In order to do this, we need to understand the pathogenesis of GVHD.

Immunobiology of GVHD

Acute and chronic GVHD are a major cause of non relapse morbidity and mortality after stem cell transplantation. Acute GVHD often presents initially with a skin rash. Anorexia, nausea, and watery/or bloody diarrhoea are typical manifestations of gastrointestinal GVHD. Liver GVHD may present with jaundice and derangement of liver function tests. Chronic GVHD has more diverse manifestations and can resemble autoimmune syndromes, such as scleroderma, with salivary and lachrymal gland involvement, and pulmonary involvement. For the purposes of epidemiological studies, acute GVHD occurs within 100 days after transplantation and chronic GVHD after this.

 GVHD is primarily a T cell mediated disease. The principal task of the immune system is to distinguish self from non-self. Major Histocompatibility Complex (MHC) molecules provide the crucial surface upon which the antigen receptors on T lymphocytes (T cell receptors or TCR) recognize foreign (non-self) antigens The MHC is highly polymorphic from individual to individual, and segregates in families in a Mendelian co dominant fashion. The genes of the HLA locus encode two distinct classes of cell surface molecules, classes I and II. Class I

molecules are expressed on the surfaces of virtually all nucleated cells at varying densities, while class II molecules are more restricted to cells of the immune system, primarily B lymphocytes, dendritic cells and monocytes. There are three different class I (HLA-A, -B, -C) and class II (HLA-DQ, -DR, -DP) antigens. HLA-A, -B and -DR antigens appear to be the most important loci determining whether transplanted cells initiate a graft versus host reaction. The normal function of these molecules on antigen presenting cells is to present antigenic fragments (in the form of linear peptides) to the CD4 positive helper T-cells (class II), or CD8 positive cytotoxic effector T-cells (class I). This process of antigen presentation consists of the binding of a single T-cell receptor to a complex on the surface of an antigen- presenting cell, consisting of the MHC molecule and a peptide fragment derived from the foreign antigen. Intracellular-derived antigens are predominantly presented via MHC-class I, whereas exogenous antigens are mainly presented via class II.

The major prerequisites for GVHD to occur are that the transplanted graft must contain immunologically competent cells and the recipient expresses tissue antigens that are recognized as foreign by the donor.[12] The first prerequisite is fulfilled by mature T lymphocytes. After allogeneic transplant, the severity of GVHD corresponds with the number of donor T cells infused[13] and T cell depletion reduces the incidence and severity of acute GVHD. Differences between host and donor MHC and minor histocompatibility antigens are the most important risk factor for initiation of GVHD. There are two forms of T-cell allorecognition, which are summarised in Fig. 1.

a) **Direct presentation of alloantigen**- The response to intact allogeneic major histocompatibility (MHC) molecules plus foreign peptide is known as direct recognition. i.e. the host antigen presenting cell (APC) presents foreign MHC/ peptide to the donor T cells. This pathway is thought to be the dominant pathway involved in the early alloimmune response as the relative number of T cells that proliferate on contact with allogeneic or donor cells is extraordinarily high compared with the number of clones that target antigen presented by self-APC

b) **Indirect presentation of alloantigen**- The indirect alloresponse is recognition of peptides from allogeneic MHC proteins presented by self MHC molecules. This occurs when donor APC process and present recipient alloantigens (e.g. from apoptotic cells) to donor T-cells a phenomenon called cross-presentation.

Minor histocompatibility antigens are polymorphisms of peptides that are presented in the context of the MHC [14]
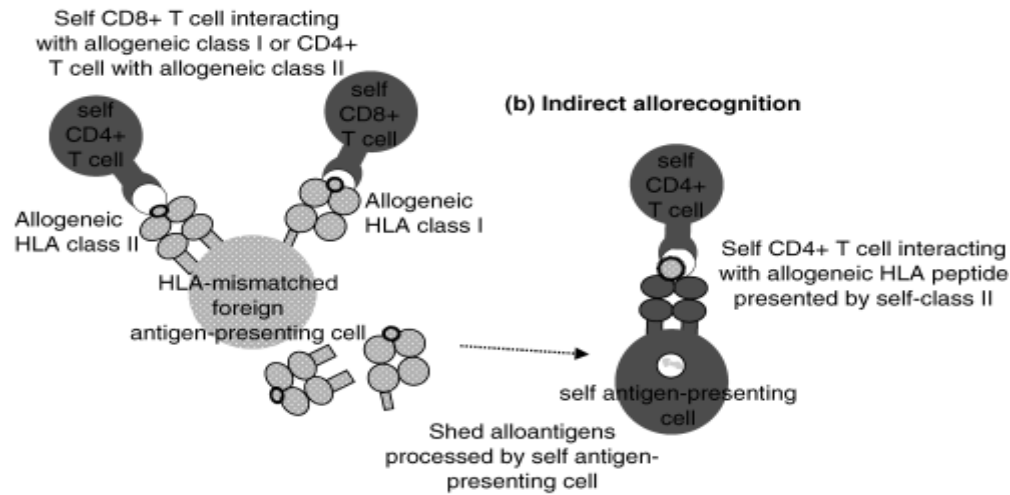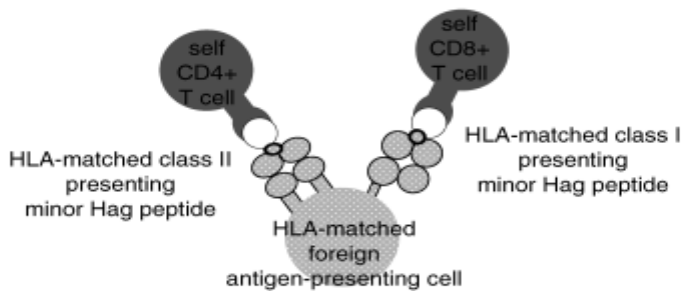
Figure 1 Pathway of T cell Recognition. Reproduced from [14] There are 2 forms of allorecognition. Host APCs presenting foreign MHC to donor T cells (a) is called direct allorecognition. Indirect allorecognition occurs when donor APCs present host MHC molecules to donor T cells (b). Minor histocompatibility antigens are polymorphisms of peptides presented in the context of MHC and explain how GVHD occurs in the matched sibling setting.

As seen in Fig 2 the pathophysiology of GVHD can be thought of as a three step process [12,15] (1)- tissue damage to the recipient by the chemo radiotherapy conditioning regimen (2) donor T-cell activation and clonal expansion (3) Effector phase with further damage to recipient tissues caused by donor T-cells.
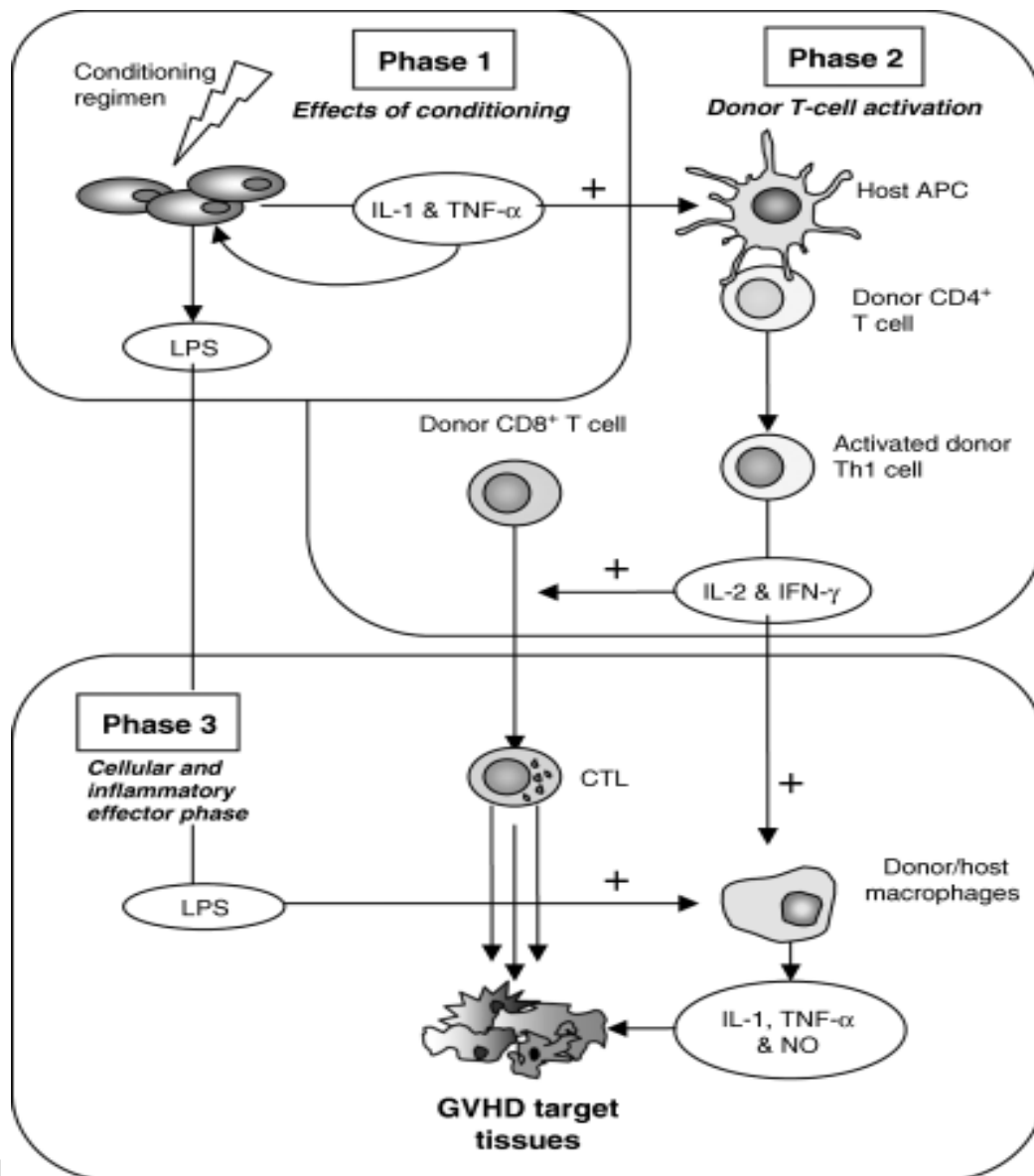
Figure 2: Three phases of GVHD. In the first phase, chemoradiotherapy damages host tissues, leading to release of proinflammatory cytokines and absorption of lipopolysaccharide across bowel mucosa. This leads to upregulation of MHC molecules and in phase 2 host APCs interact with donor T cells. The latter secrete cytokines leading to the generation of the effector phase (phase 3). In phase 3 effector CTLs damage host tissue by the FAS/FAS ligand pathway and release of granzyme B. There is further release of proinflammatory cytokies leading to a vicious cycle (' the cytokine storm')

Phase 1- Damage to host by Chemo radiotherapy

High dose chemo radiotherapy regimens damage host tissue, particularly the gut, and allows lipopolysaccharide (LPS) from bacteria in the bowel to leak into adjacent tissues and the

bloodstream. Distinct classes of conserved microbial molecules and necrotic cell elements activate Toll like receptors on various cells such as dendritic cells, NK cells, macrophages, and eosinophils, leading to the release of inflammatory cytokines such as tumour necrosis factor α (TNFα), IL-1, IL-6, IL-12[16] and interferon gamma (IFNγ), the latter promoting up regulation of MHC antigens on antigen presenting cells (APCs) and maturation of dendritic cells (DCs) As part of the innate response, neutrophils, macrophages and eosinophils migrate to damaged tissue and cause further injury. Damaged host tissues responds with further secretion of proinflammatory cytokines, such as TNF-α and IL-1, resulting in the so called "cytokine storm'.

Phase 2- Donor T cell activation

(a) Target Antigens

The precursor frequency of T cells that can recognize a mismatched MHC is very high (up to 5-10 % of the T cell repertoire)[17,18]. Mismatched MHC molecules on recipient APC are foreign to the donor T cells. In contrast, on self APCs, most of the self MHC molecules are displaying self peptides, and any foreign peptide probably occupies 1 % or less of the total MHC molecules expressed. As a result, the density of allogeneic determinants on allogeneic APCs, is much higher than the density of foreign peptide-self MHC complexes on self APCs, highlighting the importance of direct allorecognition. The abundance of recognizable allogeneic MHC molecules may allow activation of T-cells with low affinities for the foreign MHC, thereby increasing the number of T cells that can respond.

The highly polymorphic nature of the MHC implies that an allogeneic MHC molecule will differ from self MHC molecules at multiple amino acid sites. One theory of promiscuous recognition, entails that the peptide is ignored by the T cell receptor (TCR), allowing the TCR to recognize the MHC molecules themselves. Thus, each allogeneic MHC molecule can be recognized by a multiple T cell clones. However, there is evidence that TCRs recognize primarily intact peptides on MHC molecules, interacting with high specificity with each peptide MHC complex.[17] Thus, any cell may express multiple distinct peptide MHC complexes composed of different peptides bound to one or a few alleles of the foreign MHC molecules. As only a few hundred peptide MHC complexes are needed to activate a particular T cell clone, many different clones may be activated by the same allogeneic cell. This may explain the high precursor frequency of alloreactive donor T cells.

In haematopoietic cell transplantation, the principal antigenic targets of the T- cells of the graft, are the host MHC molecules if the patient and donor MHC molecules differ. However, for grafts matched at the MHC, mismatching of minor histocompatibility antigens (MHags) appears to underlie the development of GVHD. Because the manner in which a particular protein is processed is dependent upon genes outside of the MHC, two siblings, despite having identical MHC molecules, will have many different peptides in the MHC groove.[19] The identification of the particular peptides responsible for GVHD has been an area of intense research. It remains unclear how many such peptides behave as MHags antigens. Although up to 50 such antigens are estimated to exist in mice, the precise number in humans is unclear. Many potential MHags antigens exist in humans, but the actual number that may cause GVHD is probably limited. One such example is HA-1.Mismatching of HA-1 between donor/recipient pairs has been significantly associated with GVHD.[20, 21]

(b) Role of APCs

In the first stage of GVHD, the host conditioning leads to a loss of epithelial integrity, systemic exposure to microbial products and activation of the innate immune system. Toll like receptors on APCs, are sensitive to these microbial products, leading to a cascade of events that promotes maturation of dendritic cells (DCs). Very small numbers of host DCs and other APCs, surviving for the first few days after conditioning are able to prime donor T-cell activation and differentiation.[22] Naïve donor T-cells interact with host APCs, and are initially trapped within secondary lymphoid organs. There, they undergo rapid proliferation, and subsequently enter the circulation and travel to tissues such as gut and skin where they mediate GVHD.

Recipient APCs are essential for direct allorecognition, as seen when alloreactive donor NK cells kill recipient MHC class I mismatched DCs, protecting mice from GVHD.[15] The vital role of MHC Class I and II have been highlighted by a series of elegant knockout experiments. When donor CD8 T-cells were infused into a MHC mismatched mouse model lacking the $\beta2$ microglobulin (which is required for antigen presentation to occur through MHC class I), there was no GVHD. To highlight the role of donor APCs, the converse experiment was done, with $\beta2$ microglobulin deficient bone marrow infused in a mismatched setting. GVHD occurred but was less severe. This indicated that recipient APCs are required for GVHD to occur, but this may be enhanced by cross priming through engrafted donor DCs.[15] MHC class II deficient APCs were unable to elicit GVHD, in a CD4 dependent GVHD model, but GVHD occurred

when wild type APCs were infused. Thus recipient APCs are essential in the early stages post BMT to initiate GVHD, but then donor APCs participate later on to maximize the severity of GVHD.

(c) T cell Subsets in GVHD

T-cells are the main inducers of GVHD in humans, as their depletion prevents GVHD, as exemplified in haploidentical SCT.[15,23] CD4 and CD8 proteins are co-receptors for MHC class II and class I molecules, respectively. CD4+ cells appear critically involved in GVHD pathogenesis.[18,24] Following cognate interaction with activated APC, CD4^{+} T-cells are driven towards T-helper cell type 1 (Th_1)-biased cytokine production, promoting T-cell proliferation (IL-2) and further differentiation, so that very large amounts of pro-inflammatory cytokines are generated [particularly interferon γ (IFN-γ), TNFα], which induce tissue damage in a MHC-independent fashion. In contrast, donor CD8^{+} T-cells differentiate into efficient cytotoxic T lymphocytes (CTL), capable of causing host tissue damage in an MHC-I dependent fashion via their perforin/granzyme and FasL cytotoxic pathways.[23] Grafting of TH_2 polarized T-cells reduces GVHD severity and mortality.[25] However, both TH_1 and TH_2 pathways can contribute to GVHD pathogenesis, with TH_2 being particularly important in chronic GVHD. T-cells from mice deficient in signal transducer and activator of transcription 4 (STAT 4, a Th_1 associated molecule) and from mice deficient in STAT-6 (a Th_2 associated molecule) each induced distinct syndromes that were less severe, than those resulting from GVHD induced by wild type donor T-cells.[15]

(d) Role of Memory/Naïve T cells in GVHD

Peripheral T-cells can be divided broadly into 2 groups: T-cells that have never been activated by antigen (naïve T-cells), and antigen experienced T-cells (memory T-cells). Naive T-cells preferentially recirculate between blood and secondary lymphoid tissues, entering lymph nodes from the blood by crossing high endothelial venules. Naïve T-cells are characterized by the expression of CD45RA, CCR7, and CD62L+. These molecules allow naïve cells to home to lymphoid organs. [26] Upon antigen exposure naïve T-cells proliferate extensively, but lack immediate effector functions.

In contrast, memory and effector T-cells, unlike naive T-cells, can migrate efficiently into non lymphoid tissues and into sites of inflammation/ infection, subsequently entering afferent lymphatic vessels and travelling to local lymph nodes in the afferent lymph.[26] Central memory (CM) T-cells have lost the expression of CD45RA but retain CCR7 expression. They retain homing ability to lymphoid organs and display intermediate proliferative and effector capacities. Effector memory (EM) T-cells (CD45 RA-CCR7-) travel to inflamed tissues, where they proliferate poorly but have potent effector functions.[27]

Initially, naïve T-cells interact with APCs, and then proliferate and differentiate into effector T- cells, including memory T-cells. Several independent groups have intriguingly found that the naïve (CD62L$^+$) T-cells, but not memory T-cells (CD62L-) caused acute GVHD across different donor/recipient strain combinations in mouse models. [28] This may partly be explained by the ability of T-cells which are CD62 L+ to home to peripheral tissue. In HLA-matched donor pairs, only naïve CD45 RA + CD8 cells, and not memory CD45 RO+ cells demonstrated cytotoxicity to the allogeneic stimulators. Thus, the authors concluded that the minor histocompatibility alloreactive CD8 cells are found predominantly in the naïve compartment. [29] However, cytotoxicity is only one aspect of an alloresponse and other measures of alloreactivity were not tested. CCR7 deficient lymphocytes show impaired homing to lymphoid tissue and CCR7 antagonists have been shown to reduce murine chronic GVHD,[30] and in human BMT recipients, those who have received a high percentage of donor derived CD4+CCR7+ T cells (>73.5 % of CD4+ cells) in their grafts, showed earlier onset and more severe acute GVHD.[31] Thus, there is considerable evidence, particularly in mouse models that the majority of alloreactive T-cells reside in the naïve compartment.

The majority of the above data has been generated using mouse T-cells. The situation in humans may be more complex. Human memory T-cells (CD45RA-) cells proliferate and secrete IL-2 in response to alloantigen as well as naïve CD45RA+ cells, [31,32] though they had reduced cytotoxicity to alloantigen.[33] Additionally, transfer of memory T-cells and memory stem cells has been shown to promote GVHD[30,34,27] and GVHD is also more severe in male recipients of multiparous female donors, suggesting the transfer of donor memory cells.[35] Thus, in humans, the relative role of naïve and memory T-cells in the pathogenesis of GVHD is yet to be determined.

(e) T cell Cytokines

The presentation of alloantigen induces a response involving proliferation of donor T-cells and secretion of interleukin-2 (IL-2) and interferon γ (IFNγ) leading to the generation of T cytotoxic clones, as seen in Fig 2 .

IL-2 is a critical cytokine for T-cell survival, differentiation and proliferation and plays a major role in generation of T-regulatory responses. It is mainly produced by CD4+ T-cells and acts as a growth factor in an autocrine or paracrine manner promoting T-cell clonal expansion, differentiation into memory and effector T-cells, NK cell proliferation, and increased cytotoxic activity. Serum IL-2 receptor levels are elevated in acute GVHD.[36] The central importance of IL-2 signalling in GVHD is illustrated by the drugs that are used to combat it. Ciclosporin and FK506, the drugs most commonly used for GVHD prophylaxis, inhibit IL-2 production by inhibiting signalling through the T-cell receptor. Likewise, rapamycin and dacluzimab both of which inhibit signalling through the IL-2 receptor, show some efficacy in treating steroid refactory GVHD.[37]

Interferon gamma (IFNγ) activates macrophages to kill phagocytosed microbes, promotes the differentiation of naïve CD4+ T-cells to the Th_1 subset and inhibits the differentiation of Th_2 cells. IFNγ enhances the expression of MHC class I and II molecules and up regulates co-stimulators on APCs. Grafting of IFNγ deficient donor cells has been shown to lead to both accelerated and diminished GVHD depending on the conditioning regimen.[38] Thus, IFNγ appears to have both protective and pathogenic effects in GVHD. [39] One possible explanation could be due to differential effects of IFNγ on different organ systems. In a mouse GVHD model, donor derived IFNγ promoted acute GVHD by accelerating Th_1 differentiation and by augmenting inflammatory cytokine generation in the gut. However, donor derived IFNγ inhibited the development of idiopathic pulmonary syndrome by inhibiting donor cell migration and expansion within the lung and inhibited cutaneous GVHD.

One cytokine frequently implicated in the evolution of GVHD is TNF-alpha (TNFα). The secretion of TNFα in GVHD is related to endotoxin release from gut flora. Endotoxin is a well-known stimulus for TNFα production and release. Gnotobiotic mice, defined as animals free of pathogens, are protected from GVHD following BMT. Recolonization by gram-negative bacteria subsequently led to GVHD in these animals. It appears that damage to the

gastrointestinal tract from radiation and/or chemotherapy allows flora and endotoxins to enter the circulation leading to TNFα secretion from monocytes. Variations in donor or recipient genes that encode the pattern recognition receptor (NOD2/CARD15) affect the response of macrophages to bacterial toxins and can affect the incidence of gut GVHD.[40] The major cellular source of TNFα is activated mononuclear phagocytes, although antigen stimulated T-cell and NK cells can also produce it. TNF-α can (1) cause cachexia, which is a characteristic feature of GVHD; (2) induce maturation of DCs, thus enhancing alloantigen presentation; (3) recruit effector T-cells, neutrophils, and monocytes into target organs through the induction of inflammatory chemokines; and (4) cause direct tissue damage by inducing apoptosis and necrosis.[34] Donor T-cell derived TNFα has been shown to promote GVHD[18], especially in the liver and gut and is also required for GVL activity. [41] The presumed contribution of tumor necrosis factor-alpha (TNFα) to acute GVHD and the association of higher serum levels with severe GVHD[42] provide the rationale for the use of anti-TNFα antibodies (e.g. infiximab) in the treatment of GVHD[43].

IL-15 is produced predominantly by cells of the monocyte/macrophage, dendritic and stromal cell lineages and is critical for the survival of CD8 memory T-cells that are involved in GVHD. Administration of exogenous IL-15 worsens the severity of GVHD in mouse models and was associated with a dramatic expansion and activation of effector-memory CD8$^+$ T-cells.[44] Recently a new subset of CD4+ T-cells, which produce IL-17 (Th17 cells) has been characterised. These cells have been implicated in autoimmune diseases such as multiple sclerosis, and rheumatoid arthritis. Preliminary data suggests that they may have a role in acute and chronic GVHD (CD4+ T-cells from IL-17 knockout mice caused less GVHD than wild type) though further work is required to confirm this data.[45,46]

Genetic variants in cytokine secretion can also affect GVHD severity. IL-10 promotes tolerance by suppressing the release of inflammatory cytokines and inhibiting the activation of donor T-cells. Homozygosity for a common polymorphism of the IL-10 promoter increases IL-10 production and reduces the incidence of GVHD. [16]

(f) Leukocyte Migration

Donor T-cells migrate to lymphoid tissues, recognize alloantigens on either host or donor APCs, and become activated. They then exit the lymphoid tissues and traffic to the target

organs and cause tissue damage. The molecular interactions necessary for T-cell migration and the role of lymphoid organs during acute GVHD have recently become the focus of a growing body of research. However, interfering with T-cell trafficking may hinder T-cell immunity to leukaemia and viral antigens. Thus the challenge for developing leukocyte migration antagonists to prevent GVHD is selectivity.

Phase 3- Effector Phase

Effector mechanisms of acute GVHD can be grouped into cellular effectors [e.g. cytotoxic T cells (CTLs)] and inflammatory effectors such as cytokines. Perforin-dependent cytolysis and Fas-mediated apoptosis are the two important mechanisms for T-cell mediated cytotoxicity in GVHD. The principal mechanism of CD8+ CTL- mediated target cell killing is the delivery of cytotoxic proteins stored within cytoplasmic granules to the target cell, thereby triggering apoptosis of the target cells. These granules contain granzymes A, B, and C, perforin, and serglycin. The latter assembles complexes of perforin and granzyme. Perforin's main function is to deliver the granzymes into the target cell. Once inside the cell, the granzymes cleave substrates and trigger apoptosis. Mice which received perforin deficient T-cells develop all the signs of GVHD, but only after a significant delay in onset,[47] suggesting that perforin plays a role in the pathogenesis of GVHD. CTLs also kill targets by expressing FAS ligand (FasL) that binds to the death receptor Fas. This interaction activates caspases and leads to apoptosis of targets. Mice which received FasL defective T-cells develop severe cachexia, but only minimal GVHD-associated changes in liver or skin, suggesting that, FasL plays a particular role in GVHD affecting these target tissues. In a MHC class I-mismatched lethally irradiated murine model, wild type and either perforin deficient or FasL deficient CD8+ T-cells expanded early after transplantation, followed by a contraction phase in which the majority of expanded CD8+ T-cells were eliminated. In contrast, doubly deficient CD8+ T cells exhibited prolonged expansion, causing lethal GVHD.[48] Results in the doubly deficient animals suggest that both perforin and FasL play an important role in the regulation (i.e., contraction) of expanded alloreactive CD8+ T cells. Thus these molecules appear to have a dual role in GVHD.

Co stimulatory Interactions

(a) Co stimulatory Receptors

T-cells require two separate signals to enter the cell cycle:

- Signal 1 is antigen-specific and is provided by the engagement of the T-cell receptor with peptide complexed with MHC on the antigen presenting cell
- Signal 2 is provided by the interaction of one or more T-cell surface receptors with their specific ligands on the APC cell surface (co-stimulatory pathways).

Inadequate stimulation of these co-stimulatory molecules can lead to T-cell anergy and apoptosis. There are two major groups of co stimulatory receptors: the immunoglobulin super family including CD28 and inducible T-cell co-stimulator (ICOS) and the tumour necrosis factor super family including OX40, CD27, 4-1BB, CD30 and HVEM (herpes virus entry mediator). [25]

CD28 is constitutively expressed on 90 % of human CD4 cells and 50% of human CD8 cells. CD28 binds to CD80 and CD86 which are expressed on dendritic cells (see Fig 3 and Table 1). This interaction strongly amplifies weak T-cell receptor (TCR) signals, activates transcription factors, and accelerates intracellular vesicular trafficking. CD28 signalling stabilizes IL-2 mRNA and activates transcription factors NF-κβ and AP-1. These two effects increase IL-2 production by 100 fold. CD28 can promote enhanced activation and survival both of antigen presenting cells and T-cells.

In addition to these ligands that transduce a co-stimulatory or activating signal, cytotoxic T lymphocyte-associated antigen 4, or CTLA4, which also binds B7-1 and B7-2, provides an inhibitory signal. Although CD28 is expressed on resting T-cells, CTLA4 is expressed on the cell surface only after initial T-cell activation. CTLA-4 acts as a negative regulator of the CD28- CD80/86 signalling. It has a higher affinity for CD80/86 than CD28. CD28 signalling leads to increased expression of CTLA-4, which in turn negatively feeds back to inhibit the T-cell responses. The importance of CTLA4 is illustrated by the following observations:

- CTLA4 knockout mice develop massive lymphoproliferative disease, culminating in early death [49]

- The administration of blocking anti-CTLA4 monoclonal antibodies exacerbates autoimmune disease and prevents induction of T-cell anergy [50]

- Blockade of the CD28/CD80:86 interaction by administration of CTLA4-Ig greatly reduced lethal GVHD after haploidentical SCT in humans, and did not inhibit donor T cell engraftment.[30] However, CD28 is constitutively expressed on T-cells, so that targeting CD28 would be predicted to lead to extensive loss of desirable T-cells responses as well as alloreactive T-cells.

ICOS is present on resting T-cells but is up regulated on both CD4 and CD8 cells following T-cell activation and interacts with ICOS ligand which is present on dendritic cells, B cells and macrophages. [51] In mouse GVHD models, administration of ICOS deficient CD4 cells led to reduced GVHD morbidity and delayed mortality. These CD4 cells showed impaired effector functions, as characterized by reduced CD95 ligand expression, and lower levels of IFNγ and TNFα . However, the administration of allogeneic ICOS deficient CD8 cells led to increased expansion of these cells and increased cytokine release, due to impaired apoptosis of these T-cells.[52] Thus ICOS has paradoxical effects on the regulation of CD4 and CD8 alloreactive T-cells in GVHD.

OX40 (CD134) is a member of the TNF receptor super family, and interacts with OX40 Ligand, which is present on activated dendritic cells. OX40 ligation results in expression of CXC chemokine receptor 5 by activated T-cells, directing CD4+ T-cells to B cell follicles. In concert with that of 4-1BB and possibly CD30, OX40 ligation results in an increase in anti-apoptotic proteins, regulating effector persistence.[51] In mixed lymphocyte reactions OX40 has been shown to be strongly up regulated on human CD4+ lymphocytes.[53] Transfer of OX40 deficient T-cells into a lethal mouse GVHD model ameliorated GVHD compared to infusion of wild type T-cells.[54] However, in patients with acute GVHD, there was no increase in OX40 expression on T lymphocytes but there was increased OX40 expression during chronic GVHD, which declined with successful treatment.[55] More recently, OX40 has been shown to act as an inhibitory receptor on T-regulatory cells.[56] Co-transfer of T-regulatory cells to mice prevented lethal GVHD but pre-treatment of these T-regulatory cells with an agonist OX40 antibody, worsened GVHD. However, in view of OX40 and ICOS both being unregulated on activated T-cells, both would be reasonable targets to explore for allodepletion.
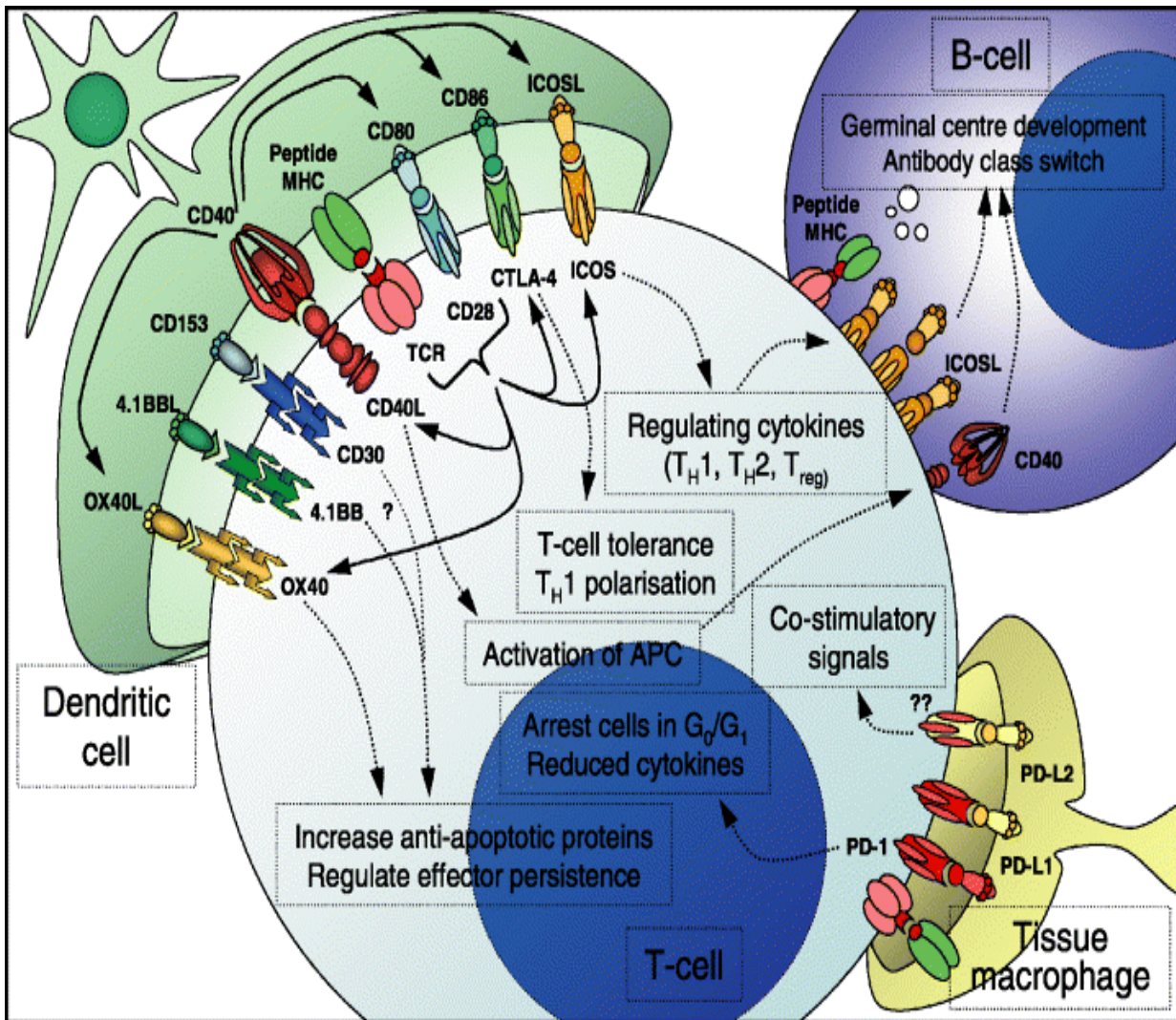
Figure 3: T cell/APC Co stimulatory Interactions. CD40 and CD28 amplify a cascade of secondary co-stimulatory pathways. Following initial TCR stimulation and CD28 ligation the expression of multiple other co-stimulatory receptors belonging to the immunoglobulin and TNF superfamilies is rapidly induced. In this way, CD28 can promote enhanced activation and survival both of antigen presenting cells and T cells, as well as B cell-mediated humoral responses. CD40 ligation results in increased ligand density of both CD80/86 and OX40L. OX40 ligation results in expression of CXC chemokine receptor 5 by activated T cells, directing CD4 T cells to B cell follicles, where the upregulation of ICOS by CD28 enables interactions favouring germinal centre development and antibody class-switching. OX40 ligation, in concert with that of 4·1BB and possibly CD30, results in an increase in anti-apoptotic proteins, regulating effector persistence. Expression of CTLA-4 is also enhanced by CD28 signalling, and favours downregulation of T cell responses and Th1 polarisation. Interactions of T cells with antigen presenting cells (APC) at sites of peripheral tissue inflammation via the PD-1:PD-L1/L2 system results in inhibitory signalling with reduced cytokine secretion and cell cycle arrest. The existence of a further co-stimulatory receptor exerting positive signalling in this system remains possible Reproduced from[51]

CD40 Ligand (CD154) is expressed primarily on activated T-cells and interacts with CD40 on antigen presenting cells. This interaction is important for antibody class switching and up regulation of CD80 and CD86 on APCs. Administration of anti-CD154 led to reduced acute and chronic GVHD in murine models, but significantly reduced GVL[30,57] Anti CD40 Ligand antibody has been shown *in vitro* to potently inhibit primary and secondary MLRs, by causing anergy.[58]

4-1BB (CD137) is up regulated on activated T-cells and NK cells and interacts with 4-1BB ligand which is expressed on APCs. CD137 is important in co-stimulation of CD8+ effector memory cells, and anti CD137 antibodies, inhibit CD4 and CD8 T-cell mediated GVHD.[30]. Hartwig *et al* demonstrated that CD8 CTL cultures, stimulated weekly with HLA-mismatched AML blasts [59] showed maximal expression of CD137 on day 1 after stimulation, with subsequent rapid downregulation. However, our method of identifying alloreactive T-cells (carboxyfluorescein succimidyl ester dye (CFSE) dilution), only detects these cells at later points in co-culture. Thus, we would be unable to characterize CD137 expression in the proliferating alloreactive T-cell population, and we therefore decided not to examine this marker.

Co-stimulatory pathway	Methods used to evaluate the role of the pathway	Results
CD28	CD28-deficient T cells, CTLA4–immunoglobulin fusion proteins, CD80- and CD86-specific antibodies, and CD80- and CD86-deficient mice	Model-dependent reduction in GVHD (more apparent for CD4$^+$ T cells)
HVEM	Soluble receptors and HVEM-specific antibody	Diminishes GVHD in Parent→F$_1$ model with sub lethal irradiation
ICOS	ICOS blockade and ICOS-deficient T cells	Promotes GVHD, effector maturation and T$_H$1-cell polarization
OX40	OX40L blockade and OX40-deficient T cells	OX40 promotes GVHD; CD4$^+$ T cell more than CD8$^+$ T cell
CD30	CD30 blockade, CD30-deficient T cells and CD30L-deficient recipients	Promotes CD4$^+$ T-cell-mediated but not CD8$^+$ T-cell-mediated GVHD
CD153 (CD30L)	Agonist antibody	Promotes CD4$^+$ T-cell-mediated and CD8$^+$ T-cell-mediated GVHD
PD1	PD1 blockade, PD1-deficient T cells	Inhibits GVHD
CD30L, CD30 ligand; CTLA4, cytotoxic T-lymphocyte antigen 4; GVHD, graft-versus-host disease; HVEM, herpes-virus-entry mediator; ICOS, inducible T-cell co-stimulator; OX40L, OX40 ligand; PD1, programmed cell death 1; T$_H$, T helper.		

Table 1:Co stimulatory Antigens and their actions Reproduced from[15]

(b) Activation Markers

Characterizing the phenotype of alloreactive T-cells is critical to enable rational approaches to allodepletion. Activated T-cells characteristically up regulate markers such as CD25, CD69, CD95, and HLA-DR.

CD25 is strongly expressed on proliferating alloreactive T-cells.[24,60] CD25 (IL-2 receptor) has three chains:α,β, and γ. Resting cells express CD25 composed of β and γ chains which bind to IL-2 with moderate affinity, allowing resting T-cells to respond to very high concentrations of IL-2. Upon activation of the T-cell, the α chain is also synthesized and this creates a receptor with a much higher affinity for IL-2. CD25 shows higher expression on T-regulatory cells than activated cells. Thus, any CD25 based allodepletion strategy will delete target T-regulatory cells. Binding of IL-2 by CD25 triggers cell proliferation and differentiation into effector cells.

CD69 is a type II integral membrane protein belonging to the family of C- type lectin receptors. It has been shown to form a complex and negatively regulates with sphingosine 1 phosphate recpetor-1 (S1P1). It acts downstream of IFNα/β to inhibit the egress of lymphocytes from lymphoid organs.[61] It is one of the earliest detectable cell surface markers detectable when resting lymphocytes are stimulated by mitogen. In MLRs, it has been detected at 24 hours, rising to a peak at 96 hours and then plateauing.[62]

CD95 (Fas antigen) is a 45 kDa cell surface type 1 membrane protein member of the tumour necrosis factor (TNF)/nerve growth factor receptor family. Once triggered by its cognate antigen, Fas ligand, Fas initiates a series of events that lead to apoptosis of the cell. This process involves the formation of the death inducing signalling complex, consisting mainly of Fas –associated death domain and caspases 8 and 10 proteins. Mutations in the Fas gene have been shown to be responsible for autoimmune lymphoproliferative syndrome (ALPS), a disorder characterized by autoimmunity, and lymphoproliferation.[63] Fas has been shown to be up regulated in mouse models of GVHD[64], and on CD8+ cells in patients with acute GVHD.[65] In MLRs Fas expression is low on Days 1-3 but then is up regulated rapidly peaking on day 5.[66] Susceptibility of lymphocytes to apoptosis in MLR cultures using anti Fas antibodies increases above baseline on Day 5 and peaks on day7.[66] Transfection of antigen presenting cells using Fas ligand has been shown in a mouse model to induce clonal deletion of antigen specific T-cells.[67]

CD71 is a type II transmembrane glycoprotein largely expressed on proliferating cells including proliferating T-cells where it is involved in iron homeostasis.[68] Iron is involved in essential cellular functions such as energy transport and DNA synthesis. Transferrin is the

main protein involved in serum iron transport and iron uptake is essentially dependent on receptor-mediated endocytosis, involving the transferrin receptor. CD71 is homodimeric receptor (180 kDa) which allows internalization of iron-bound transferrin in clathrin-coated pits. In endosomal vesicles, iron is then released by compartment acidification (pH 5–5.5), while apo-transferrin and CD71 are recycled into the blood or to the cell surface, respectively. CD71 expression is strictly regulated by intracellular iron level: CD71 mRNA is stabilized and abundant in iron-deficient cells to increase extracellular iron uptake. This post-transcriptional regulation is allowed by the presence of iron responsive elements (IRE) in the 3'-untranslated region of the CD71 transcript that are recognized by two iron regulatory proteins.

CD71 has been shown to be important in the pathogenesis of GVHD. Nguyen *et al*. demonstrated in a MLR[69] that there was strong up regulation of CD71 on alloreactive T-cells, peaking on days 7-9 with CD71 showing best correlation with T-cell proliferation. Transferrin is a critical growth factor for lymphocytes *in vitro*. [70] Proliferating thymocytes and lymphocytes express high levels of the transferrin receptor, and anti CD71 antibodies inhibit thymocyte and lymphocyte proliferation and differentiation *in vitro*. Administration of anti-CD71 antibodies have been shown in mismatched mouse model to significantly prolong pancreatic islet graft survival[71]. This was associated with a reduction in IL-2, IFNγ levels and a rise in IL-10. Drobyski et al demonstrated that administration of gallium (a metal that binds to transferrin, depriving proliferating cells of iron) potently inhibited MLRs and prolonged survival in a mouse model of GVHD.[72] The low level of CD71 expression on resting lymphocytes and its significant up regulation in a MLR make it an important marker to examine in allodepletion.

HLA-DR is a class II molecule and is up regulated on activated T lymphocytes in mixed lymphocyte cultures peaking between days 7-9.[69]

(c) Signal Transduction by the TCR Complex

Following T-cell activation, four major biochemical events occur within the cytoplasm. These include the following:

- Hydrolysis of membrane bound inositol phospholipid
- Increases in cytoplasmic calcium
- Tyrosine phosphorylation of a variety of proteins

- Increases in protein kinase C activity.

T-cell signalling pathways co-ordinately activate the transcription of genes that are silent in naïve T-cells. When T-cell activation occurs, lck, a src tyrosine kinase phosphorylates ITAM (immunoreceptor tyrosine based activation motifs) residues found on the CD3ζ and CD3 complex. This in turn leads to activation of another tyrosine kinase called Zap 70. This leads to activation of important downstream pathways such as Ras-Map kinase pathway, the protein kinase C pathway and the calcium-calcineurin pathway. These in turn lead to up regulation of transcription factors such NFAT (nuclear factor of activated T-cells), NF-$\kappa\beta$, and AP-1. These stimulate the expression of various genes involved in T-cell responses such- as IL-2.

Calcineurin dephosphorylates cytoplasmic NFAT, permitting its translocation to the nucleus where it binds to the IL-2 promoter sequence and then stimulates transcription of IL-2 mRNA. NF-$\kappa\beta$ is another important nuclear transcription factor that is activated by several different signals that include TNF, IL-1, and LPS. In the resting cell, this molecule is found in a heterodimeric form in the cytoplasm bound to inhibitors of $\kappa\beta$ (I$\kappa\beta$s). Signal induced degradation of the I$\kappa\beta$s frees the NF$\kappa\beta$, permitting it to enter the nucleus where it binds to its specific binding site. Transcription of a number of different genes including MHC class I, and IL-2 then ensues.

Phenotypic Characterization of Proliferating Alloreactive T cells

As outlined above alloreactive T-cells have been identified by a variety of phenotypic endpoints, including expression of activation markers/co-stimulatory molecules, cytokine secretion in response to alloantigen and proliferation. While alloreactive T-cells have multiple phenotypes, proliferation in response to alloantigens is their most basic hallmark. To monitor T-cell proliferation, T-cells can be labelled with carboxyfluorescein succimidyl ester (CFSE) dye. CFSE is a membrane permeable dye that binds to intracellular proteins, and upon cell division, the cell fluorescence decreases by half. Therefore by following the CFSE dim cells, one can analyse the dividing cells Using this technique, Godfrey et al[24] identified alloreactive T-cells flow cytometrically by co-culturing CFSE stained CD4+ cells with alloreactive PBMCs or dendritic cells. Two distinct populations, the CFSE Dim or dividing alloreactive T-cells, and the CFSE bright or non-dividing, non alloreactive T-cells were discernible. Dendritic cells were superior stimulators of alloreactive T cell proliferation than PBMCs as they were able to recruit a larger frequency of T-cells to divide in response to alloantigen. (Fig 4a, b)

No of Cells (vertical axis label for A, B)

CFSE Intensity →

CD25 (vertical axis label for C, D)

CFSE Bright CD25 +   CFSE Bright CD25-
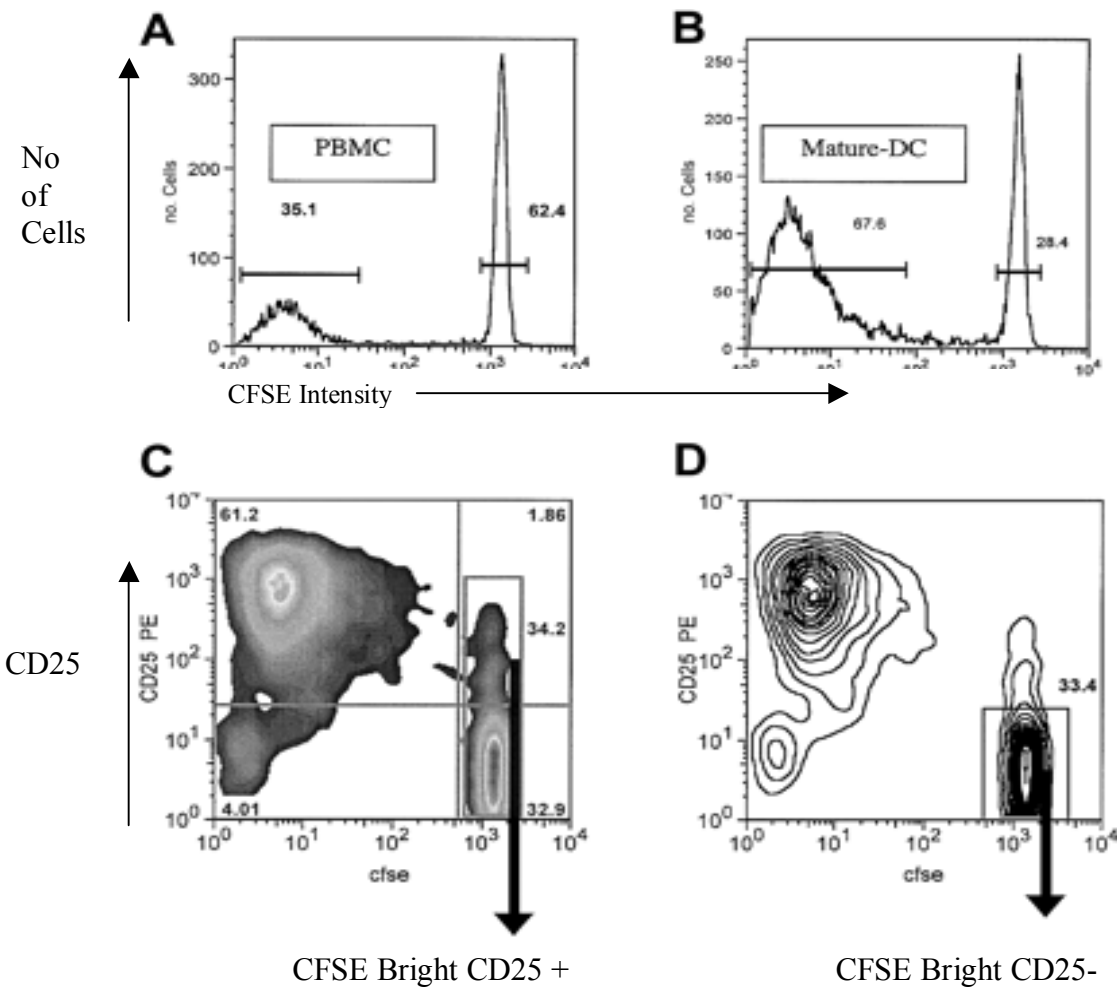
Figure 4: Clear separation of CFSE Dim and CFSE Bright in allogeneic MLRs. CFSE labelled CD4+ cells were co cultured with HLA mismatched PBMCs or dendritic cells. The no of CFSE Dim cells after 7 days in the DC co cultures was almost double that of the PBMCs co cultures. Thus, dendritic cells are superior in stimulating alloreactive T cell proliferation compared to PBMCs. The majority of CFSE Dim cells are CD25 + (C,D) There remain CD25- CFSE dim cells (Figure C). There are also CFSE Bright CD25+ cells. The nature of the latter are unknown. Reproduced from[24]

Thus, by labelling T-cells with CFSE, it is possible to identify alloreactive T-cells in a MLR, allowing us to investigate their phenotype. Furthermore, flow cytometric depletion of CFSE-dim T-cells almost completely abrogated *in vitro* alloreactivity in secondary MLRs and markedly reduced GVHD in an MHC Class II disparate murine model. FACS analysis of the CFSE dim population showed that this strongly expressed CD25 whilst the CFSE bright population had weak staining of CD25 (5 % of cells) (Fig 4c,d). It was unclear whether this latter population were anergic T-cells, regulatory T-cells or T-cells which were about to

divide. CFSE-based precursor frequency analysis indicates that approximately 5% (range, 3.1%-6.5%) of the CD4^{+} T-cells are recruited into cell division by the mature DCs which is significantly higher than that obtained by limiting dilutional assays.[18]

Infusion of CFSE bright T-cells into an animal model of GVHD led to prolonged survival compared to infusing CFSE dim or unmanipulated PBMCs[24]. However infusing CFSE Bright (non alloreactive T cells), into a mouse leukaemia model, did not support donor engraftment or enhance tumour control compared to a bone marrow, though it did reduce GVHD. [73] This suggests that depletion of alloreactive T-cells may compromise GVL responses, and that novel methods of preserving this are required.

Graft versus Leukaemia

The rationale for using immunotherapy to prevent and/or treat the re-emergence of malignancy is based in part upon the following observations

1. There is an increased relapse rate after syngeneic transplants (where there is no alloreactivity) or after T-cell depleted BMT [74]
2. Association between GVHD and a lower risk of relapse. Relapse is related to the HLA disparity between donor and host and the number of T-cells infused.[75]
3. The infusion of donor leukocytes into patients who have relapsed following an allogeneic HCT has directly resulted in a GVL effect, especially in chronic myeloid leukaemia (CML)

Donor lymphocyte infusions (DLI) can restore durable remissions in patients with haematological malignancies (particularly myeloid) who have relapsed post BMT. This effect is most impressive in chronic phase CML (cytogenetic response of between 70-80 % in chronic phase), EBV lymphoproliferative disease (LPD) and paediatric juvenile myelomonocytic leukaemia. DLI shows lesser degrees of activity in acute myeloid leukaemia (AML), myelodysplasia, myeloma, Hodgkin's lymphoma, chronic lymphocytic leukaemia, and Philadelphia positive acute lymphoblastic leukaemia.[76] The efficacy of DLI also reflects a complex equation between the nature of the leukaemia, the tumour burden, the kinetics of leukaemic cell growth, the potential for alloreactivity and the T-cell dose. Up to 50 % of patients experience significant GVHD with DLI, but a proportion do experience GVL without

GVHD, suggesting that GVL can be separable from GVHD, particularly when DLI are administered in a dose escalating fashion.[77-79]

The cell populations capable of recognizing and lysing malignant targets can be divided into two broad categories based upon the mechanism of cellular recognition: cytotoxic T-cells (CTLs) and natural killer (NK). T-cells do so in a HLA-restricted manner, and NK cells kill via the presence or absence of receptors such as KIRs. It is thought that T-cells mediate their GVL effect in three ways;[80]

A) Direct killing of leukaemia cells by perforin and granzyme attack from cytotoxic lymphocytes ($CD4^+$, $CD8^+$ and NK cells);

B) Apoptotic death through the Fas/Fas ligand pathway ($CD4^+$ and $CD8^+$ T cells),

C) Cytokine-mediated leukaemia cell death (e.g. IFNγ , TNFα) or control of proliferation (mainly $CD4^+$ cells)

It now appears that as well as initiating the alloresponse, $CD4^+$ T-cells are also critical in the effector phase of GVL. $CD4^+$ cells produce cytokines with a wide spectrum of biological activities: production of IL-2 and IL-12 recruits NK cells and $CD8^+$ T cells into the immune responses and augments their antitumor cytotoxicity. IFNγ and TNFα inhibit leukaemia cells directly and both cytokines up regulate MHC and Fas antigen expression rendering targets susceptible to cytotoxicity by T-cells. $CD4^+$ cells therefore have a role both as effectors and as orchestrators of the GVL response.

Targets for the GVL Effect

Cytotoxic T-cells (CTLs), recognize malignant cells as foreign when unique antigens are expressed by the tumor. CTL responses in GVL are directed against 3 broad classes of antigen:

- Minor histocompatibility antigens
- Tumour associated antigens
- Viral Antigens

(a) Minor Histocompatibility Antigens

One of the most important T-cell targets in GVL reactions are minor histocompatibility antigens. Minor histocompatibility antigens (mHAgs) are highly immunogenic peptides which

are presented in a HLA restricted manner. Polymorphisms in these mHAgs between donor and host can result in GVHD and GVL. GVHD can be seen in male recipients of HLA identical grafts from female donors, due to recognition of minor antigen (HY) encoded by the Y chromosome. Conversely, these mHAgs are responsible for graft rejection in female recipients of HLA identical grafts from male donors. Some mHAgs are present ubiquitously (e.g. HY), and thus CTLs directed against these antigens will result in GVL and GVHD. Others, such as HA-1 and HA-2 are only present on haematopoietic tissues, and thus should only elicit GVL responses, though in fact HA-1 mismatching has been shown to be associated with GVHD.[20]

HA-1 and HA-2 are both encoded by biallelic gene systems with one being immunogenic, and the other nonimmunogenic.[81] The immunogenic peptides encoded by HA-1^H and HA-2^V are presented on HLA-A2 molecules and then recognized by HLA-A2 restricted CTLs, whilst the non immunogenic peptides, are functionally silent due to poor presentation. Thus, while HA-1 and HA-2 are potential targets of immunotherapy, the applicability of this approach would be limited to patients who are HLA-A2 positive and who carry at least one immunogenic variant that have a donor who is homozygous for the nonimmunogenic allele. It is estimated that only 10-15 % of sibling transplants expose a GVL effect of HA-1 mismatching.[75] In one study where there was a HA-1/HA-2 mismatch between donor and recipient, 33 % of CTL clones generated after DLI were specific for HA-1 and HA-2. The other 67 % of leukaemia reactive CTLs were of unknown specificity.[75] This shows that the immune system reacts against a variety of antigens to eradicate leukaemic clones. A clinical vaccine trial with HA- 1 or HA-2 peptides after HSCT in HLA-A2+ve patients is currently in progress.

(b) Tumour Associated Antigens

Tumour associated antigens are those antigens that are expressed in cancer cells, but at low levels or not at all, in normal tissue. Whilst considerable effort has been made to identify tumour specific neoepitopes e.g. created by leukaemic fusion genes such as BCR-ABL, there is little evidence that such epitopes are processed and presented *in vivo* or that they are significant targets for a GVL response. However, an increasing body of evidence suggests that over expressed tumour associated antigens may be used as targets to augment GVL responses. Proteinase 3 is a tumour associated antigen and is over expressed in CML and AML. PR1 is a HLA-A2 restricted peptide derived from proteinase 3. PR1-specific CTLs have been shown to kill myeloid leukaemic colonies that over express proteinase 3, but not normal marrow cells. [82]

Increased numbers of high avidity PR1 CTLs have been seen in CML patients who develop cytogeneic remission in response to IFNα.[75] Low frequencies of PR1 CTLs are also found in healthy donors.[83]

Other tumour associated antigens include Wilm's Tumour Protein (WT1), which is over expressed in myeloid malignancies. WT1, a zinc-finger transcription factor, was initially described as a tumor-suppressor gene in childhood Wilms' tumor. WT1 is abundantly over expressed in most human leukaemia cells, including AML, CML and acute lymphocytic leukaemia, with higher levels associated with a worse prognosis. Leukaemia stem cells express between 10- to >100-fold more WT1 protein than normal $CD34^+$ cells. T-cells can distinguish this difference in protein expression, as $CD8^+$ CTLs generated against WT1 lyse leukaemic $CD34^+$ but not normal $CD34^+$ cells, and inhibit growth of leukaemic but not normal myeloid colonies.[84] Thus, like PR3, WT1 might serve as a useful target for adoptive T-cell therapy. Phase 1/II clinical trials using peptide vaccines with PR1 and WT1 in HLA-A2 patients with AML and CML are currently underway.[85] Of note, allodepletion strategies using LCLs, (which do not express myeloid antigens) as APCs, have been shown to preserve potential anti-myeloid tumor T-cell responses.[86]

(c) Viral Antigens

Latent EBV infection is associated with non-Hodgkin's lymphoma, Burkitt's lymphoma, NK cell lymphoma, lymphoproliferative disease, Hodgkin's lymphoma, and nasopharyngeal carcinoma, making adoptive T cell strategies targeting EBV a potential option. DLI and adoptive transfer of EBV-specific CTLs have led to remissions in post transplant lymphoproliferative disease and Hodgkin's disease.[76,87,88]

Improving Anti-leukaemic and Anti-viral Responses post Haploidentical BMT

As highlighted above, the requirement for rigorous T-cell depletion in haplo-SCT to avoid GVHD leads to a delay in immune reconstitution leading to a high mortality from viral infections/relapse. There have been numerous approaches to improving immune reconstitution after haplo-SCT. As the frequency of allo-reactive T-cells is logs higher than anti-viral/anti-leukemic T-cells, unmanipulated DLI cannot be used, without the risk of severe GVHD.[89]

Thus, there is a need to addback T-cells responsible for the GVL and anti-viral responses, but without allo-reactive T-cells that cause GVHD. There are several approaches to this problem:

(a) Adoptive Transfer of T cell precursors

(b) Alloanergisation

(c) Antigen Specific CTLs

(d) DLI transduced with suicide gene

(e) Allodepletion

(a) Adoptive Transfer of T cell Precursors

One way to minimize GVHD after adoptive immunotherapy is to transfer T-cell precursors rather than mature T-cells. These T-cell precursors will progress through the thymus, and the alloreactive T-cells will be deleted, allowing T-cell reconstitution but without GVHD. [90] Using this approach, mouse T-cell precursors generated *in vitro* from stem cells (isolated on the basis of culturing mouse stem cells on mouse stroma expressing Notch-1 ligand), were transferred to HLA mismatched mice after lethal irradiation and T-cell reconstitution was assessesed.[90] Compared to mice receiving stem cells alone, mice receiving T-cell precursors, showed enhanced thymic reconstitution and a rapid recovery of host-tolerant CD4 and CD8 populations with normal T-cell repertoires, cytokine secretion and proliferative responses to 3rd party antigen. These mice did not develop GVHD, reflecting depletion of alloreactive T-cells in the thymus, leading to tolerance. Significant GVL effects were seen in mouse lymphoma models that received these precursors and such mice had superior survival compared to control mice. This approach is attractive as it uses the host's thymic system to deplete alloreactive T-cells, but thymic activity in adults is limited and further studies on whether it is similarly possible to isolate and transfer human T-lymphoid progenitors under GMP conditions are required.

(b) Alloanergisation

Interest in the manipulation of the CD28:B7 pathway in transplantation has focused on the administration of CTLA4-Ig, which is a recombinant fusion protein that contains the extracellular domain of soluble CTLA4 combined with an IgG1 heavy chain. CTLA4-Ig has a higher affinity for the B7 molecules than CD28, resulting in blockade of the CD28-B7 interaction and hence T-cell anergy *in vitro*. Guinan *et al.* used this approach by co-culturing T-replete donor marrow cells with recipient mononuclear cells *ex vivo* in the presence of CTLA4-Ig.[91]. 12 patients with predominantly high risk malignancies underwent haploidentical transplants using alloanergised grafts. All patients received at least 10^7 CD3+ cells/kg with the bone marrow i.e. 3 logs more than routinely transferred with a haploidentical graft. The precursor helper T-cell frequency against recipient stimulators was reduced by between 1-3 logs, but third party responses were not significantly reduced. All patients engrafted. The incidence of GVHD was impressively low given the number of T-cells infused, as only three of the eleven evaluable patients developed acute GVHD ($\geq$ grade 2), and only one went onto develop chronic GVHD. There were four deaths due to infection (2 from aspergillus, 1 bacterial, 1 from toxoplasmosis). 5 of the 12 were alive at the time of analysis. The low survival rate was partly due to the high risk cohort, many of whom had been heavily pre-treated. However, effect of alloanergisation on desirable anti-viral/ anti-leukaemic responses was not formally assessed and it is possible that the high rate of infectious deaths reflects a negative effect of CTLA-4 Ig on bystander T-cells responsible for such responses. Further, anergy can be reversed e.g. by addition of exogenous IL-2 so that it is possible in the appropriate cytokine milieu, anergised alloreactive T-cells could cause GVHD. Unfortunately, further clinical studies were not possible since a GMP CTLA4-Ig is not available. However, clinical studies using newly developed similar agents are shortly to begin in The US.

(c) Antigen Specific CTLs
The feasibility of adoptive immunotherapy to improve immunity post –HSCT has been demonstrated by studies where *ex vivo* generated CTL, directed against viral antigens have been infused post HLA- matched SCT.

1. Adoptive Immunotherapy for EBV

By repetitively stimulating PBMCs with lethally γ-irradiated EBV-transformed autologous B lymphoblastoid cell line (LCL), EBV-specific CTL lines can be generated *in vitro* which recognize the immunogenic type 3 antigens, chiefly EBNA-3. Using such an approach, Rooney *et al* infused retrovirally marked donor-derived EBV CTLs. After infusion of these CTLs, there was a 4 log rise in the precursor frequency of EBV CTLS in the patients' blood, and the EBV viral load decreased by 2-4 logs. None of these patients developed EBV post transplant lymphoproliferative disease (PTLD), compared to 11 % in historical control group. No GVHD occurred following the infusion of these CTLs, and the neomycin gene could be detected up to 7 years after the infusion, indicating long term persistence of these CTLs.[92] A number of other groups have replicated this data and over 100 patients have been treated with EBV CTLs post HSCT to date. The results show clearly that prophylactic infusion of donor-derived EBV CTLs is safe and effectively prevents PTLD in HSCT patients at high risk of this complication. Smaller numbers of patients with established PTLD have also been treated and this too appears effective, although sometimes associated with significant local inflammatory reactions at the site of disease. The major limitation of this approach is the time taken to generate CTLs (10 weeks) and the resources needed to do this under GMP conditions. Together with the apparent efficacy and simplicity of pre-emptive therapy with rituximab to prevent PTLD, this has limited the broader use of this approach.

2. Adoptive Immunotherapy for CMV

A number of studies have investigated whether adoptive immunotherapy with CMV CTLs [93] can be used to prevent opportunistic CMV infection after allogeneic HSCT. [94] [95,96] Initial studies with CD8+ CTL clones,[93] demonstrated that this approach could be used to safely augment CMV-specific immunity post-SCT but this approach was extremely laborious and responses were poorly maintained in the absence of CD4 T-helper cells. Subsequently, shorter culture protocols utilizing CMV-lysate pulsed dendritic cells or monocytes as stimulators have been used to generate polyclonal CTL lines, comprising both CD4 and CD8 populations.

Infusion of CMV CTLs into 16 patients (matched related/unrelated BMTs who were CMV PCR positive), [94] after the first detection of CMV viraemia, led to a massive *in vivo* expansion of CMV CTLs, (3-5 logs), within days of adoptive transfer with low rates of GVHD and

secondary reactivation. Similar results were obtained by Einsele *et al* [95]. These studies demonstrate adoptive immunotherapy with CMV-specific CTLs to be feasible, safe and provide evidence that this approach augments *in vivo* cell-mediated immunity to this virus. However, demonstration of clinical efficacy in preventing CMV associated complications or secondary reactivations will require much larger randomised studies.

Whilst the 4 week period of co-culture required for the generation of CMV-specific CTLs by *in vitro* repetitive stimulation, enables CTLs to be generated pre-emptively in response to viral reactivation, the existing culture protocols are too complex and laborious for use outside highly specialised centres. For adoptive immunotherapy to be more broadly applicable, simpler, robust and more rapid protocols for isolation of viral-specific T-cells are needed. One novel approach to isolating CMV-specific T-cells exploits the secretion of IFNγ by both CD4+ and CD8+ T-cells following stimulation with purified pp65, the immunodominant antigen of CMV. Virus-specific T-cells can then be selected using the immunomagnetic beads (the so called 'γ-capture system'). Preclinical studies have demonstrated that it is possible to select CMV-specific T-cells with reduced alloreactivity using this system[97] and clinical studies utilising this approach are now underway.

In an alternative approach to simplify the generation of CMV-specific CTLs, Moss *et al* developed a technique of selecting CMV CTLs by staining with HLA- peptide tetramers, followed by magnetic bead selection. [98] CMV-specific CD8+ cells were infused directly into nine patients within 4 h of selection. CMV-specific CD8+ T cells became detectable in all patients within 10 days of infusion, and were persistent in the patients studied. CMV viremia was reduced in every case and eight patients cleared the infection, including one patient who had a prolonged history of CMV infection that was refractory to anti-viral therapy. Though this approach significantly shortened the time to infusing CMV CTLs, it is limited to donors who have the appropriate HLA type, and the durability of such responses in the absence of CD4 + helper T-cells remains to be determined.

3. Adoptive Immunotherapy for Adenovirus

Adenoviral infections are significant causes of morbidity and mortality after HSCT, especially after T-depleted haploidentical transplants. Though adenovirus specific-CTLs have

been generated by repetitive stimulation of donor-T cells with APC transduced with an adenoviral vectors[89], there has only been one clinical trial of adenovirus specific-CTLs infused post HSCT, which used the IFNγ capture system to isolate adenovirus (ADV)-specific T-cells. Though highly specific antigen responses were demonstrated *in vitro* when the expanded adenovirus- specific T-cells underwent restimulation with ADV-pulsed target cells there was still significant anti host reactivity.[99] Using this approach in mismatched unrelated donor HSCTs, Feutchinger *et al*, infused adeno-specific T-cells (doses 10^3/kg) into 9 paediatric patients who were adenovirus PCR positive[100]. In 5 patients, clearance of the virus was associated with adenovirus T-cell expansion but one patient developed GVHD. Concurrent use of anti-viral agents in this study, and any possible recovery of donor derived adenovirus-specific CTLs, independent of infused CTLs, makes it hard to determine the efficacy of this approach. Further studies are needed to demonstrate the safety and efficacy of this approach, particularly in the context of haploidentical HSCT.

4. Adoptive Immunotherapy for Aspergillus

By repetitively stimulating donor APC pulsed with CMV/aspergillus antigens, T-cell clones to aspergillus and CMV have been generated.[101] T-cell clones which reacted to host antigens were discarded but non reactive clones were pooled and infused post haploidentical BMT. In 46 control transplant recipients who did not receive adoptive therapy, pathogen-specific T-cells occurred in a low frequency and displayed a low interferonγ /high interleukin-10 production phenotype. In the 35 recipients who received a single infusion of CMV/aspergillus T-cells (dose range of 10^5 to 10^6 cells/kg) there was a high-frequency of T-cell responses to pathogens, which exhibited a protective high interferon-γ/low interleukin-10 production phenotype within 3 weeks of infusion. Frequencies of pathogen-specific T-cells remained stable over time, and were associated with control of Aspergillus and cytomegalovirus antigenemia and infectious mortality.[101] However, one patient who received 3 x 10^6 /kg T cells developed GvHD, and generation of antigen specific clones is highly labour intensive and required prolonged culture thus limiting this approach to prophylactic infusions in high risk patients.

5. Trispecific CTLs

Leen *et al* infused trispecific CTLs (recognising CMV, EBV, adenovirus), to adult and paediatric patients undergoing matched unrelated or related donor transplants without any significant toxicities or GVHD.[102] These CTLs were generated by repeatedly stimulating donor PBMCs with EBV LCLs transduced with a chimeric adenovirus CMVpp65 vector. The CMV and EBV CTLs expanded post infusion, but the adenovirus CTLs only expanded in those patients who had adenoviral infection. Infusion of these CTLs led to a reduction in viral DNA to all three viruses in patients with infection, resolution of adenoviral pneumonia in one case, and was associated with enhanced viral immunity in IFNγ ELISPOT assays. However, *in vitro* IFNγ release and cytotoxicity responses were far higher for CMV responses that adenovirus. This may reflect competition among the various immunogenic components leading to a single viral antigen dominating the immune response. This study demonstrated that it is possible to generate effector T-cells against multiple pathogens in a single co-culture However, as with the above approaches, the generation of trispecific CTLs involves prolonged cell culture (10 weeks) and it is too labour intensive to enable this approach to be routinely used in large numbers of patients. In contrast, allodepletion has been shown to provide anti-viral responses to all three viruses and is simpler, and more robust than such approaches.

(d) Donor Lymphocyte Infusion and Suicide Gene therapy

A suicide gene codes for a protein able to convert a non toxic prodrug into a toxic product. Thus, the transfer of a suicide gene into donor lymphocytes can allow the selective elimination of transduced lymphocytes should GVHD occur. The most effective current suicide gene is the Herpes simplex thymidine kinase (HSV-TK). The HSV-TK protein converts ganciclovir to a phosphorylated compound which is toxic to cells, by inhibiting DNA chain elongation. A phase 1/2 clinical study of adoptive transfer of HSV-TK transduced donor T lymphocytes at doses of 10^5/kg-10^8/kg to patients affected by disease relapse after matched related or mismatched related HSCT demonstrated [103,104] that 35 % or patients achieved a complete remission, and 29 % a partial response. The anti-tumour effect was strongly correlated with the *in vivo* expansion of TK+ cells. Administration of ganciclovir led to complete elimination of the TK+ cells and successfully treated GVHD.

Although these preliminary studies suggest that this approach can safely be used improve immune reconstitution after SCT, genetic modification of T lymphocytes with retroviral

vectors requires polyclonal activation which may compromise affect third party and antiviral responses, particularly to EBV.[27,76] Treatment of CMV infection with ganciclovir also led to the elimination of HSV-TK T-cells. 3 of 17 patients died of viral infections after day 100, suggesting that this approach may affect the anti-viral immunity of the transduced lymphocytes.[105] Furthermore, this approach was limited by host immune responses to the HSV-TK, which occurred in a number of cases, [104,106] resulting in elimination of the transduced T-cells.

The above trials have used a retroviral vector system which relies on preactivation of T-cells with CD3/28 stimulation to allow efficient entry of the viral vector into the T-cells. However this preactivation impairs anti-viral and third party activity of the transduced T-cells. [107,108] Lentiviral vectors may serve as an alternative because they can transduce non cycling cells. T-cells can be induced in G1 phase by stimulation with IL-7, and transduced within 48 hours. A shorter culture time with less expansion, preservation of the naïve phenotype and unskewed TCR repertoire would favour maintenance of T-cell function. Lentiviral vectors may also reduce the risk of insertional mutagenesis because of an alternative insertion profile.[109] Qasim *et al* developed a lentiviral HSV-TK construct for transduction of T-cells in a cytokine culture without preactivation of the T-cell receptor. In contrast to polyclonally activated T-cells, efficient transduction was obtained with preservation of anti-viral [107], and third party responses and there was minimal up regulation of T-regulatory numbers.

In view of the immunogenicity of the HSV-TK, alternate suicide genes have been investigated. The most promising of these utilises a chimeric protein consisting of caspase 9 fused to human FK506 binding protein (FKBP) which appears not to be immunogeneic. Adding the drug AP1903, dimerizes the chimeric protein and leads to cell death. [110] Brenner *et al* transduced CD25 immunotoxin allodepleted donor T-cells with a retroviral construct consisting of an inducible caspase 9 (iCasp9) suicide gene, and a selectable marker (truncated CD19). The residual donor T-cells were efficiently transduced, expanded, and subsequently enriched by CD19 immunomagnetic selection to >90% purity. These engineered T-cells retained anti-viral specificity and functionality, and contained a subset with regulatory phenotype and function. Activating iCasp9 with a small-molecule dimerizer rapidly produced >90% apoptosis. However, concerns remain about the basal toxicity of iCasp9 to T-cells in the absence of dimerizer and this approach has not yet been tested clinically.

An alternative approach to safely administering DLI in the haploidentical setting involves infusing regulatory T-cells (Tregs). T-regs are functionally defined as T-cells that inhibit immune responses by influencing the activity of other cell types. High CD4 (+) FOXP3 (+) T-cell counts in the donor graft have been associated with a reduced risk of GVHD. [111] In a mouse leukaemia model, transfer of donor Tregs suppressed the proliferation of alloreactive donor T-cells, without affecting GVL effects. [112] This was associated with a reduction in the expression of CD25 on alloreactive T-cells, reduced proliferation of donor T cells, and reduced serum levels of IFNγ and TNFα. Transfer of Tregs in established GVHD was less effective, suggesting that early transfer is required for an optimum effect. This data suggested that GVHD is a cytokine dependent process highly sensitive to T regs activity, whilst the anti-leukaemic activity mediated by the perforin lysis pathway was relatively insensitive to the effect of T regs.

However, whilst this data looked very promising it is unlikely that such a clear delineation in humans is possible. The major risk of the therapeutic use of T regs is generalised immunosuppression compromising desirable anti-viral and anti-leukaemic responses. Whilst the induction of T regs occurs after specific activation, they then appear to suppress proliferation of all T-cells regardless of their antigenic specificity.[113] The high ratio of T regs: effector T cells used in mouse BMT studies (1:1) above, would not be feasible clinically for adoptive transfer without *ex vivo* expansion. This is compounded by the fact that the best marker of human T regs is an intracellular marker FoxP3, making cell sorting to obtain a pure Treg population difficult. The long term survival of *in vitro* selected and expanded Tregs *in vivo* is not known.[113] Nevertheless, the Stanford group under Robert Negrin are planning a clinical study looking at adoptive transfer of Tregs + DLI post SCT.

(e) Selective Allodepletion

Allodepletion strategies aim to selectively deplete alloreactive T-cells after stimulation in an *ex vivo* mixed lymphocyte reaction (MLR) and then infuse the residual donor T-cells with the aim of improving anti-viral and anti-leukaemic responses post-SCT. The advantages of this approach are that alloreactive T-cells are permanently removed, and that it has the potential to improve immunity to multiple pathogens simultaneously. Strategies to deplete alloreactive T-cells include negative selection of donor T-cells expressing activation markers (e.g. CD25,

CD69, HLA-DR)[114-118], killing activated T-cells by photodynamic purging[52], inducing Fas mediated apoptosis[119], or chemotherapy agents.[120,121]

1. Allodepletion Targeting Activation Markers

(a) CD25 Based Allodepletion

CD25 is highly expressed on alloreactive T-cells and there have been a variety of methods targeting this antigen, including immunotoxins or immunomagnetic depletion. RFT5-SMPT-dgA is a CD25 immunotoxin consisting of a mouse IgG1 anti CD25 conjugated via a hetero-bifunctional crosslinker to a chemically deglycosylated ricin A chain (dgA). Depletion of CD25+ alloreactive T-cells by the addition of this immunotoxin led to a greater than 98% reduction in residual proliferation to host cells in the HLA mismatched setting whilst retaining proliferative responses to CMV, and Candida, and cytotoxic T-lymphocyte precursor (CTLp) frequencies against CMV/EBV infected targets.[122] Furthermore precursor frequencies of anti-leukaemic T cells against myeloid leukaemias were not reduced with use of this immunotoxin. [122,123] Differences in the techniques used, such as the type of stimulator cells used and the methods used for assaying residual alloreactivity against host (see Discussion) may account for the variability observed in residual anti-host responses after CD25 based allodepletion.

Vitetta *et al.* compared the effectiveness of 2 CD25 immunotoxins RFT5-SMPT-dgA and Ontak, (a fusion protein of IL-2 and diphtheria toxin), and anti CD25 microbeads, in their ability to delete CD25+ alloreactive T cells. [124] They showed that RFT5-SMPT-dgA, CD25 beads or a combination of RFT5 and CD25 beads were equally effective in depleting alloreactive CD4 + CD25 expressing T-cells without affecting third party responses. Ontak however, depleted CD4+CD25+ cells poorly. RFT5-SMPT-dgA was more effective than CD25 beads or Ontak at depleting CD8+CD25 expressing alloreactive T cells. Ontak again was poor at depleting CD8 + CD25 + alloreactive T-cells. These data suggest that CD25 depletion with RFT5 immunotoxin or immunomagnetic beads is superior to Ontak, but it should be noted that functional analysis of residual alloreactivity after secondary stimulation of allodepleted cells was limited in this study.

CD25 is expressed on T regulatory cells, and thus one potential concern is that, by depleting the T regulatory cells, this would lead to an increase in GVHD and autoimmunity. This has not been seen in clinical studies to date (see below), probably because effective allodepletion, leads to a low incidence of alloreactive effector cells, and therefore concomitantly depleting T regulatory cells in this situation will not lead to GVHD.

One critical issue for all allodepletion strategies targeting activation markers is the choice of host antigen-presenting cells: if antigen presentation is ineffective this will result in less activation and hence less effective depletion of host-reactive T-cells. Our group has previously demonstrated that using HLA mismatched EBV transformed lymphoblastoid cell lines (LCL) as antigen presenting cells rather than PBMCs, gave a more consistent depletion of *in vitro* alloreactivity using the CD25 immunotoxin (CD25IT).[86]   There was an average 15 fold decrease in proliferation in primary MLRs using this approach (see Fig 5B) and a residual proliferation to host was significantly lower (mean 0.8 %) after stimulation with LCL than PBMCs (mean 8.5 %) in the same donor-recipient pairs (Fig 5C).

A

CD3/25   2.8%   11.3%   0.88%

CD4/25   2.5%   9.2%   0.58%

CD8/25   0.19%   2.26%   0.4%

Donor PBMC   Donor PBMC +   Donor PBMC+
alone   host LCL   Host LCL + IT

B

C
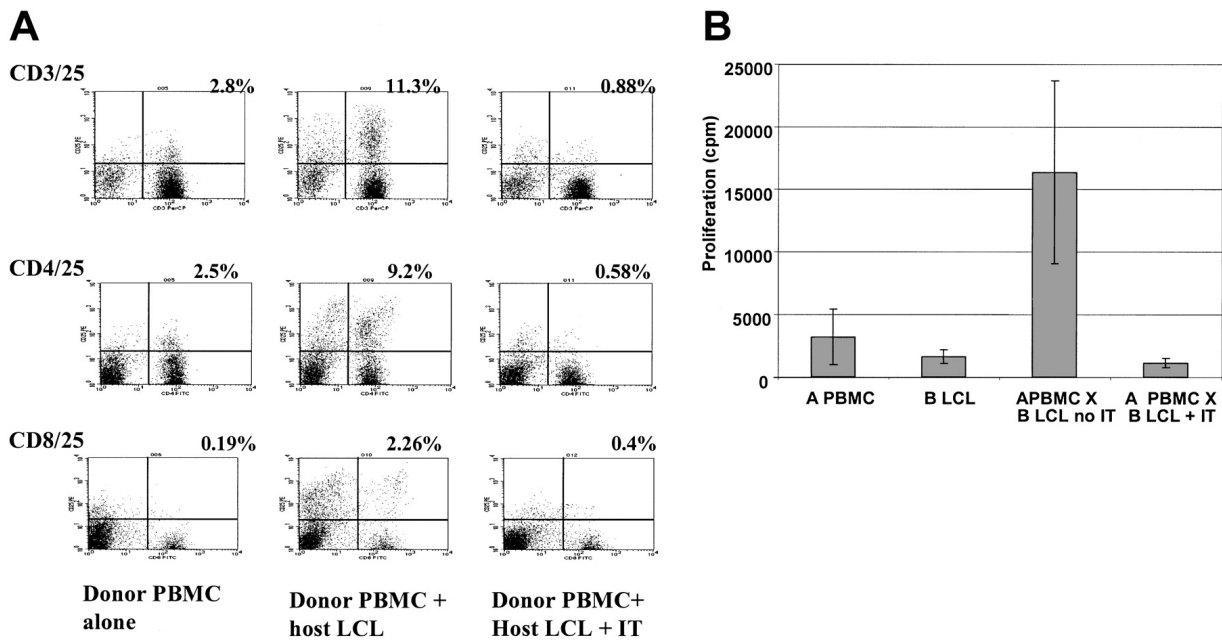
	Responder							
Stimulator	1	2	3	4	5	6	7	Mean ± SD
Allo-PBMCs	0	14.8	0	27.4	0	17.4	0	8.5 ± 11.3
Allo-LCLs	4.8	0	0.7	0	0	0	0	0.8 ± 1.8

Figure 5: **Depletion of alloreactive T cells by anti-CD25 immunotoxin.** (A) FACS analysis showing increased expression of CD25 (y axis) on CD3/4/8$^+$ T cells after activation with HLA-mismatched LCLs and effective depletion of CD3$^+$/CD25$^+$, CD4$^+$/CD25$^+$, and CD8$^+$/CD25$^+$ cells following treatment with anti-CD25 immunotoxin. The figure shows a representative FACS analysis from 6 different donor-recipient pairs. The percentage of double-positive cells is indicated. (B) Primary mixed lymphocyte reaction showing a mean 15-fold decrease in proliferation in response to HLA-mismatched LCL stimulators after treatment with anti-CD25 immunotoxin (IT). Results are the mean ± SD of 5 haplo-identical donor-patient pairs each assayed in triplicate. (C) Residual proliferation in 7 donor-patient pairs after allodepletion with anti-CD25 immunotoxin following stimulation of donor PBMCs with PBMCs (R/S 1:1) or LCLs (R/S 40:1) from the same HLA-mismatched recipient. Residual proliferation was calculated using the formula in "Results" and was significantly higher after stimulation with PBMCs ($P < .05$). Reproduced from [86]

As relapse and viral reactivation are the major causes of mortality post haploidentical transplantation, determining whether T-cell responses to such antigens following allodepletion is of paramount importance. Using CD25 immunotoxin based allodepletion, Amrolia *et al* demonstrated that following allodepletion, anti-viral responses to adenovirus and CMV following allodepletion were preserved on HLA tetramer (see Fig 6) and IFNγ ELISPOT

assays.[86] The use of LCLs as APCs, led to a partial reduction in EBV-specific responses, but significant activity was retained through the non shared haplotype.
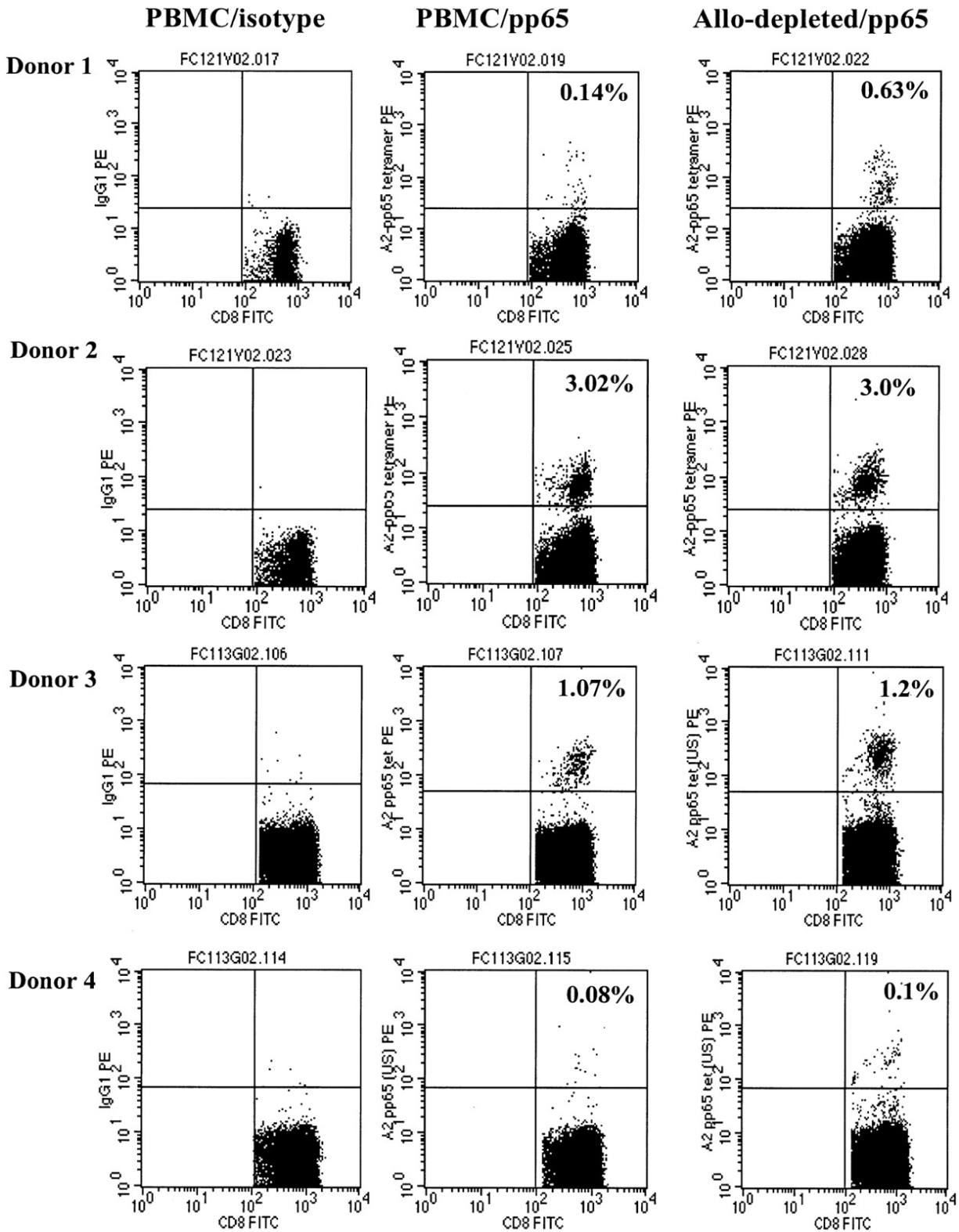
Figure 6 : CMV-specific CD8$^+$ T cells are not deleted by allodepletion. The figure shows FACS analysis following staining of either unmanipulated PBMCs (right and centre columns) or allodepleted cells (left column) from 4 HLA-A2–positive, CMV-seropositive donors with IgG PE (left column) or an HLA-A2–CMV pp65 tetramer (centre and right columns). The percentages of tetramer-positive cells as a proportion of CD8$^+$ cells with isotype subtracted are shown. Reproduced from [86]

As LCLs do not express myeloid or leukaemic antigens, allodepletion using this approach did not affect the frequency potential myeloid tumour antigens such as the PR1 epitope of proteinase 3. (See Fig 7) Thus, CD25IT based allodepletion preserved anti-viral responses *in vitro* to CMV, adenoviral antigens and potential myeloid tumour antigens.
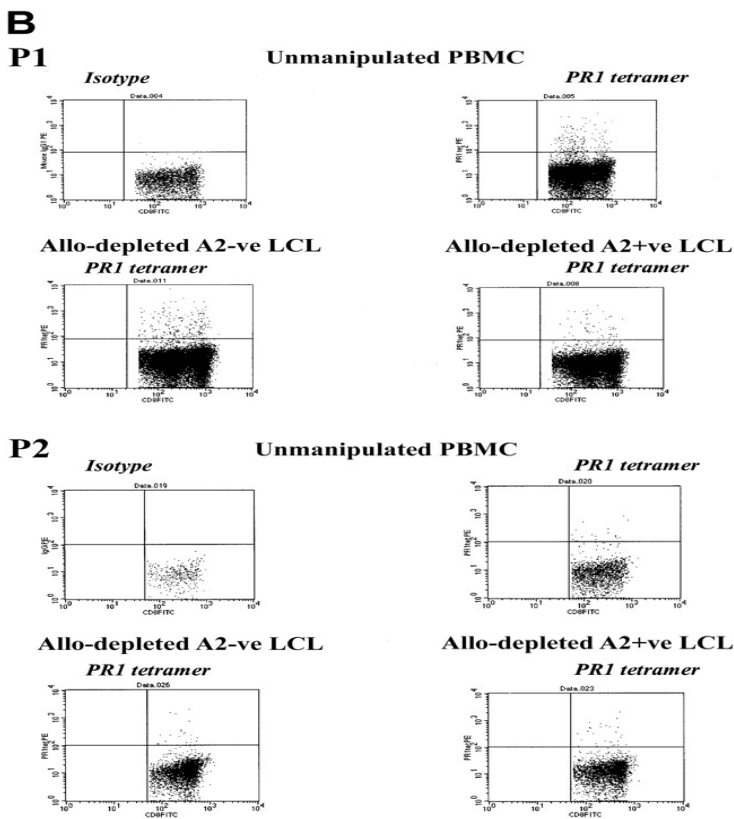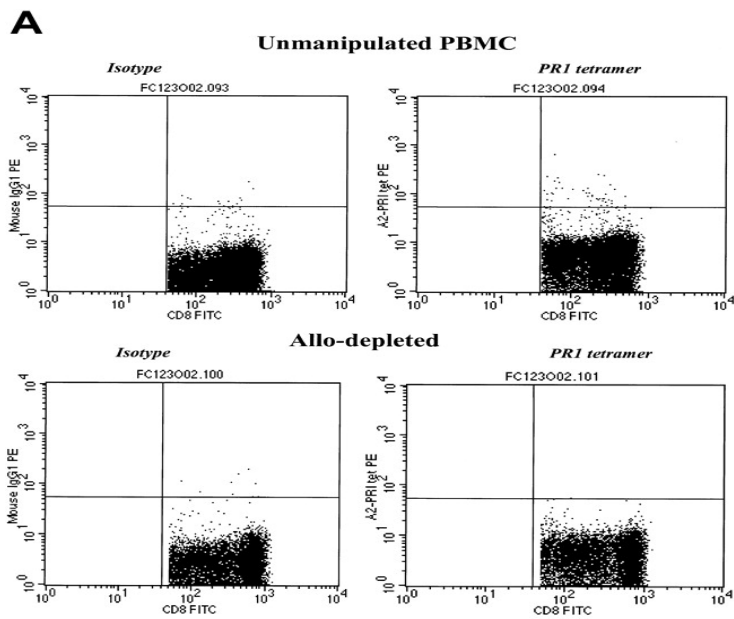
Figure 7: CD8⁺ T cells specific for the myeloid tumor epitope PR1 are retained after allodepletion after stimulation with mismatched LCLs but not CML PBMCs. (A) FACS analysis following staining with HLA-A2–PR1 tetramer of unmanipulated PBMCs (top row) or allodepleted PBMCs (bottom row) from a patient with CML. In each case isotype controls are shown on the left and tetramer-stained cells on the right. Allodepletion was performed after stimulation with allogeneic HLA-A2–positive PBMCs from a mismatched donor with CML. (B) FACS analysis following staining with HLA-A2–PR1 tetramer of unmanipulated PBMCs or allodepleted T cells from 2 HLA-A2–positive patients with CML. Allodepletion was performed after stimulation with either HLA-A2–positive or –negative LCLs. The percentages of tetramer-positive cells as a proportion of CD8⁺ cells (isotype subtracted) are shown. Reproduced from [86]

Despite almost complete depletion of CD25+ve T-cells with CD25IT based allodepletion, residual alloreactivity against host stimulators was often detectable in the allodepleted T-cells. Rested CD25IT generated allodepleted T-cells demonstrated a median 17-fold reduction in IFNγ release in an ELISPOT assay, when stimulated with host antigen-presenting cells compared to unmanipulated PMBCs,[125] but there were still significant residual responses to host in 7/12 donor recipient pairs (see Fig 8). This suggests, that combining CD25-based with alternative methods of allodepletion may be necessary to enhance allodepletion and hence enable higher T-cell doses to be infused in order to improve anti-viral and anti-leukaemic responses.
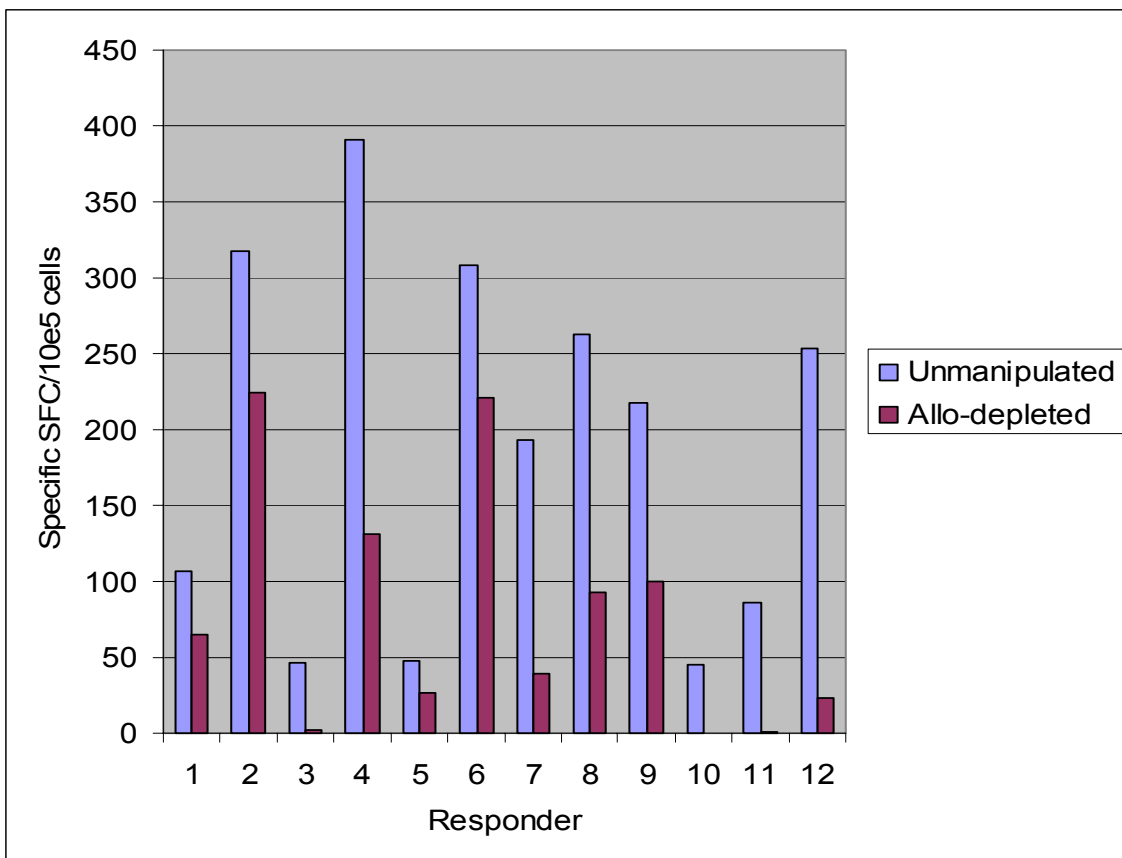
Figure 8: Significant Residual IFNγ Responses to Original Stimulator remain in many donor recipient Combinations despite CD25IT Allodepletion. Normal donor PBMCs were co-cultured with HLA mismatched LCLs and a day 3 CD25 IT allodepletion. In 12 donor recipient pairs, on day 5 thawed unmanipulated PBMCs or rested CD25IT allodepleted PBMCs were stimulated with host LCLs in an IFNγ ELISPOT assay. Results are expressed as Spot forming cells SFC/10^5 PBMCs. Reproduced from [125]

(b) Non CD25 based Allodepletion

CD69 is one of the earliest markers to be up regulated in a MLR (within 24 hours). A number of groups have targeted CD69 to deplete alloreactive T-cells and have demonstrated a mean residual alloreactivity of between 1.5 % to 25 % with maintenance of third party responses after CD69 allodepletion.[116,117] Anti CMV and EBV activity and responses against potential tumour antigens (e.g. WT-1) were maintained in the allodepleted fractions. Furthermore, unlike CD25 based allodepletion, CD69 allodepletion does not affect T regulatory cells.[126]. Koh *et al* demonstrated that infusion of CD69 allodepleted donor T-cells led to significantly enhanced survival compared to unmanipulated donor T-cells in a MHC mismatched mouse model.[127]

This has encouraged investigators to examine combined anti CD25/69 depletion, with the aim of enhancing existing CD25 based allodepletion strategies. Depletion of CD25 or CD69 alone led to a 60 % reduction in HTLp frequency against host stimulators but targeting CD25 and CD69 using immunomagnetic allodepletion, led to a 80 % reduction in residual alloreactivity in secondary MLRs, and a 80 % reduction in HTLp frequencies, compared to unmanipulated controls, with preservation of anti-3rd party responses.[115] Van Dijk *et al* depleted alloreactive T-cells expressing CD25, CD69, CD71 and HLA-DR using an immunomagnetic allodepletion and this resulted in a 1 log reduction in alloantigen specific helper T lymphocyte precursor (HTLp) frequency without affecting third party responses.[118]

Numerous other activation antigens have also been targeted for allodepletion strategies. 4-1BB (CD137) is upregulated on activated T-cells early in a MLR and CD137 is important in co-stimulation of CD8 effector memory cells. Anti CD137 antibodies, inhibit CD4 and CD8 T-cell mediated GVHD. Hartwig *et al* generated CTL cultures by stimulating CD8+ lymphocytes with single HLA mismatched AML blasts or renal carcinoma cells.[59] On Day 21 of the culture, the lymphocytes were stimulated with HLA-negative K562 cells transfected with the disparate HLA-Class 1 cDNAs. After 24 hours, the CD137 positive alloreactive T-cells were depleted using MACS technology. CD137 negative cells and unmanipulated CTL cells were then stimulated with the original AML blast/renal carcinoma cells or the K562 cells expressing the disparate HLA antigen in a 2º IFNγ ELISPOT. In 15 donor-recipient pairs, the allodepleted fraction showed a median residual activity of 9.5 % to the transfected K562 cells, and 58%

residual activity to the leukaemic/ carcinoma cell line compared to sensitized non depleted PBMCs from the same donor. Chromium release cytotoxicity assays showed that the allodepleted fraction lost reactivity to the single HLA mismatched K562 lines but maintained cytotoxicity against the leukaemia/carcinoma cell lines. Anti-EBV and CMV activity was maintained despite allodepletion as assessed by tetramer and IFNγ ELISPOTS. However, unlike CD25 or CD69 a clinical grade CD137 antibody does not exist limiting this approach. Furthermore, a significant residual alloreactivity was still evident to the single class MHC mismatched K562 cells after CD137 allodepletion. The effectiveness of CD137 allodepletion in haploidentical mismatched donors is not known. These studies demonstrate that there are a host of different approaches to targeting alloreactive T-cells based on their expression of activation markers, but these have not been compared with each other and the optimal method and marker is yet to be determined.

2. Activation Induced Cell Death

Activation induced cell death is an important physiological pathway to control the expansion of activated T-cells. CTLs kill targets by expressing FAS ligand (FasL) that binds to the death receptor Fas. This interaction activates caspases and leads to apoptosis of targets. Alloreactive T-cells up regulate the Fas antigen, and upon addition of a Fas Ligand agonistic antibody in an *ex vivo* MLR, there was a 80 % reduction in proliferation in a 2º MLR, compared to controls.[119,128] This approach maintained third party activity, and in mouse model of GVHD, transfer of CD95 allodepleted T-cells prevented lethal GVHD, compared to untreated T-cells. Another approach has been to transfect APCs, with a Fas Ligand vector, to therefore lead to clonal deletion of alloreactive T-cells expressing the Fas antigen. [67] The transfected APCs, led to a large decrease in the T-cell proliferation to the original stimulator in MLR, but responder cells were able to maintain third party responses. This decrease was specific to Fas-Fas ligand interaction, because when responder T-cells from Fas knockout mice were used, the Fas ligand transfected APCs, had no effect on the proliferation. Limitations of this approach include the lack of a GMP grade CD95 agonistic antibody, and the possibility of inducing fatal hepatic damage with a CD95 antibody,[129] due to expression of CD95 expression on hepatocytes.

3. Photodynamic Purging

Photodynamic purging (PDP) involves the administration of a photosensitizing agent, followed by activation of the agent by light of the appropriate wavelength. One such agent, TH9402 is taken up by T-cells and is then actively extruded by the multi drug transporter P-glycoprotein 170 (P-gp170). However, p-gp170 is inactivated upon T-cell activation, leading to a selective retention of the dye in the mitochondria of activated T-cells. Following exposure to visible light (512 nm), the dye becomes cytotoxic leading to the generation of free radicals which resulting in cell death. The capacity of TH9042 to reduce alloreactivity is primarily due to depletion of CD25 positive alloreactive T cells, but may not affect CD25+FoxP3 + regulatory T-cells.[130] Some CD25+ve T cells remain after treatment, though whether they are residual alloreactive T-cells or regulatory T-cells is not known. PDP treatment also removes B cells, and this may also account for account for some of the effect of photodynamic purging.

Thus this approach could lead to the selective deletion of alloreactive T-cells, which become activated in a MLR, leaving resting T-cells intact. [52] Allodepleted donor T-cells generated using PDP demonstrated a significant reduction in IFNγ release in ELISPOT assays when compared to sensitized non PDP treated PBMCs but had a residual response to host of over 6 times higher than that of unmanipulated PBMCs.[33] Though the IFNγ ELISPOT showed that PDP treated donor cells still had significant alloreactivity, when these cells were infused into a mouse model, there was no clinical GVHD. Moreover, adoptive immunotherapy with PDP treated donor T-cells in a mouse tumour model, demonstrated improved survival, prevented relapse, and promoted immune reconstitution.[33] More recently, Mielke *et al* demonstrated a 4 log reduction in residual alloreactivity to host following PDP purging of allodepleted donor T-cells in a clinically applicable system.[131] Third party responses were maintained but anti-viral responses in the aforementioned studies were not systematically examined. In this regard, Perruccio *et al* observed large decreases in T-cell responses to viral/fungal antigens, using a similar PDP approach.[132]

Preliminary results of a phase 1 clinical study of adoptive transfer of PDP treated donor T-cells after haplo-SCTs in 13 high risk adult patients with haematology malignancies, have been reported by Roy *et al* [133]. Patients received escalating DLI with donor PDP treated T-cells at doses of 10^4/kg to 10^5/kg. Anti host cytotoxic T lymphocyte precursors (CTLp) were reduced

by 1.5 log after PDP treatment, whilst activated CD25 + T cells were depleted by over 90 %. Of 11 evaluable patients, none developed acute GVHD, whilst 3 developed chronic GVHD. 5 patients died (4 due to infections including 1 EBV LPD), and the other of relapse.[133] Immune reconstitution was disappointingly poor, with a relatively high mortality from infectious deaths, suggests that PDP may affect the anti-viral function of the residual allodepleted T-cells. Other clinical studies of photodynamic purging with higher doses of allodepleted T-cells are currently ongoing.

4. Chemotherapy Based Methods of Allodepletion

Alloreactive cells are characterized by their ability to proliferate in response to alloantigen. With this in mind several groups have looked at adding chemotherapeutic agents to *ex vivo* MLRs, to preferentially kill proliferating cells. Fludarabine, is an adenine nucleoside analogue that inhibits DNA synthesis when incorporated into the replicating chain. It also has potent immunosuppressive effects including profound depletion of CD4+ cells. In a MHC-mismatched murine SCT model Waller *et al* treated mismatched donor lymphocyte infusions (DLI) with fludarabine *ex vivo* for 24 hours, prior to infusion.[120] Recipients of fludarabine treated DLI did not develop GVHD and had superior engraftment and chimerism analysis compared to T-cell depleted bone marrow. In contrast, mice receiving unmanipulated DLI with bone marrow showed high rates of GVHD. The mechanism of fludarabine's actions appeared to be a selective depletion of naïve T-cells, whilst preferentially preserving memory T-cells. [134] While such an approach is conceptually attractive, fludarabine is very potently immunosuppressive, and such a strategy would require careful dose titration experiments to minimize effects on desirable anti-viral and anti-leukaemic T-cell responses.

Other groups have targeted proliferating T-cells directly in an *ex vivo* MLR. Addition of trimetrexate, an anti folate drug,[135] inhibited proliferation in a MLR due to apoptosis in the proliferating cells. Combining trimetrexate mediated allodepletion with a CD25 immunotoxin (Ontak) was superior to either alone in inhibiting proliferation to host stimulators in a primary MLR. This combination did not affect proliferative responses to third party or Candidal antigens. This approach is potentially clinically applicable, but again would require careful titration to avoid toxicity to desirable T-cell responses. However, the CD25 immunotoxin used was Ontak, which has been shown to be inferior to ricin based CD25 immunotoxin.[124]

Bortezomib is a reversible proteosome inhibitor.[136] By inhibiting proteosome activity and thus preserving I$k\beta$ activity, it inhibits the translocation of NF-$k\beta$ into the nucleus. Bortezomib inhibits proliferation *in vitro* MLRs, [55,121] due to selective apoptosis of the proliferating alloreactive T-cells. [55,136] Blanco *et al* used this approach to selectively delete alloreactive T-cells.[55] This study however, was limited by the assays used to assess residual alloreactivity. The percentage of viable CD25+ T-cells in the bortezomib treated MLR was lower than in the CD25-ve fraction but 55 % of CD25+ve alloreactive cells still remained viable. CD69 expression after secondary stimulations was used to assess residual alloreactivity but CD69 is only expressed on a minority of alloreactive T-cells. The effect of bortezomib on reducing CD69 expression in the 2º MLR was modest (7.1 % in unmanipulated T-cells vs 2.9 % in the bortezomib MLR). There was no comment in the paper about the effect on desirable anti-leukaemic or anti-viral responses, though 3rd party responses were decreased suggesting that this approach may target bystander cells.

In mouse GVHD models, administration of bortezomib on days 0-3 post BMT along with allogeneic T-cells, led to improved survival due to decreased GVHD compared to addback of unmanipulated T-cells.[121] However, delayed administration of bortezomib (days 5-7 post BMT), led to enhanced gut GVHD, and increased mortality.[137] Histopathological examination of the mice that received delayed administration of bortezomib, showed significant increases in TNFα receptor transcription in gut cells and increased serum TNFα, IL-1, and IL-6. Thus, the effects of bortezomib on GVHD, are critically dependent on its timing. PS-1145 is a selective inhibitor of NF-$k\beta$, and delayed administration of this agent protected mice from lethal GVHD and did not cause gut toxicity.[136] This suggests that toxicity of delayed administration of bortezomib is due effects from proteosome inhibition other than NF-$k\beta$ inhibition.

Clinical Trials of Allodepleted Donor T cells Generated with CD25 Immunotoxin (RFT5)

Andre-Schmutz *el al* performed a clinical study of add back CD25 immunotoxin (RFT5-SMPT-dgA) allodepleted donor T-cells after HLA mismatched BMT.[138] They treated 15 paediatric patients in this study, 13 of whom had haploidentical transplants, and 2 received matched unrelated donors. Five patients had high risk haematological malignancies, the rest were transplanted for metabolic/immunodeficiency disorders. Donor PBMCs were co-cultured with irradiated host PBMCs for 3 days *in vitro*. Harvested cells were then treated with an overnight incubation with the CD25 immunotoxin. The efficacy of depletion was assessed by flow cytometric analysis of CD3+CD25+ expression and by residual proliferation to host in a primary MLR. Allodepleted T-cells were only infused after engraftment, if there was no GVHD, and no residual anti thymocyte globulin detected. Doses infused ranged between 1 -8 x 10^5 T cell/kg. GVHD occurred in 4 patients (two patients developed had grade 1 GVHD of the skin, two others developed grade 2 of gut and skin). One of the latter went onto develop chronic GVHD of the skin. Onset of GVHD did not correlate with the number of T-cells infused but rather with residual proliferation to host in 1° MLRs. The 4 patients who developed GVHD all had a residual proliferation of greater than 1%, thus suggesting that targeting CD25-ve alloreactive T-cells may reduce the incidence of this complication.

Preliminary studies on immune reconstitution suggested that this was enhanced following transfer of allodepleted donor T-cells compared to historical controls. Infusion of allodepleted T-cells led to enhanced CD3, CD4 and CD8 recovery, particularly in those patients who had viral reactivations. Responses to PHA were also enhanced. These T-cells had a memory phenotype and a diverse T-cell receptor repertoire. Two patients who had active CMV infection, showed strong cytolytic activity against CMV infected targets, 4 weeks after infusion of allodepleted T-cells, suggesting that T-cells encountering their cognate antigen show considerable expansion. Although viral specific immunity was not systematically assessed, one case of adenoviraemia, and another of EBV lymphoproliferative disease, who were both resistant to conventional treatment, responded following infusion of allodepleted T-cells.

Three of the five leukaemic patients died of relapse. Eight patients were alive and well with a median follow up of 24 months. This study showed that administering allodepleted T-cells generated using CD25 immunotoxin was safe and that deleting T regulatory cells did not lead to enhanced GVHD. However, in view of the high mortality from relapse enhanced allodepletion is needed, thus allowing higher doses of T-cells to be infused to augment anti-leukaemic responses.

Solomon *et al.* used CD25 immunotoxin (RFT5-SMPT-dgA) based allodepletion in an adult trial in the HLA-matched setting. Sixteen patients with a median age of 65 and advanced haematological malignancies underwent reduced intensity transplants using HLA-identical sibling donors. G-CSF mobilised peripheral blood stem cells were T-cell depleted by CD34 selection. Donor T-cells from the negative fraction were then incubated with irradiated host T-cells (OKT3 immunomagnetically selected) in a 1:1 ratio. [139] The immunotoxin was added after 24 and 48 hours of co-culture, and cells were harvested at 72 hours. The stem cells and the allodepleted product were co-infused on day 0 of the procedure. The median T-cell dose infused was 1.0×10^8/kg (range 0.2-1.5 x 10^8/kg). Successful depletion of alloreactivity was observed in 9 out of 11 patients as tested in helper T lymphocyte precursor (HTLp) assays. There was a ½ log mean reduction in the HTLp frequency against host stimulators after allodepletion, though with a large range (range 2-11 fold). The rates of acute grade II-IV GVHD observed were relatively high (46 % ± 13 %) but grades III-IV GVHD was uncommon (12 ± 8 %). Seven out of 14 patients developed chronic GVHD. The likelihood of GVHD was inversely correlated to the post depletion HTLp frequency. i.e. patients who had retained high HTLp precursor frequencies after CD25 allodepletion developed GVHD. There was no relationship to the starting HTLp frequency. There were 5 relapse deaths, with a 2 year probability of relapse of 56 %. The major flaw in this study was that the host antigen presenting cells used (expanded T-cells) may induce suboptimal activation of CD25 expression in alloreactive T-cells, particularly in the absence of HLA-mismatch, and hence inadequate depletion of alloreactivity. Nonetheless, these data suggests that targeting alternative CD25-ve markers may be valuable in enhancing existing CD25 based allodepletion.

Further analysis of this cohort showed effective T regulatory cell reconstitution at 1 month post BMT.[140] Thus acute GVHD occurring in this cohort was unlikely to be due to removal of CD25 positive T-regulatory cells, but rather due either to inadequate up regulation of CD25, or

the presence of CD25 negative alloreactive T-cells.[140]   This emphasizes the need to characterize the phenotype of alloreactive T-cells that do not express CD25.

Follow up on immune reconstitution in these studies was limited and there was no formal assessment of anti-viral immunity. Equally it was unclear how many allodepleted cells were needed to improve immune reconstitution without causing GVHD in the haploidentical setting. To address this, Amrolia *et al* performed a trial in 16 paediatric haploidentical transplants recipients, comparing immune reconstitution after addback of 2 dose levels of allodepleted T-cells. 8 patients were treated at dose level 1 (10^4/kg/dose), comparable to the number of unmanipulated T-cells given in a standard haploidentical graft, whilst the other half received a higher dose of 10^5/kg/dose.[141] Each arm received 3 doses of allodepleted T-cells. 12 of the patients had high risk haematological malignancies, and 6 of these were not in remission. Host LCL were co cultured with donor PBMCs for 72 hours prior to the addition of CD25 immunotoxin (RFT5-SMPT-dgA).   Each patient was scheduled to receive 3 doses of allodepleted T-cells at either dose level on days +30, +60, and +90 post transplant, providing their was no evidence of grade II or greater GVHD, or until total circulating T-cell numbers were greater than 1000/µl. The primary aims were to compare toxicity (i.e. GVHD), and immune reconstitution between the two dose levels. Secondary outcomes were frequency/outcome or viral infections, and day 100 and 1 year overall/disease free survival. The efficacy of allodepletion was assessed by FACS (residual % of CD3+CD25) and residual proliferation in 1º MLR. The residual percentage of CD3$^+$/CD25$^+$ cells in the infused allodepleted cells ranged from 0.01% to 0.27% (median 0.08%), and the residual proliferation against host cells in the primary MLR ranged from 0% to 3.1% (median, 0.02%).

Error! Bookmark not defined.

Most (13 of 16) patients completed their scheduled infusions: the remainder did not because of GVHD (*n = 2*) or autologous reconstitution (*n = 1*). Immunosuppression was withdrawn prior to infusion of the allodepleted products. Two patients developed GVHD, one at dose level 1 (after 1 infusion Grade IV skin GVHD), and at dose level 2 (Grade II skin GVHD after 2 infusions). Both patients went onto develop chronic GVHD, affecting the liver in one, and skin and mouth in the other. This incidence of GVHD is equivalent to what one would see in standard T-cell depleted haploidentical transplants without T-cell addback. There was no relationship between the occurrence of GVHD and residual proliferation to host in this study.
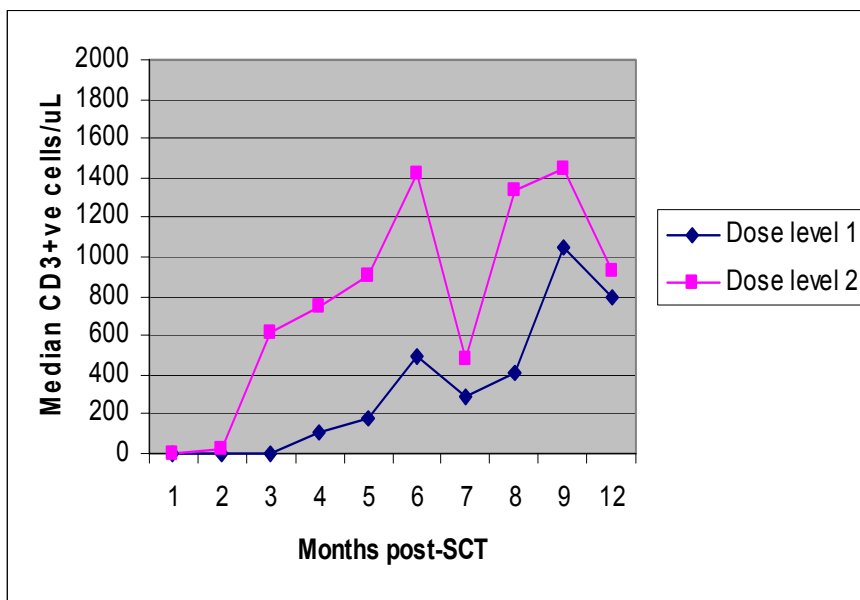
Figure 9: Enhanced CD3 Recovery in dose level 2 patients between 3-5 months post SCT. Donor PBMCs were cocultured with host LCLs and on day 3 a CD25 allodepletion was done using CD25IT. These allodepleted donor T cells were infused at specific time points at either 10^4/kg in one cohort or 10^5/kg in the other cohort. Those who received dose level 2 (10^5/kg) had superior T-cell recovery compared to dose levels 1 (10^4/kg) Reproduced from [141]

With regards to immune reconstitution, CD3 numbers were significantly higher in dose levels 2 (10^5/kg) patients between 3-5 months post BMT, compared to dose level 1(10^4/kg). (See Fig 9). This was true in both the CD4 and CD8 compartments. Area under the curve analysis also showed that CD3, CD4, and CD8 recovery at 4 and 6 months was significantly improved.

There was no difference in B and NK-cell recovery between the 2 groups. The majority of recovering T-cells in the dose levels 1 group were of a naïve phenotype (CD45RA+, CCR7+), whilst in dose level 2 the majority of T-cells had an effector memory phenotype (CD45RA-, CCR7-). Effector memory T-cells were significantly higher in dose level 2 between 3-5 months post BMT. This is important, as effector memory cells responses are likely to be long lived.

To determine whether the improved immune reconstitution was due to infusion of the allodepleted T cells, or due to naïve precursors passing through the thymus, T-cell receptor signal joint excision circles (TRECs) were analyzed by real time polymerase chain reaction (PCR). TRECs were detected at low levels in 3 of the 5 patients assessed on dose level 1 at 4 and 6 months. TRECs were undetectable in 4 out of 4 dose level 2 patients who were analyzed at these time points. This suggests that the improved T-cell reconstitution observed in patients treated at dose level 2 was due to the infusion of allodepleted T-cells.

Tetramer and IFNγ ELISPOT assays were used to analyze viral-specific immunity. At dose level 1, none of the 6 evaluated patients had a significant CD8+tetramer population recognising EBV epitopes up to 12 months post BMT, despite the fact 2 had viral reactivations. In contrast, at dose level 2, tetramers were detected in 3 of the 4 evaluated patients, shortly after viral reactivation. Similarly for CMV, none of the 6 evaluable patients at dose level 1 had tetramer positive populations before 9 months post BMT, despite 5 of them having viral reactivations. At dose level 2, 2 of the 4 patients had tetramer positive populations detected as early as 2 months post BMT, again in one of these in response to viral reactivation. These data suggests that at doses of 10^5/kg there is considerable peripheral T-cell expansion in response to the T-cells seeing their cognate antigen. Likewise, using IFNγ ELISPOTs to assess the function of these viral-specific T-cells, EBV immunity in dose level 2 was of a greater magnitude and occurred earlier than in dose level 1. Only 2 of 7 evaluable dose level 1 patients had a significant response (defined as >200 spot forming cells/10^6 PBMCs) to EBV LCL, compared to 4 out of 6 treated at dose level 2. Once again, in general these responses were correlated with viral reactivation. Similar data was obtained for CMV.

Nine patients (of the 16 at risk) had CMV reactivation. They were treated with ganciclovir ± foscarnet though none developed CMV related disease. Six patients had EBV reactivations, but none needed treatment with rituximab, or developed lymphoproliferative disease. There were three probable fungal infections which resolved with antifungal therapy. There were 2 cases of adenoviraemia. In one patient, this led to fatal disease of the liver. The other patient did not clear the virus despite anti-viral treatment with cidofovir and ribavarin and 3 doses of allodepleted T-cells at dose level 2, and he was subsequently given a single dose of allodepleted T-cells at 2.5 x 10^6/kg on a compassionate basis, with rapid clearance of the virus. This suggests that infusing higher doses of allodepleted donor T-cells is required to combat pathogens which have a low precursor frequency (e.g. adenovirus). Another patient had progressive multifocal leucoencephaolpathy due to JC virus, which showed a significant clinical and radiological improvement following infusion of allodepleted T-cells at dose level 2. This patient showed marked improvements in cognitive function, and motor skills, associated with improvement in their T-cell numbers.

At a median follow up of 33 months, 7 patients in this study have relapsed and 5 are alive and disease free. Thus this study showed that infusions of allodepleted T-cells at dose level 2 post haplo-SCT led to;

- **Low incidence of GVHD**
- **More rapid recovery of T-cells with memory phenotype**
- **Absence of TRECs + diverse TCR repertoire**
- **Accelerated recovery of viral-specific immunity and a low incidence of infection related deaths**
- **Clinical responses**

However, clearly relapse remains a major problem. Selective allodepletion will deplete T-cell responses against the mismatched HLA alleles and ubiquitous minor histocompatibility antigens presented by the shared HLA alleles. Nonetheless, anti-leukaemic activity may be retained after allodepletion [86,122,142] In particular, T-cell responses to potential myeloid tumour antigens are preserved by virtue of their lack of expression on the LCL used as stimulators. [86]Likewise, allorestricted responses against tumour, haematopoietic-specific and minor histocompatibility antigens presented through the non-shared haplotype[143-146] should also be retained. It is unclear how many allodepleted donor T-cells would be required to confer clinically relevant anti-leukaemic responses in the haploidentical setting: because of the possibility of allorestricted responses, this may in fact be considerably lower than the numbers of DLI required to induce GVL after HLA-matched SCT. Because of the limitations of experimental models, it may be that this question can only be answered in clinical studies.

Likewise, the fact that the patient who cleared adenovirus only did so after receiving 2.5 x 10^6 T cells/kg suggests that the protective response against pathogens with a low T cell precursor frequency may require larger doses of allodepleted T-cells to be infused. Our in vitro data suggests that a median 17 fold reduction in alloreactivity (as assessed by ELISPOT) after CD25 immunotoxin based allodepletion, together with the fact that 2/16 patients did develop GVHD, suggests that it may not be safe to give larger doses of allodepleted T-cells using this strategy alone. Thus, in subsequent studies we need to enhance depletion of allo-reactive cells so that can add back sufficient T-cells for protective anti-leukemic responses without causing GVHD.

Adoptive Immunotherapy with Genetically Modified Donor T cells

As noted above, the major cause of mortality following adoptive transfer of CD25 allodepleted donor T-cells after haploidentical transplantation was relapse.[141] Potentially, the anti-leukaemic activity of allodepleted T-cells could be augmented by redirecting the specificity of T-cells. One approach to do this is by transducing allodepleted donor T-cells using chimeric T-cell receptors. The advantage of redirecting the specificity of allodepleted T-cells using chimeric TCRs is that the latter are not MHC restricted.

Chimeric T cell Receptors

Tumour cells evade recognition and elimination by immune effectors by a variety of ways:

1) low or absent expression of tumour antigens
2) expression of antigens that are shared with normal cells, so that the immune system has become tolerant
3) down regulation of MHC molecules
4) defective pathways of antigen presentation
5) absence of co-stimulation
6) secretion of inhibitory molecules e.g. IL-10, transforming growth factor-β
7) expansion of T regulatory cells

Chimeric T cell receptors (ChTCR) can potentially overcome some of the above difficulties. ChTCR are artificial T-cell receptors constituted by an antigen recognizing antibody molecule linked to a T-cell triggering domain. The most common form of these molecules are fusions of single-chain Variable fragments (scFv) derived from monoclonal antibodies, fused to CD3-zeta endodomain (see Fig 10). Such molecules result in the transmission of a zeta signal in response to recognition by the scFv of its target. An example of such a construct is 14g2a-Zeta, which is a fusion of a scFv derived from hybridoma 14g2a (which recognizes disialoganglioside GD2). When T-cells express this molecule (usually achieved by oncoretroviral vector transduction), they recognize and kill target cells that express GD2 (e.g. neuroblastoma cells). To target malignant B cells, investigators have redirected the specificity of T-cells using a chimeric immunoreceptor specific for the B-lineage molecule, CD19. The main advantages of this approach are:

1. It overcomes the lack of immunogeneic tumour antigens on leukaemic blasts by redirecting T-cells to surface molecules they express.

2. It enables targeting of tumour cells in a HLA-independent fashion, so that a single vector can be used to treat all patients expressing the surface molecule (in contrast to transfer of exogenous TCR)

3. It overcomes tumour evasion by down regulation of HLA molecules or defects in antigen processing

T cell mediated function should be more effective that that of infused monoclonal antibody as cytokine release at the site of a tumour can lead to amplification of the anti-tumour response. Redirected T-cells can also home to tumour sites, proliferate locally, and penetrate solid tumours. Critically, eradication of tumours, by adoptive immunotherapy will depend on the transformation of tumour CTLs into memory long lasting CTLs.

Figure 10: Structure of Chimeric TCR. A chimeric TCR consists of a monoclonal antobody binding domain complexed to the signalling regions of the TCR. This enables the recptor to bind independent of the HLA. Upon binidng to it cognate antigen, signalling through the TCR leads to T-cell proliferation and differentiation.
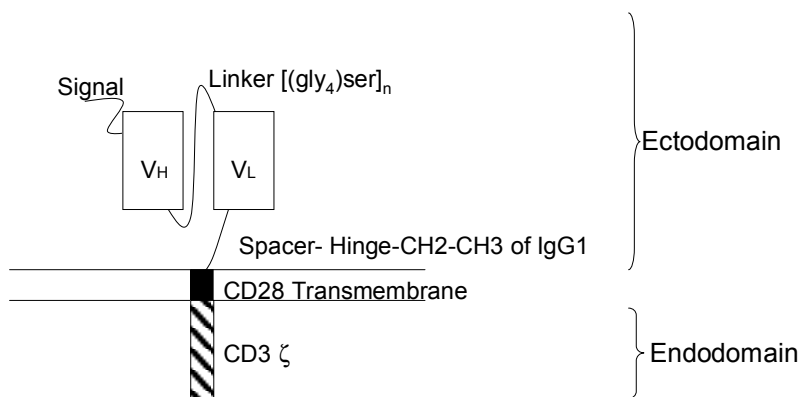
The extracellular domain consists of [147] (see Fig 10)

- **Ectodomain (Signal sequence)** - to allow entry into the endoplasmic reticulum and transport to the cell membrane. This is essential if the receptor is to be glycosylated and anchored in the cell membrane. Generally, the signal peptide natively attached to the

amino-terminal is the component most commonly used (e.g. in a scFv with orientation light-chain - linker - heavy chain, the native signal of the light-chain is used)

- **Ectodomain - antigen recognition region**

The antigen recognition domain is usually a scFv. This consists of variable domains of a monoclonal antibody, linked together as a single chain Fv (scFv). Most scFvs are generated from mouse hybridomas. Connecting the V_H and V_L is a linker $[(gly_4)ser]_n$, which is at least 12 amino acids in length, which allows the correct stereotactic orientation of the scFv.[148]

There are however many alternatives. An antigen recognition domain from native TCR alpha and beta single chains have been described, as have simple ectodomains (e.g. CD4 ectodomain to recognize HIV infected cells) and more exotic recognition components such as a linked cytokine (which leads to recognition of cells bearing the cytokine receptor). In fact almost anything that binds a given target with high affinity can be used as an antigen recognition region.[148] Importantly, if the antigen recognition domain is derived from a monoclonal antibody, this may potentially be immunogeneic, as may junctional regions within the ChTCR.

- **Ectodomain (The Spacer region)** - A spacer region links the antigen binding domain to the transmembrane domain. It should be flexible enough to allow the antigen binding domain to orient in different directions to facilitate antigen recognition. A spacer region separating the antigen binding region and the signalling domain seems to be necessary to enable optimal function of constructs The simplest form is the hinge region from IgG1 though alternatives include the CH_2CH_3 region of immunoglobulin , portions of CD3 or from the hinge –CH2- CH3 of the human IgG 1 molecule.

- **Transmembrane Domain**- This is a hydrophobic alpha helix that spans the membrane. Generally, the transmembrane domain from the most membrane proximal component of the endodomain is used. Different transmembrane domains result in different receptor stability. The CD28 transmembrane domain results in a highly expressed, stable receptor. [148]

- **Endodomain**- consists of the signalling component of region of the TCR (usually CD3ζ). CD3-zeta contains 3 immunoreceptor tyrosine-based activation motifs (ITAMs). This transmits an activation signal to the T cell after antigen is bound. CD3-zeta may not provide a fully-competent activation signal and additional co-stimulatory signalling is needed. For example, chimeric CD28 and OX40 can be used with CD3-Zeta to transmit a proliferative / survival signal, or all three can be used together[149]

A variety of haematological malignancies could be targeted using an approach involving a ChTCR e.g. CD19 and CD20 have been targeted for B cell lymphoid tumours, and CD30 for lymphomas.

Targeting CD19 on Malignant B cells

The CD19 molecule is a 95 kDa membrane glycoprotein, found on human B lymphocytes at all stages of maturation, but usually disappears upon differentiation to terminally differentiated plasma cells.[150] It is expressed on most ALL, CLL and B cell lymphomas. CD19 is rarely lost during the neoplastic transformation and is not expressed on haematopoietic stem cells, or normal tissues outside the B lineage. CD19 is also not shed into the circulation and therefore there is no soluble CD19 to compete with binding to a CD19 ChTCR.

Human peripheral blood T-cells transduced with a retroviral vector carrying a CD19-TCRζ have been shown to potently kill and to secrete Th_1 cytokines in response to CD19+ve leukaemic cell lines and B-ALL blasts *in vitro*. [151] CD19chTCR redirected T-cells, traffic to the bone marrow and have led to the eradication of established Burkitts lymphoma and prolonged survival in a SCID-beige mouse tumour model.[152] Incorporation of a suicide gene (HSV-1 TK) construct resulted in their elimination following ganciclovir. [147]

Limitations of Chimeric TCRs

Targeting endogenous antigens such as CD19 would lead to a loss of B cells and a reduction in humoral immunity necessitating immunoglobulin infusions, thus limiting this approach to high risk ALL patients. There also remain concerns over the persistence of these genetically modified T-cells *in vivo*. Adoptive transfer of *ex vivo* expanded CD4 ζ modified syngeneic

CD8+ T cells in HIV infected twin pairs, showed *in vitro* cytotoxicity, but failed to induce objective clinical responses. The observed lack of antiretroviral activity was associated with a rapid decline in gene marked cells following transfusion. Although 1st generation chTCRs were able to kill *in vitro* and produce IFNγ, they showed diminished IL-2 production and thus poor proliferation and survival. [153] Many tumours lack co-stimulatory molecules so that when the chTCR interacts with its receptor, it will get 1 signal, but no co-stimulatory signal 2. This will lead to poor proliferation and anergy or cell death. As noted above, the incorporation of a co-stimulatory domain *in cis* may improve the expansion and survival of T-cells transduced with chimeric chTCRs.

The short life span of chTCRs *in vivo* may be due to a host immune response to the foreign proteins on the receptor. The immunogenicity of recombinant receptors can be reduced by using human antibody fragments as recognition domains.[153] Transfer of antigen specific CD4+ helper cells has been shown to be vitally important in CTL function. Co-administration of CD4+ and CD8+ chTCRs in HIV patients showed persistence for at least 1 year, but no significant therapeutic effects were seen. Thus transfer of CD4 + antigen specific T-cells may help with persistence, but effective anti-tumour activity will require T- cell proliferation, and establishment of memory T-cells.

So far transduction of T-cells with chTCRs has been with retroviral transduction. The ability of retroviruses to integrate into the host cell chromosome raises the possibility of insertional mutagenesis and oncogene activation. Acute leukaemia has developed in 4 of 9 children treated with gene therapy for X-linked SCID in France[154] and 1 out of 10 in the UK. This adverse event was attributed to the integration of the retrovirus into the LMO2 locus, a key transcription factor in T-lymphoid progenitors, resulting in aberrant expression of this gene and uncontrolled proliferation of T-lymphoid blasts in 2 patients. However, in these studies CD34 selected haematopoietic progenitors were transduced. There have been no reported cases of insertional mutagenesis in patients followed up over an extended period following treatment with retrovirally transduced mature lymphocytes. Likewise, there have also been no reported cases of insertional mutagenesis, in patients treated with T cells retrovirally transduced with a chimeric TCR to treat HIV or neuroblastoma.

Oncoretroviral transduction uses high doses of IL-2, OKT3 and/or CD28 stimulation to maximize T-cell proliferation. This however converts naïve T-cells into an effector phenotype, and has been associated with a reduction in anti-viral and third party immunity.[107] Thus, any transduction of allodepleted T-cells with a chTCR may need a gentler stimulation in order to preserve the phenotype of the T-cells, and thus not compromise on anti-viral immunity. Furthermore, an inverse relationship between the acquisition of effector functions and the capacity to mediate tumour reduction *in vivo* (in mouse models) has been demostrated[155]. Fully differentiated tumour CTLs showed potent *in vitro* cytotoxicity, but naïve T-cells showed poor *in vitro* cyotoxicity. *In vivo*, however, tumour shrinkage was greatest when naïve T-cells were infused and poorest when fully differentiated T-cells were administered. Possible reasons for this discrepancy could lie in the ability to naïve T-cells to home to tumour sites, due to expression of CD62L, proliferate and secrete IL-2, express co-stimulatory molecules, and express less proapoptotic molecules than fully differentiated CTLs[155]. Thus, to maximize tumour activity as well as maintaining third party activity and minimize insertional mutagenesis, we have investigated a lentiviral gene transfer of a chimeric TCR into allodepleted T-cells. It has been shown that it is possible to efficiently transduce T-cells with a lentiviral vector using less intensive pre-stimulation with low dose IL-2 or IL-7 with preservation of the phenotype and anti-viral immunity of transduced T-cells.[107] Additionally, using SIN (self inactivating) lentiviral vectors, will minimize the risks of insertional mutagenesis .[147]

Enhancing Chimeric TCR Signalling

The CD3ζ signalling domain appears insufficient to fully activate of manipulated T-cells to proliferate. Integration of the signal transduction domain of the co-stimulatory molecules (2nd generation ChTCR) e.g. CD28, ICOS, CD134, or CD137 enhances the proliferative properties of gene modified cells, leading to greater secretion of IL-2, and prolonged survival.[149,153] The signalling characteristics of ChTCR can be further improved by linking *in cis* more than one co-stimulatory domain or a combination of co-stimulatory and co-receptor domains to the TCRζ chain (3rd generation ChTCR) e.g. linking the CD28 with the OX40 domain markedly increased proliferation, cytokine release and effector function. Moreover, the combination of ζ-chain together with the co-receptor (lck) and co-stimulatory (CD28) signals in a single receptor has been demonstrated to enhance ChTCR sensitivity and potency.[147]

An alternative approach is to use dual specific T-cells. Dual specific EBV T-cells recognize EBV antigens through their native TCR, and the ChTCR target (e.g. CD19) through the ChTCR. Using this approach, the engagement of the native TCR in vivo by recurrent EBV infections is capable of constantly, stimulating the ChTCR redirected T cells. EBV CTLs transduced with a retrovirus carrying the CD19ζ transgene specifically lysed and secreted IFNγ in response to CD19+ cell lines and primary ALL blasts in a MHC unrestricted fashion.[151] Similarly, bispecific CD19 redirected influenza specific CTLs have been shown to safely mediate regression of Daudi lymphoma tumors in NOD/SCID mice and this was enhanced by vaccination with T antigens presenting cells modified to present influenza antigens.[156]

Lentiviral Vectors

The lentivirus, when compared to gammaretroviruses has a more complex genome and consequently a more complex replication cycle. In addition to gag, pol and env genes, they encode two regulatory gene, tat and rev, essential for efficient viral gene expression and four accessory proteins termed vpr, vpu, nef, and vif. Lentiviral vectors (LVs) which are based on lentiviruses, such as HIV-1, can integrate a copy of their genome into the DNA of host non-dividing cells. This ability is particularly advantageous in T-cell gene therapy since cells may be transduced without extensive prestimulation, thus avoiding prolonged *ex vivo* culture that may result in loss of proliferative and homing ability, and reduction in anti-viral responses. Initial packaging constructs for these vectors maintained the accessory genes; however these proteins were shown to be dispensable for efficient transduction and integration of lentiviral vectors and were therefore deleted in second generation packaging constructs [157]. The deletion of accessory genes also increased the safety of these vectors, since any replication competent virus generated during vector production would lack the essential factors for HIV-1 virulence *in vivo*. A further safety measure was achieved by the production of third generation packaging constructs in which the *rev* gene was placed on a separate plasmid to that of the *gag-pol* genes and the *tat* gene was removed altogether [158]. (see Fig 11)
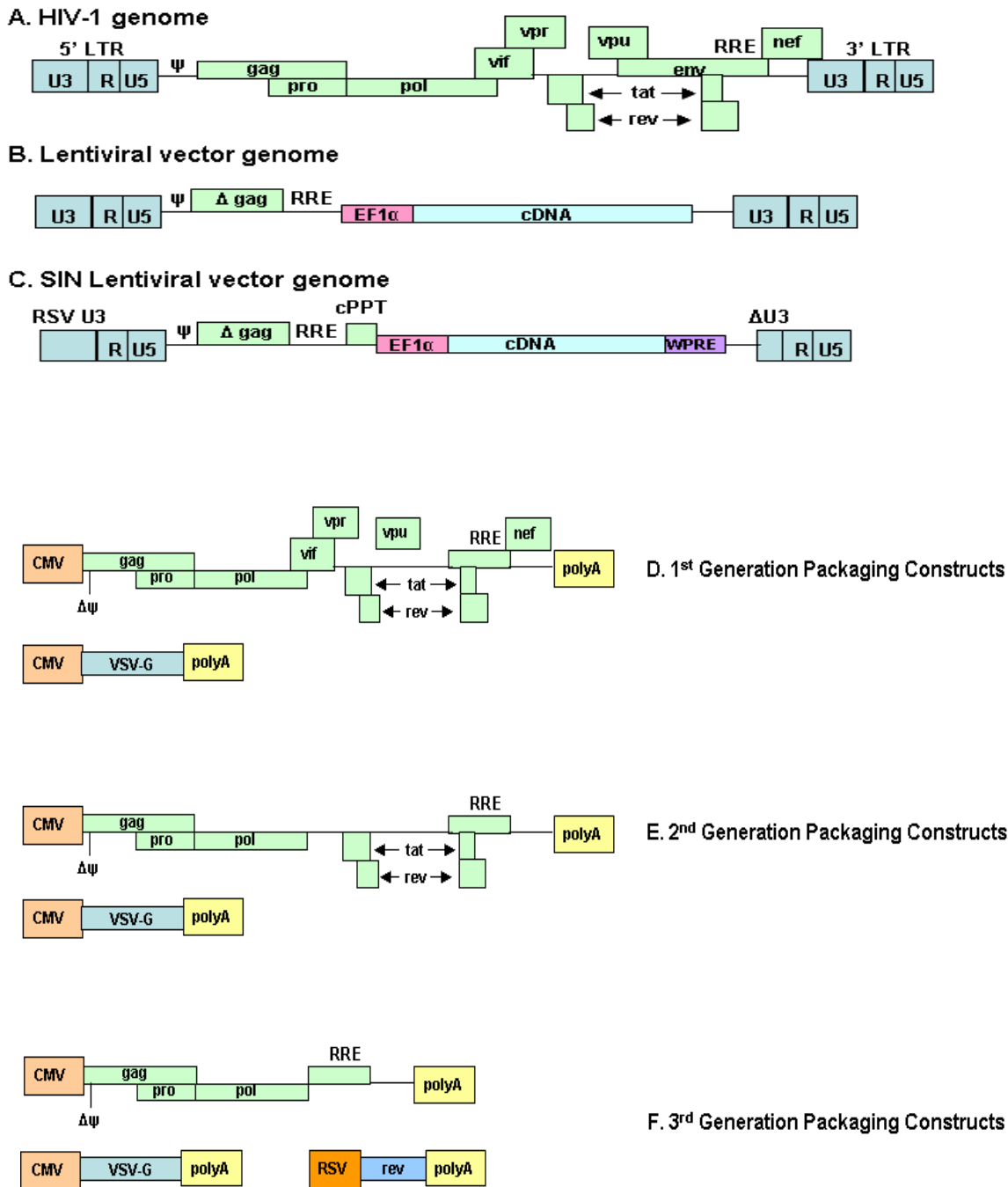
Figure 11: Lentiviral vector genomes and packaging constructs. (A) Genome of a wild-type HIV-1. (B) Wild-type LTR lentiviral vector genome in which the cDNA of a therapeutic gene is regulated by an internal promoter (e.g. EF1α). (C) Genome of a self-inactivating (SIN) lentiviral vector containing a modified 5'LTR in which the U3 region has been replaced by the constitutive RSV promoter. The vector contains a cPPT and WPRE to enhance vector potency and transgene expression. (D, E and F) First-, second- and third generation packaging constructs containing sequences from HIV-1 and the VSV-G envelope, used in conjunction with A, B or C. (ψ - packaging signal) Adapted from [159]

The viral tat protein acts as a potent transcriptional transactivator of the HIV-1 LTR and is therefore required for high titre virus production [160]. High vector titre in the absence of *tat* was found to be possible however by replacement of the U3 region in the 5'LTR with a constitutively active heterologous promoter such as that from the Rous sarcoma virus (RSV)[158]. The viral *rev* gene product functions as a nuclear export factor; the protein binds to a RNA motif, the Rev-response element (RRE), and promotes cytoplasmic export of unspliced and spliced transcripts and hence must be provided during vector production[160]. To optimize LVs, the incorporation of central polypurine tract sequences (cPPT) and post transcriptional regulatory elements, such as woodchuck post transcriptional regulatory element (WPRE) have been shown to increase the infectivity of replication incompetent viral particles and augment gene expression

One major limitation of lentiviral vectors is the lack of effective packaging cell lines, so that in general transient lentiviral supernatants are generated. The risk of insertional mutagenesis with lentiviral vectors may be lower than that of oncoretroviral vectors. Cattoglio and colleagues examined retroviral integration site (RIS) preferences in $CD34^+$ cells and concluded that gamma-retroviruses carry a higher risk for insertional mutation than lentiviruses.[161] Retroviruses have integration-site preferences. Studies in HeLa and CD34 cells have shown a gamma-retroviral bias for transcriptional start sites (29% of total) and for actively transcribed genes. Lentiviral integrations were also biased toward actively transcribed genes, but for intragenic rather than transcriptional start sites. Additionally, it was found that many of the recurrent gamma-retroviral integrations were cancer associated (i.e. proto-oncogenes). In contrast, recurrent lentiviral integrations did not involve a statistically significant number of cancer-associated genes. To improve the safety of LVs, more recent constructs have been developed called self inactivating vectors (SIN). These are characterised by a large deletion in the U3 region of 3′ long terminal repeat (LTR) of the DNA used to produce the vector RNA which is ultimately transferred to the 5′ LTR of the proviral DNA during reverse transcription, and leads to the inability of producing full length vector RNA. Thus, the risk of triggering cellular oncogenes by the enhancer activity of the LTR is diminished. Another advantage of SIN vectors is that the expression of the transgene can be restricted to specific cell targets depending on the internal promoter.[162] A recent pilot study with lentiviral engineered T-cells

that expressed an anti-sense HIV vector in HIV infected patients showed no evidence for insertional mutagenesis after 21–36 months of observation.[163]

Pseudo typing Lentiviral Vectors

The glycoprotein of the Vesicular stomatitis virus (VSV-G) is most commonly used to pseudo type lentiviral vectors and expands vector tropism since the receptor for VSV-G, although still undetermined, appears to be ubiquitous in all cell types. Furthermore, VSV-G pseudo typed vectors can be efficiently concentrated by ultracentrifugation, enabling the production of serum-free, high-titre vector particles. However, VSV-G is associated with cytotoxicity limiting the concentrations of vector which can be used without reducing target cell viability. VSV-G pseudo typed late generation LVs can transduce T lymphocytes that have progressed from G0 into the G1 phase of the cell cycle without becoming committed to proliferation.[162] The efficient transduction of human T lymphocytes by these vectors does not require cycling cells but a certain degree of activation. Stimulation with IL-2, -7 and -15 drives the progression to the G1 phase of the cell cycle without inducing further progression or proliferation. Gene transfer without TCR triggering reduces or eliminates the risk of selection or phenotypic alteration of cells in culture;

Transduction of Human PBMCs with Lentiviral Vectors

Qasim *et al* demonstrated that lentiviral vectors readily transduced peripheral blood lymphocytes (PBLs) cultured in IL-2 (100 IU/ml) or IL-7 (5 ng/ml) for 96 h at an efficiency of approximately 20–25% using a multiplicity of infection (MOI) of 20.[107] There was no advantage combining cytokines or using higher MOI, but full activation using anti-CD3/CD28 microbeads plus IL-2 (100 IU/ml) led to more efficient gene transfer (consistently above 50%). Cells cultured in IL-2 or IL-7 maintained their cell surface phenotypes, with preservation of the CD4 and CD8 subset proportions and naïve (CD27$^+$CD45RO$^-$) phenotype, whereas CD3/CD28-stimulated cells were highly activated (CD25$^+$), with a memory phenotype (CD27$^+$CD45RO$^+$).

One concern arising from previous clinical trials of adoptive immunotherapy with suicide gene transduced T-cells, was the relatively high rates of viral infections following transplantation. In addition, the incidence of GVHD was lower than might have been expected, and questions have been raised about the functional potential of T-cells after full activation and extensive *ex vivo* expansion. Using the lentiviral vector system, Qasim *et al* found that cytokine-stimulated cells (in particular IL-7) showed preservation of responses against CMV-pulsed dendritic cells (DCs) at levels comparable to fresh PBMCs, whereas these responses were diminished in CD3/CD28-stimulated cells.[107] Recent studies that have indicated that expansion of $CD4^+CD25^+$ regulatory T cells (Tregs) following activation with anti-CD3/CD28 may be partly responsible for suppression of effector function of transduced T-cells.[107]. Assessment of FoxP3 levels following transduction showed substantially increased levels in fully activated cells, with minimal alteration in IL-2- or IL-7-cultured cells. The increased numbers of FOXP3+ Tregs in CD3/CD28-activated cell cultures is consistent with the reduced proliferation detected in response to CMV or allo-stimulation.[107] Thus, T-cells cultured in cytokines IL-2 or IL-7 are amenable to lentiviral-mediated gene transfer, and although the cells undergo division, they retain their phenotype, anti-viral responses, alloreactive potential and regulatory numbers.

Haploidentical SCTs are compromised by a high rate of infectious death and malignant relapse. Allodepletion using CD25 based strategies has been shown to enhance immune reconstitution. However, the problems of leukaemic relapse and morbidity and mortality from adenoviraemia remain a problem. Our data has shown that increasing doses above 10^6/kg of allodepleted donor T-cells is effective against adenovirus. Furthermore, despite CD25 allodepletion, there remains significant residual alloreactivity against host. Thus, by enhancing existing CD25 based allodepletion, this may enable us to give higher doses of allodepleted donor T-cells, and thus get better anti-viral and anti-leukaemic responses but without GVHD.

Statement of Aims

The aims of this study are;

1. To characterize the activation marker and cytokine profile of alloreactive T-cells identified using CFSE dye dilution. In particular we have focused on determining the phenotype of the CD25 negative, proliferating alloreactive cells to provide a rational basis for enhancing allodepletion with CD25-based strategies.

2. Based on these studies, we have compared residual alloreactivity after depletion of alloreactive cells expressing these markers to determine if this leads to enhanced allodepletion compared to CD25-based strategies. We have also determined if anti-viral T-cell responses are preserved following our refined allodepletion method.

3. Using lentiviral transfer of chTCR CD19-CD3ζ transgene we have redirected the specificity of allodepleted T-cells generated using the optimal strategy identified from the above studies, to determine if this approach can augment anti-leukaemic activity of allodepleted T-cells.

CHAPTER 2

Materials and Methods

MATERIALS AND METHODS

Reagent Suppliers

Chemicals were obtained from Sigma, Mo, USA, and media purchased from Invitrogen, unless otherwise stated. Restriction endonucleases and their appropriate buffers were purchased from Promega. Cytokines were purchased from R&D systems (Minneapolis, MN).

Buffers and Solutions

All buffers were prepared in double distilled water (ddH$_2$O). Sterile solutions used ddH$_2$O autoclaved at 121ºC for 15 min and filtered through a 0.22μm syringe tip filter (Millipore). Compositions of buffers and solutions are listed below.

Buffer	Ingredients
Acetate Buffer	4.6 mls 0.1 M acetic acid, 11.0mls 0.1M sodium acetate, 46.9 mls Milli-Q water
Cell Dissociation Buffer Invitrogen (13151-014)	
Orange G loading buffer	10Mm Tris Ph 7.5, 50Mm EDTA, 10% Ficol 400, 0.4 % Orange G (Sigma (861286))
Flow Cytometry staining buffer (FACS buffer)	PBS, 0.5 % (w/v) BSA (Sigma A9418)
Flow cytometry fixing buffer (FACS fixing buffer)	FACS buffer + 1 % Paraformaldehyde (Sigma 441244)
PBS Invitrogen (14190-094)	
Luira-Bertani Broth (LB)	1% (w/v) tryptone peptone, 0.5 %(w/v) yeast extract (Becton Dickinson), 170mM NaCL, pH 7.0. Ampicillin (Sigma A9393) was added at 10 mg/ml where indicated (LB-Amp)

MACS Buffer	PBS, 2mM EDTA, 0.5 % BSA
TAE (X 50)	0.2M Tris, 1 M glacial acetic acid (BDH) 50mM EDTA, pH8.0
PBS 0.05 % Tween	50 ul of Tween 20 (Sigma) per 100 mls of PBS
APC Complex solution	PBS/ 0.1 % Tween 20
ELISPOT Coating buffer	1.59 g Na2CO3, 2.93g NaHCO3, 200mg NaN3, Up to 1 L with sterile water 9.6 pH, Sterile filter 0.22μm

Media and Solutions used for Tissue Culture

Media and their supplementary ingredients used in tissue culture are presented.

Medium	Supplements
AIM V Invitrogen (12055-091)	
Cell Genix DC media TCS Cellworks (2005)	1% L-glutamine
Complete DMEM Invitrogen (61965-026)	10%(v/v) FCS (Sigma F7524) and 10μg/ml penicillin/streptomycin (Invitrogen 15140-122)
Complete RPMI with glutamax (RF10) Invitrogen (61870-010)	10%(v/v) FCS and 10μg/ml penicillin/streptomycin
Optimem Invitrogen (31985-047)	
CTL media	45 % RPMI hyclone (Hyclone SH30096.02), 45 %clicks media (Irvine Scientific 9195), 1 % L-glutamine, 10 % fetal calf serum hyclone (Hyclone SH30070.03) and 10μg/ml penicillin/streptomycin
Trypsin Invitrogen 25300-062	

Cell Lines used in Study

Cell Line	Origin	Supplier	Medium
EBV- lymphoblastoid (LCLs)	Normal donor B cells	ICH, London	RF10
293Ts	Human Embryonic kidney	ICH, London	Complete DMEM
Ramos	Human Burkitt's lymphoma	Baylor College of Medicine	RF10
K562 cells	Human erythroleukaemia	Baylor College of Medicine	RF10
K562 GFP+CD19+	K562 stably transduced with CD19 GFP vector	Dr. Martin Pule	RF10
K562 GFP+ CD19-	K562 stably transduced with a GFP vector	Dr. Martin Pule	RF10

Cytokines used in Study

Cytokine	Source	Catalogue Number
Recombinant Human GM-CSF	R&D	215-GM-010
Recombinant Human IL-2	R&D	212-IL-010
Recombinant Human IL-4	R&D	214-IL-010
Recombinant Human interferon gamma (IFNγ)	R&D	285-IF-100
Recombinant Human Tumour necrosis alpha (TNFα)	R&D	210-TA-010
Recombinant Human IL-7	R&D	207-IL-005
Prostaglandin E2	Sigma	P5640

Antibodies Used in this study

All antibodies were purchased from Becton and Dickinson unless otherwise stated. A list of the antibodies utilised in this study is listed below

Antibody	Use	Clone
Mouse Anti Human CD3 Fitc	Functional Characterisation of alloreactive T-cells, assessing allodepletion efficacy and assessing lentiviral transduction	SK7
Mouse Anti human CD3 Pe	Functional Characterisation of alloreactive T-cells, assessing allodepletion efficacy and assessing lentiviral transduction	SK7
Mouse Anti Human CD3 APC	Functional Characterisation of alloreactive T-cells, assessing allodepletion efficacy and anti-viral studies	UCHT1
CFSE (Invitrogen)	Functional Characterisation of alloreactive T-cells	Catal No. C34554
Mouse Anti Human CD69 Pe	Functional Characterisation of alloreactive T-cells	FN50
Mouse Anti Human CD71 Pe	Functional Characterisation of alloreactive T-cells and assessment of allodepletion	M-A712
Mouse Anti Human CD71 Biotinylated	Performing CD71 allodepletion	M-A712
Mouse Anti Human HLA-DR PeCY5	Functional Characterisation of alloreactive T cells	TU36
Mouse Anti Human OX40 PeCY5	Functional Characterisation of alloreactive T-cells	ACT35
Mouse Anti Human ICOS Pe	Functional Characterisation of alloreactive T-cells	DX29
Mouse Anti Human CD95 PeCY5	Functional Characterisation of alloreactive T-cells	DX2
Anti Human CCR7 APC R&D systems	Functional Characterisation of alloreactive T cells	150503
Anti Human CD45RA PeCY5 R&D systems	Functional Characterisation of alloreactive T-cells	5H9
Mouse Anti Human TNFα Pe	Functional Characterisation of alloreactive T-cells	MAb11
Mouse Anti Human IFNγ APC	Functional Characterisation of alloreactive T-cells	B27
Mouse Anti Human IL-2 APC	Functional Characterisation of alloreactive T-cells	5344.111

Mouse Anti Human CD25 Pe	Functional Characterisation of alloreactive T-cells	2A3
Mouse Anti Human CD25 PeCy5	Functional Characterisation of alloreactive T-cells and assessment of allodepletion	M-A251
Goat Anti Human IgG Fcγ Cy^{tm}5 Jackson Laboratories	Assessment of Lentiviral Transduction	Catal No 109-176-008
Mouse Anti Human CD8 Fitc	Anti-viral Studies	SK1
Anti Human CMV pp65 HLA2 A2-NLV PE pentamer Proimmune	Anti-viral Studies	Catal No HCMVpp65 495-504
Anti Human CMV pp65 HLA2 B7-TPR PE pentamer Proimmune	Anti-viral Studies	Catal No HCMV pp65 417-426
Anti Human EBV LMP-2 A2-CLG PE pentamer Proimmune	Anti-viral Studies	EBV-LMP-2 426-434
Mouse Anti Human CD14 Pe	Assessment of Dendritic cell maturity	M5E2
Mouse Anti Human CD83 Pe	Assessment of Dendritic cell maturity	HB15e
Mouse Anti Human CD86 Pe	Assessment of Dendritic cell maturity	IT2.2
APC isotype IgG1 κ		MOPC-21
Pe isotype IgG1 κ		MOPC-21
Fitc isotype IgG1 κ		MOPC-21
PeCY5 isotype IgG1 κ		MOPC-21
Mouse Anti Human CD19 Pe	Assessment of CD19 positivity on cell lines	4G7
7-AAD	Assessment of viability of CD19 positive targets	

Kits

Plasmid Megaprep/Maxiprep Kit	Invitrogen (K2100-07)
QIAquick gel extraction kit	Qiagen (287706)
QIAquickPCR purification kit	Qiagen (28106)
DNA Quick Ligase Kit	New England Biolabs (M2200S)

PCR

Primers	Invitrogen
Taq DNA polymerase, dNTPs, buffer	Promega (M7660)
Restriction enzymes	New England Biolabs(NEB)
BamHI	NEB R0136L
EcoR1	NEB R0101L
Bgl II	NEB R0144S
NotI	NEB R0189S

Buffer 1	NEB B7001S
Buffer 2	NEB B7002S
Buffer 3	NEB B7003S
Buffer 4	NEB B7004S
BSA	NEB B9001S

Bacteria

Escherichia coli strains used:

| DH5α Competent cells | NEB C2987H |

Centrifuges

Microcentrifuge	Heraeus Biofuge Fresco
Tabletop centrifuge	Sorvall Legend RT
Superspeed centrifuge	Sorvall Evolution RC
Ultracentrifuge	Sorvall Discovery SE

Isolation of Responder and Stimulator Cells for MLR cultures and Generation of Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines (LCLs)

Ethical approval for the study was obtained through the non-clinical institutional review board at University College London. T-cells and dendritic cells (DC) were isolated from peripheral blood or single donor buffy coat preparations from healthy donors with informed consent $CD3^+$ T-cells were isolated by positive selection of peripheral blood mononuclear cells (PBMC) with CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, DE) according to manufacturer's instructions. PBMCs were incubated with CD3 microbeads for 15 minutes at $4°$ C (20 µl of beads/10^7 PBMCs), and washed in MACS buffer (PBS containing 2 mM EDTA and 0.5 % bovine serum albumin). Labelled cells were then passed over immunomagnetic LS columns, washed twice with MAC buffer and the positive fraction eluted after removal of LS columns from the MIDI MACS device. FACS analysis showed the cells to be 99 % routinely pure.

Dendritic cells were generated from $CD14^+$ PBMC isolated by immunomagnetic selection (Miltenyi Biotec). PBMCs were incubated with CD14 microbeads for 15 minutes at $4°$ C (20 µl of beads/10^7 PBMCs), and washed in MACS buffer (PBS containing 2 mM EDTA and 0.5

% bovine serum albumin). Labelled cells were then passed over immunomagnetic LS columns, washed twice with MAC buffer and the positive fraction eluted after removal of LS columns from the MIDI MACS device. These were cultured at a concentration of 10^6 cells/ml in CellGenix DC Media (CellGenix Technologies, Illinois) in 6 well plates supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (R & D systems, GM 215-010), (800U/ml final) and IL4 (1000 U/ml final) cytokines (R&D Systems, Minneapolis, MN systems, 214-IL-010). Cells were cultured for 1 week and matured with tumour necrosis factor (TNFα; 10 ng/mL final) (R&D Systems 210-TA-010) and prostaglandin E2 (1μg/mL final) (Sigma P5640) for 2 days. The phenotype and purity of the dendritic cells was verified by FACS staining using phycoerythrin (PE) conjugated monoclonal antibodies specific to human CD14, CD83 and CD86 (BD Biosciences CA, USA). Dendritic cells were irradiated at 30 Gy prior to being used as stimulators for MLRs.

For LCL generation, 100μl (5 x 10^6 PBMCs) were infected with concentrated supernatant from the B95-8 EBV-producer cell line (100μl), in the presence of ciclosporin 1μg/ml (Sigma C3662) to inhibit EBV specific CTLs that would prevent LCL outgrowth. LCLs were cultured in RF10 medium consisting of RPMI 1640 (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The cells were plated on a 96 well plate at a density of 5 x 10^5/well. Cultures were fed with half volume medium exchange weekly. When clumps were seen, they were progressively transferred to a 24 well plate, and then a T25 flask. LCL were irradiated (70 Gy) prior to being used as stimulators for MLRs

CFSE Staining and MLRs

Purified CD3$^+$ T-cells were labelled with 2.5 μM CFSE (Invitrogen, Carlsbad, CA C34554) for 15 minutes at 37° C. The reaction was stopped by quenching with AB serum (Sigma Aldrich, Dorset, UK) and then the cells were washed twice in RPMI 1640 containing 10 % AB serum. CFSE-labelled T-cells were co-cultured with HLA-mismatched allogeneic irradiated DC at a responder: stimulator ratio of 5:1. A negative control consisting of CD3$^+$ CFSE stained T-cells alone was used. Cells were cultured at a concentration of 2x10^6/ml in AIM V serum free media (Invitrogen, Carlsbad, CA).

Flow cytometric Staining and Analysis

FACS were performed using PE/PerCP/APC- conjugated monoclonal antibodies specific for human CD3, CD25, CD69, CD71, HLA-DR, OX40, ICOS, CD95, CD45RA, CCR7, interferon-γ (IFN-γ), tumour necrosis factor alpha (TNFα), and interleukin-2 (IL-2). All antibodies were purchased from BD Biosciences (San Jose,CA, USA) except for CCR7 (R & D systems, Minneapolis, MN). Brefeldin (1 µg/ml) (BD Biosciences) was added for a period of 8 hours. All tubes were stained with CD3 and CD25, allowing gating on the CFSE-dim, CD3$^+$, CD25^{-ve} population. Staining was performed on samples taken from MLR cultures at days 0, 1, 3, 5, and 7. Voltages were determined by acquiring events on unstained co-cultures. Corresponding isotypic monoclonal antibodies were used to determine cut off points for positive populations. Co-cultures were stained with positive controls (either CD3 FITC, CD3 PE, CD3 PeCy5, CD3 PERCP or CD3 APC) and isotypic monoclonal antibodies to determine appropriate compensation controls. All tubes contained CFSE, CD3 and CD25 except cells which were stained with CD45 RA and CCR7 PerCP, which were co stained with CD3 and CFSE. This allowed gating on the CFSE Dim CD25 negative population. 2x10^5 cells were incubated with human AB serum for 20 minutes at 4°C and then were stained with antibody for 30 minutes at 4 ° C and then washed twice in FACS buffer (PBS and 1 % calf serum) and were then resuspended in 1 % paraformaldehyde for 20 minutes.

To simultaneously analyse surface molecules and intracellular cytokines, samples were first stained for surface antigens, then fixed with 2% paraformaldehyde (incubated PBMCs for 20 minutes in paraformaldehyde) to stabilize the cell membrane. They were then washed twice in FACS buffer and permeabilized with 0.5 % saponin to allow anti-cytokine antibodies to stain intracellularly (incubate PBMCs with saponin at room temperature for 10 minutes). PBMCs were then washed in saponin and were incubated with anti cytokine antibodies resuspended in a total of 50 µl of saponin. They were then incubated at 4ºC for 30 minutes and washed twice in saponin and resuspended in FACS buffer. Samples were acquired using 4-colour flow cytometry on a FACS LSR (BD biosciences). Analysis was done using WinList software (Verity Software House). The proliferative index and precursor frequencies were derived using ModFit LT software (Verity software House). For FACS analysis in allodepletion experiments, a CyAn flow cytometer (Dako, Fort Collins, CO) was used to acquire data and Summit v4.1 software (Dako) to analyze data.

Generation of Allodepleted Donor T cells and Comparison of Allodepletion Methods

Normal donor peripheral PBMCs and HLA-mismatched irradiated (70Gy) recipient LCLs were resuspended at 2×10^6/ml in AIM V media. PBMCs were co-cultured with or without LCLs at a responder: stimulator ratio of 40:1 in T-175 flasks for 3 days. For comparison of CD25 immunomagnetic bead and CD25 IT depletion, on day 3 of the MLR, co-cultures were split into 2 arms. Anti-CD25 microbeads (20 µl microbeads/10^7 PBMCs, Miltenyi Biotec, Bergisch Gladbach, Germany 130-092-983) were added to half the co-culture and 4 µg/ml CD25 IT (RFT5-SMPT-dgA) was added to the other half. CD25 immunomagnetic negative selection was performed according to manufacturer's instructions using LD columns and depletion using CD25 IT was performed overnight as follow; co cultures were harvested and resuspended at 10^7/mL in immunodepletion medium consisting of AIM V supplemented with 20 mM ammonium chloride (Sigma, St Louis, MO) to improve the bioactivity of the immunotoxin with pH adjusted to 7.75 using Na HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma). 0.22 µm filtered RFT5-SMPT-dgA anti-CD25 immunotoxin was then added and the next morning co-cultures were washed twice and then resuspended at 2×10^6/mL in AIM V. The CD25^{-ve} fractions were resuspended at 2×10^6/ml in AIM V. Unmanipulated donor PBMC:LCL co-cultures and donor PBMCs alone were used as controls. 2×10^5 cells from unmanipulated and allodepleted day 3 co-cultures were sampled in triplicate for FACS analysis and primary proliferation assays. The remaining cells were rested in AIM V medium in 24 well plates at 2×10^6 per well for 2 days prior to secondary stimulation in secondary MLRs and enzyme-linked immunospot (ELISPOT) assays.

For combined CD25/71 allodepletion, day 3 PBMC:LCLs co-cultures were washed and resuspended in 60 µl of FACS buffer (PBS with 1 % calf serum) per 10^7 PBMCs. Biotinylated anti-CD71 antibody (BD Biosciences 555535) was added (20 µl of antibody/10^7 PBMCs) for 15 minutes at 4ºC. Cells were then washed and labelled with anti-CD25 beads and anti-biotin beads (Miltenyi Biotec 130-090-485) using 20 µl of antibody/10^7 PBMCs for 15 minutes at 4ºC. Immunomagnetic depletion of CD25/71 labelled cells was then performed on LD columns according to manufacturer's instructions. For combined CD25/45RA depletions, PBMCs were labelled with CD25 and CD45RA beads (Miltenyi Biotec 130-091-092), and depletions were done according to manufacturer's instructions. Aliquots from the allodepleted negative fractions were analysed flow cytometrically. The remainder of the negative fraction

was resuspended in AIM V media at 2 x 10^6/ml and rested for 2 days and were then analysed for residual alloreactivity in secondary MLRs or IFN-γ ELISPOT assays.

Scale Up studies

In order to optimize our scale up studies of CD25/71 allodepletion we first compared allodepletion between clinical grade CD25 immunomagnetic beads (Miltenyi 274-01) with a biotinylated CD25 and clinical grade anti biotin beads (Miltenyi 173-01). Day 3 PBMC: LCLs co-cultures were split into 2 arms: CD25/71 allodepletion was performed as above for one half of the co-culture. The remaining half was washed and resuspended in PBS (60µL/ 10^7 PBMCs). Biotinylated anti-CD25 (gift of Dr. Marina Cavazzana-Calvo, 2 µg/10^8 PBMCs) and biotinylated anti-CD71 (BD biosciences) were added to the remainder for 15 minutes at 4ºC. The co-culture was washed and antibiotin beads were added and the depletion was performed using LD columns as described above.

In order to determine if our data using CD25/71 allodepletion could be replicated using CliniMACs device (Miltenyi 151-01), we performed scale up studies in the cell therapy laboratories at Great Ormond Street Hospital. Donor PBMCs were co cultured with irradiated host DCs (R:S 10.1) in cell culture bags (Miltenyi Biotec 200-074-301) and a combined CD25/71 allodepletion (using biotinylated CD25+71) was done on day 4 using a CliniIMACs device using depletion programme 1.2. Two sets of CliniMACs tubing were used to perform the depletion; CLINIMACS depletion tubing set (Miltenyi 266-01) and TS tubing (Miltenyi Biotec 161-01). Samples were taken after depletion for FACS to determine the efficacy of depletion, and for sterility testing. The allodepleted cells were rested for 2 days and restimulated to host/3rd party in a 2º MLR.

Proliferation assays

Primary proliferation assays were performed by pulsing unmanipulated or allodepleted co-cultures on day 5 with 1 µCi ^{3}H-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK) per well for 16 h. ^{3}H-thymidine incorporation was measured with a MicroBeta TriLux (Perkin-Elmer Weiterstadt, Germany). Data are presented with the mean cpm of triplicate responder alone and stimulator alone subtracted from mean cpm of test cultures.

To assess residual alloreactivity in secondary MLRs, 5 days after the initial co-culture 2×10^5 allodepleted T-cells were restimulated with 5000 irradiated LCLs from either the original stimulator or HLA mismatched 3rd party. Controls consisted of unmanipulated PBMC which were frozen on day 0 and thawed on the day of plating, allodepleted T-cells alone or LCLs alone. After 5 days, cells were pulsed with ^{3}H-thymidine and uptake assayed the next day

IFN-γ ELISPOT assay

To further assess residual alloreactivity, on day 5 after primary stimulation, 2×10^5 allodepleted donor T-cells or thawed, unmanipulated donor PBMCs were plated per well in the presence of 2×10^5 irradiated (30 Gy) host or 3rd party LCL stimulators in triplicate for 18 - 24 hours at 37°C. Controls consisting of 2×10^5 responders or stimulators alone were also plated. MAHAS4510 plates (Millipore, Billerica, MA) were coated with anti–IFNγ capture antibody MAB91 DIK (Mabtech, Cincinnati, OH) overnight and blocked with RF10 medium for 1 hour at 37°C. 2×10^5 allodepleted donor T cells or thawed donor PBMCs per well were plated in the presence of 2×10^5 irradiated stimulators in triplicate wells for 18 to 24 hours at 37°C. Controls consisting of 2×10^5 responder alone, and 2×10^5 stimulators alone were also plated. Plates were cultured for 18-24 hours and then washed and incubated for 2 hours at 37°C with biotinylated–anti-IFNγ detection antibody 7-B6-1 (Mabtech). Avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was added for 1 hour at room temperature and spots developed with 3-amino-9-ethylcarbazole (AEC, Sigma) substrate mix. The numbers of spots were counted using a plate reader (Bioreader 3000, Bio-Sys GmbH, Karben, Germany), the means of triplicate wells calculated and expressed spot-forming cells per 10^5 cells. The mean number of specific spot-forming cells was calculated by subtracting the mean number of spots produced by responder alone and stimulator-alone wells from the mean number of spots in test wells for each dilution.

Assessment of Antiviral Immunity

For pentamer analysis, 10^6 PBMC or allodepleted T-cells were co-stained with CD8 FITC, CD3 PerCP and either isotype PE control antibody or PE-conjugated pentamer appropriate to the donors' HLA restriction. The following pentamers were used to detect virus-specific CD8+ T cells (ProImmune, Oxford, United Kingdom): *CMV pp65*- HLA-A*0201-NLVPMVATV

(A2-NLV), and HLA-B*0702-TPRYTGGGAM (B7-TPR); *EBV- LMP-2-* HLA-A*0201-CLGGLLTMV (A2-CLG). The PBMCs were stained with 5 µl PE-labelled pentamer for 15 min at room temperature, washed and co-stained for surface expression of CD8-APC and CD3-PerCP. PBMCs from donors with known positive populations served as positive controls and PBMCs from normal donors negative for the restricting HLA-type were used as additional negative controls. A total of 200 000 events in the lymphocyte gate were analyzed where possible and the percentage of tetramer-positive cells in the $CD3^+/CD8^+$ lymphocyte gate was expressed as a proportion of the $CD8^+$ cells with the isotype control subtracted. For a population to be labelled as positive, at least 50 $CD3^+CD8^+$ tetramer-positive cells with the staining characteristics of the positive control population had to be acquired. The percentage of tetramer-positive cells in the $CD3^+/CD8^+$ lymphocyte gate was expressed as a proportion of the CD8+ ve cells.

Functional responses to CMV, EBV and adenovirus were analyzed in IFN-γ ELISPOT assay. The following stimulators were used to monitor antiviral responses of the allodepleted PBMCs: To assess EBV responses, unmanipulated PBMCs or allodepleted PBMCs were co-cultured with irradiated (70Gy) autologous LCLs. To assess CMV responses, unmanipulated PBMCs or allodepleted PBMCs were co-cultured with irradiated autologous PBMCs pulsed with pp65 pepmix (1 µg/ml).The pp65 pepmix was purchased from JPT Peptide Technologies GmbH (Berlin, Germany) and consists of 138 different pp65 peptides. Autologous PBMCs were co incubated with pp65 pepmix for 1 hour at 37ºC, washed twice, then resuspended at 2 $x10^6$/ml in AIM V and then irradiated to 30Gy. We examined the response to adenovirus by stimulating unmanipulated PBMCs or allodepleted PBMCs with autologous PBMC transduced with Ad5f35-GFP; The Ad5f35-GFP vectors were purchased from Baylor College of Medicine (Houston, TX). Autologous PBMCs were transduced with the Ad5f35GFP (Multiplicity of infection MOI 20) for 2 hours at 37ºC washed twice, resuspended at 2 $x10^6$/ml in AIM V and then irradiated at 30Gy. The titre of the AD5f35GFP was 1 x 10^{10} plaque forming units/ml. To control for GFP responses, autologous PBMCs were pulsed with Vaccinia-GFP (MOI 3)

PCR amplification of CD19 chTCR Sequences

The ScFv CD19ζ plasmid was supplied by Dr. Martin Pule. The CD19 chimeric TCR (CD19R) transgene consists of the variable domains of the CD19 specific murine monoclonal antibody FMC-63 assembled as a single chain variable fragment (ScFv), in frame with a

sequence encoding the human IgG1 hinge CH2-CH3, the human CD28 transmembrane domain, and the cytoplasmic signalling domain of the human CD3ζ. The CD19R transgene was subcloned into the Not 1 and Bam H1 sites of the pHR –SIN-SE lentiviral vector[164] to create the construct CD19R/ pHR –SIN-SE (Fig 39).

The 1946 base pair fragment of the CD19 chTCR was amplified by PCR. Not 1 restriction restriction sites were added to the 5′ end of the reverse primer, and a Bgl II restriction site was added to the 5′ of the forward primer so that the fragment could be subcloned into the Not 1 and Bam H1 sites of the pHR –SIN-SE lentiviral vector (gift of Dr. Martin Pule).

Forward 5′GATTCGGCACTGAGATCTGCCACCATGGAGACCGACACCCTGCTGC 3′
Reverse 5′AGCCTGGACACTGCGGCCGCACGCGTCATCTGGGTGGCAGGGCCTG 3′

PCR

PCR reactions were performed in a total volume of 25μl containing 100ng template DNA, forward and reverse primers at 0.5μm, dNTPs, each dNTP at 200μM and 2.5U of Taq DNA polymerase in the appropriate buffer (Promega). The PCR was performed using an Eppendorf AG 22331 Themocycler utilising the following programme set at 35 cycles: 98ºC for 2 minutes, 98ºC for 30 seconds, 67ºC for 2 minutes, and 72ºC for 3 minutes, and then hold at 4ºC. PCR products to be used for cloning procedures were purified using a QIAquick PCR purification kit (Qiagen) as per the manufacturer's instructions.

Enzyme digestion of Plasmid DNA

Plasmid DNA (typically 0.5-5μg) was digested in a final volume of 25-50 μl of 10 x buffer (supplied by manufacturer and diluted x1 with distilled water) and bovine serum albumin (0.1 mg/ml). The amount of enzyme used contained an excess of 5-10 units/μg DNA according to manufacturer's instructions (Promega). The digest was incubated at 37ºC for 3 hours. The pHR –SIN-CSGW lentiviral vector was digested with BamH1 and Not1 excising the GFP sequence and creating overlapping ends for the CD19 chTCR. The amplified CD19 chTCR fragment was digested with Not 1 and Bgl II. The DNA digestion was verified by agarose gel electrophoresis.

Agarose Gel Electrophoresis and Gel Purification of Fragments

DNA fragments were resolved by electrophoresis thorough 1 % agarose gels in 1 x TAE buffer (40 mM Tris-acetate, 5mM EDTA). To prepare the gels, agarose was dissolved in 1x TAE buffer by boiling in a microwave, and after cooling, ethidium bromide was added at 0.5μg/ml for visualisation of DNA. DNA samples were mixed with Orange G loading buffer (10Mm Tris Ph 7.5, 50Mm EDTA, 10% Ficol 400, 0.4 % Orange G) before loading onto agarose gels. A 1kb plus ladder DNA ladder (bioline) was loaded onto each gel to enable size determination of DNA fragments. Gels were electrophoresed using a voltage of 50-100 V (up to 150 mA) and the separated DNA fragments subsequently visualised by exposure to ultra-violet light using the UVIdoc gel documentation system. Following electrophoresis DNA fragments were excised from agarose gels using a clean scalpel blade under ultra-violet light. The DNA was extracted from agarose using a QIAquick gel extraction kit (Qiagen) as per manufacturer's instructions.

Ligations

Ligations were performed using a vector to insert ratio of ratios of 1:3 or 1:6 (100 ng vector DNA) in a final volume of 20 ul with 1.5 ul of quick ligase (NEB). The reaction was incubated for 5 minutes at room temperature. A vector only control sample was also included to provide an estimate of the re ligated plasmid. The ligation reaction was immediately transformed into competent E.Coli DH5 alpha cells (NEB)

Bacterial Transformation

Competent DH5α E.Coli were transformed by heat shock. 25 μl of competent cells were slowly thawed on ice and mixed with 1-10ng of DNA from the ligation mix. The cells/DNA were incubated on ice for 30 minutes and then heat shocked by placing the mixture for 35 seconds at 42ºC and then transferred back onto ice. 250 μl of LB media was then added to the cells, and the mixture was transferred to a 5ml tube. The cells were shaken at 250 rpm at 37ºC for 1 hour, after which they were diluted in LB media and spread on LB ampicillin agar plates. The plates were incubated overnight at 37ºC, after which colonies were picked using sterile 20 μl pipette tips and grown overnight in 5 ml liquid cultures.

Small Scale Plasmid DNA Preparation

Plasmid DNA was prepared using Qiagen Mini-Prep kits as per manufacturer's instructions from overnight single colony inoculums. Test cuts with EcoR1/Not1 (predicted size 2.5 kb and 8.5 kb), EcoR1/BamH1 (predicted size 1.3 kb and 9.7 kb) and Not1/BamH1 (predicted size 1.15 kb and 9.86 kb) were done to determine clones with the correctly inserted CD19R fragment.

Large Scale Plasmid DNA Preparation

For large scale plasmid DNA preparation 500 ml LB media containing ampicillin (50µg/ml-Sigma) was inoculated with 500 ul of a fresh 5 ml culture and incubated overnight at 37ºCwith agitation (250 rpm). Plasmid DNA was subsequently prepared using Qiagen Mega-Prep kits as per manufacturer's instructions. After overnight culture at 37ºC with shaking, bacteria were pelleted, by centrifugation at 4000xg for 10 minutes and resuspended in the presence of 100µg/ml RNAase. Plasmid was purified by alkaline lysis of bacteria, followed by binding of plasmid DNA to anion exchange resin columns under appropriate low salt and pH conditions (Qiagen Maxi/Mega Prep Kits). The columns were washed to remove non-DNA fractions and the plasmid DNA was eluted using a high salt solution and then desalted by precipitation with isopropanol. The plasmid DNA pellets were washed in 70 % ethanol and then resuspended in pyrogeneic-free water. Correct assembly of the CD19 chTCR was verified by DNA sequencing. Plasmid DNA concentration was calculated by measuring the absorbance of light at a wavelength of 260nm (A_{260}) using a nano dropND-1000 spectrophotometer with a 0.2 mm path length. At this wavelength 50µg/ml of double stranded DNA has an absorbance of 1.

Lentivirus Preparation and Transductions

Lentivirus Production

1.2×10^7 293 T cells grown in complete DMEM were seeded in 175 cm^2 tissue culture flasks the day before transfection. Lentiviral vector DNA (40µg) and packaging plasmids pMDG.2

carrying the envelope transgene (VSV-G) (10μg) and pCMVΔ8.74, carrying the lentiviral transgene (gag-pol)(30μg) were added to 5 mls OPTI-MEM, filtered through a 0.22μm filter, and combined with 5 ml filtered OPTI-MEM supplemented with 1 ul 10mM polyethylenimine (PEI) transfection reagent. The transfection reaction was incubated at room temperature for 20 minutes during which time the 293T cells were washed once with OPTI-MEM media. The 10 mls DNA/PEI complexes were subsequently added to the cells and they were then incubated at 37ºC/5%CO$_2$ for 4 hours, after which the media was replaced with 14 mls of complete DMEM. Viral supernatant was harvested at 48 and 72 hours post transfection, filtered through a 0.22μm filter and concentrated by ultracentrifugation at 23,000 rpm for 2 hours. Lentiviral pellets were resuspended in 100μlm serum free media (OPTI-MEM), stored on ice for 20 minutes and then snap frozen in aliquots at -80ºC.

Titration of Lentiviral Supernatants

Virus transduction was determined by transduction of 293 T cells. 1 x 10^5 cells were seeded in complete DMEM in 24 well plates and left to adhere overnight. The media was subsequently aspirated and serial dilutions of lentiviral supernatant in a total volume of 100μl of optimem were added. 72 hours post transduction cell dissociation media was added to the 293 T cells and the infectious viral titre was determined by analysis of transgene positive cells by flow cytometry. The titre of the virus was determined as below:

No of virus agents/ml= % of transduced cells x no of cells in the well
_____

Vol of virus used to infect cells

Wells giving 5-15 % transduction was used to determine titre.

Transduction of PBMCs and Allodepleted T cells

PBMCs were resuspended at a concentration of 1 x 10^6 /ml in CTL media (45% Click's medium, Irvine Scientific, Santa Ana, CA, 45% RPMI 1640, Hyclone, Logan, UT and 10% FCS, Hyclone) in 24 well plates. IL-2 (R&D Systems 212-IL-010) was added on day 0 only at 100 u/ml and incubated at 37ºC/5 % CO$_2$ for 4 days. At 96 hours concentrated lentiviral supernatant (MOI 150, volume 7.5 μl- from soups which have given titre of >10^{10} viral

agents/ml) was added directly to 1ml of cell culture and the cells were subsequently analysed for transgene expression 96 hours later.

For positive controls, PBMCs were transduced after polyclonal stimulation with agonistic anti CD3 (1μg/ml) (eBiosciences 16-0037) and agonistic anti CD28 monoclonal antibodies (1μg/ml) (caltag laboratories CD2800). They were resuspended in CTL media at a concentration of 1 x 10^6/ml in 24 well plates. On day 1, IL-2 was added at 50u/ml, and then on day 2 further IL-2 was added at 100u/ml and concentrated lentiviral supernatant was added at a multiplicity of infection (MOI) of 20.

To transduce allodepleted PBMCs, Day 3 CD25/71 allodepleted donor T cells were resuspended in CTL media[165] at a concentration of 10^6/ml supplemented with IL-2 100 u/ml (R &D systems) in 24 well plates. On day 7, the PBMCs were transduced using a multiplicity of infection (MOI) of 150 and were harvested on day 11. Expression of the CD19R transgene was determined flow cytometrically as described below. Mock transduced allodepleted cells were treated in the same way except no lentiviral soup was added on day 7.

Flow cytometric Analysis of Transgene Expression

A goat anti-human IgG FcγCy^{tm}5 antibody (Jackson ImmunoResearch, West Grove, PA) was used to detect cell surface expression of CD19chTCR. This antibody binds to the human IgG1 CH2-CH3 hinge of the CD19R. 1.5 μg of antibody was added to 2 x 10^5 PBMCs for 30 minutes at 4ºC followed by two washes before acquisition. For positive controls PBMCs polyclonally stimulated with agonistic anti CD3/28 antibodies were used, whilst allodepleted PBMCs which were mock transduced were used as negative controls. For FACS analysis, a CyAn flow cytometer (Dako, Fort Collins, CO) was used to acquire data and Summit v4.1 software (Dako) to analyze data.

Functional Assays of Anti-Leukemic Responses

Measurement of Granzyme B and IFNγ production

MAHAS4510 plates (Millipore, Billerica, MA) were coated with anti–granzyme B capture antibody GB10 (Mabtech, Cincinnati, OH) or IFNγ capture antibody overnight (described above). They were then washed and blocked with RF10 medium for 2 hour at 37°C. Triplicate samples of αCD19ζ TCR transduced or mock transduced allodepleted donor T cells were co cultured with $CD19^{+/-}$ tumor cell lines (K562, K562 stably transduced with GFP or a CD19-GFP transgene, Ramos), autologous/allogeneic LCLs or 1° ALL blasts at a responder: stimulator ratio of 1:1. After 18 hours granzyme B production or IFNγ was assessed in a granzyme B ELISPOT/IFNγ ELISPOT assay. Controls consisting of 1×10^5 responders or stimulators alone were also plated. Plates were cultured for 18-24 hours and then washed and incubated for 2 hours at room temperature with biotinylated–anti-granzyme B detection antibody GB11 (Mabtech) or IFNγ detection antibody. Avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was added for 1 hour at room temperature and spots developed with 3-amino-9-ethylcarbazole (AEC, Sigma) substrate mix. The plates were read and the number of specific spot forming cells was determined.

Cytotoxicity assays

Cytotoxic specificity was determined in a standard ^{51}Cr release assay. 3×10^6 target cells were labelled with 100 µCi ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at 37°C. ^{51}Cr-labelled K562 cells (CD19 positive, negative and non transduced) and LCLs were plated at 5×10^3 cells per well, respectively, and cultured with transduced and non transduced PBMCs at different concentrations (effector to target ratios: 30:1, 5:1 and 1:1) in 96-well U-bottom plates. One percent Triton X-100 (Sigma-Aldrich) was added to measure maximum release and target cells were incubated alone to assess spontaneous release. After 4 h of incubation at 37°C, plates were spun and 25 µl supernatant were harvested and transferred to 96-well Wallac isoplates (Perkin-Elmer, Weiterstadt, Germany) and mixed with 150 µl OptiPhase Supermix Cocktail (Perkin-Elmer). Counts were measured on a MicroBeta TriLux (Perkin-Elmer) and the percent specific lysis was calculated as ([experimental release - spontaneous release] / [maximum release - spontaneous release]) × 100.

Flow Based Cytotoxicity assay

An alternative cytotoxicity assay was based on assessment of cell viability using FACS. Normal donor PBMCs were transduced/mock transduced in media supplemented with OKT3 (1μg/ml) anti CD28 (1μg/ml) and IL-2 (100U/ml) to serve as a positive control, or IL-2 100u/ml only as described. Transduced/mock transduced PBMCs were co-cultured with an equal number of K562 GFP+CD19+ or K562GFP+CD19- cells. After a week, the number of viable GFP+ cells was determined by staining with 7AA-D. A fixed number of trucount beads (BD biosciences) was acquired to ensure that comparison could be made between the different co-cultures. The number of GFP+ viable (7AA-D negative) cells was then determined in each co-culture and the percentage cytotoxicity was determined by dividing the number of viable targets cells in the presence of transduced PBMCs by the number in the mock transduced control co-culture.

Statistical Analysis

A Wilcoxon matched pairs test was used to determine statistical differences between samples (GraphPad Software Version 5.0, San Diego, CA). Data pertaining to flow cytometry is expressed as mean ± SD, whilst ELISPOT and MLR data is expressed as median and range.

Chapter 3

Functional Characterisation of Alloreactive T cells

Aims

1. To characterize the activation marker and cytokine profile of proliferating alloreactive T-cells identified using CFSE dye dilution.

2. To determine the phenotype of the CD25 negative proliferating alloreactive cells to provide a rational basis for enhancing allodepletion with CD25 based strategies

Introduction

Adoptive immunotherapy with allodepleted donor T-cells generated using CD25 IT [141] at a dose of 3 x 10^5/kg accelerated T-cell reconstitution and recovery of CMV and EBV-specific immunity after haplo-SCT. However, the rate of leukaemic relapse rate was high, resulting in an overall survival of only 5/16 patients. This may be explained by the high-risk nature of this patient group and the low precursor frequency of leukaemia-reactive T-cells within the infused allodepleted T-cells. Additionally, 2 patients died of adenovirus associated complications, including 1 who had persistent adenoviraemia despite 3 infusions of allodepleted donor T-cells at 10^5/kg, which cleared after a single infusion at 2.5 x 10^6/kg. Importantly, no patient had detectable T-cell responses to this virus before 9 months post-SCT. These data suggest that larger doses of allodepleted T-cells may be necessary to confer protective responses to pathogens which evoke low frequency T-cell responses in the donor and for a graft-versus-leukaemia (GVL) effect. Our *in vitro* data indicates that significant residual alloreactivity persists after CD25 IT mediated allodepletion[125]. While the incidence of significant acute and chronic GVHD was low in the clinical study, this was nonetheless observed in 2 cases, raising concerns about the safety of administering larger doses of allodepleted donor T-cells in the haploidentical setting. Thus in order to develop this approach further, there is a pressing need to enhance the degree of depletion of alloreactive cells, to enable add back of sufficient T-cells for protective anti-leukemic and anti-infective responses without causing GVHD.

Activated T-cells express a variety of surface markers, including CD25, CD69, CD71, CD95, CD137, CD147, OX40, ICOS, HLA-DR, and secrete Th_1 cytokines IL-2 and IFN-γ. As such, a plethora of potential targets and methods now exist for allodepletion strategies[59,114-118] but there is no data on the relative expression of these targets on alloreactive T-cells to enable

identification of the optimal targets for allodepletion. The data above suggest that a significant minority of alloreactive T-cells are retained after CD25-based allodepletion, suggesting that CD25 may be expressed only in a subset of alloreactive T-cells and raising the possibility that targeting other molecules, perhaps in combination with CD25, could enhance allodepletion. In order to rationally design strategies for enhanced allodepletion, we have functionally characterized the phenotype of alloreactive T-cells. While alloreactive T-cells have multiple phenotypes, proliferation in response to alloantigens is their most basic hallmark. We have identified proliferating alloreactive T-cells using CFSE dye dilution. Godfrey et al[24] have shown that flow cytometric depletion of CFSE-dim T-cells almost completely abrogates *in vitro* alloreactivity in secondary MLRs and markedly reduces GVHD in an MHC Class II disparate murine model. While it is not practical to use this approach clinically, we have used this method to systematically characterise the expression of cytokines, effector molecules and activation markers on proliferating alloreactive T-cells, in particular those that are CD25 negative.

In this chapter we have characterised the phenotype of proliferating alloreactive T cells against a wide range of activation markers and intracellular cytokines. In particular we went onto examine the phenotype of proliferating CD25-ve alloreactive T-cells.

Optimisation of Intracellular Cytokine Staining

To simultaneously analyse surface molecules and intracellular cytokines, samples were first stained for surface antigens, then fixed with 2% paraformaldehyde to stabilize the cell membrane. They were then washed twice in FACS buffer and permeabilized with 0.5 % saponin to allow anti-cytokine antibodies to stain intracellularly. PBMCs were then washed in saponin and were incubated with anti cytokine antibodies resuspended in a total of 50 µl of saponin. They were then incubated at 4ºC for 30 minutes and washed twice in saponin and resuspended in FACS buffer before acquisition. Optimisation of intracellular staining required careful dose titration of anti human IFNγ, TNFα and IL-2 antibodies. To determine this, human PBMCs were stimulated for 6 hours with PMA 0.1µg/ml (Sigma Aldrich), and 1µg/ml of ionomycin (Sigma Aldrich). Brefeldin (a golgi inhibitor which leads to an accumulation of intracellular cytokines, preventing their secretion) at 1µg/ml (BD biosciences) was also added

to culture. Optimal staining was achieved at the following concentrations of antibodies: IFNγ (B27 clone) at $0.2\mu g/10^6$ PBMCs, TNFα (MAB11 clone) at $0.5 \mu g/10^6$ PBMCs, and IL-2 (5344.111 clone) at $0.02 \mu g/10^6$ PBMCs.

After optimizing the intracellular cytokine antibody dose titration, we then went onto compare golgi inhibitors, brefeldin and monensin to determine which one was superior for detecting intracellular TNFα, IL-2 and IFNγ. PBMCs were incubated with PMA/ionomycin for 6 hours, with brefeldin or monensin added at the beginning of culture. The percentage of CD3 positive cells staining for each cytokine was then determined. (See Table 2)

	Brefeldin 1μg/ml	Monensin 2μM
CD3 IL-2	13.3	16.8
CD3 TNFα	31.5	22.6
CD3 IFNγ	15.6	14.7

Table 2: Golgi inhibitor Brefeldin is superior to monensin in detecting intracellular TNFα in activated PBMCs. % of T cells expressing each cytokine is shown. *n=3* (mean of the three values shown)

As can be seen from table 2, brefeldin led to far more TNFα significantly improved detection of TNFα with little difference between IL-2 and IFNγ accumulation between the different golgi inhibitors. Therefore for future experiments, brefeldin was used.

Subsequently, the optimal duration of incubation of brefeldin was determined. PBMCs were stimulated with PMA/ionomycin and co-cultured with brefeldin for 4, 6, or 8 hours and the percentage of CD3+ T-cells staining for each cytokine and cell viability (by trypan staining) was determined. (See Table 3).

	4 hours	6 hours	8 hours
CD3 IL-2	4.3	12.5	20.5
CD3 TNFα	10.2	30.8	38.9
CD3 IFNγ	12.3	14.8	23.8
Viability	96 %	94 %	91 %

Table 3: 8 hour incubation with brefeldin maximized intracellular cytokine detection without compromising cell viability % of T cells expressing each cytokine is shown *n=3* (values shown are mean)

As can be seen from Table 3, incubation for 8 hours leads to far more intracellular cytokine being detected, without the viability being affected. Increasing the time of incubation with brefeldin beyond 12 hours significantly affected the viability as did increasing the concentration above 1µg/ml. Thus, for future experiments an incubation time of 8 hours with brefeldin was chosen.

Finally a comparison was made between the efficacy of different cell permeabilization agents, the commercial PD wash (BD biosciences, CA, USA) and saponin 0.5 % (PBS and 1 % calf serum and 0.5 % saponin, filtered through 0.22 µm filter).
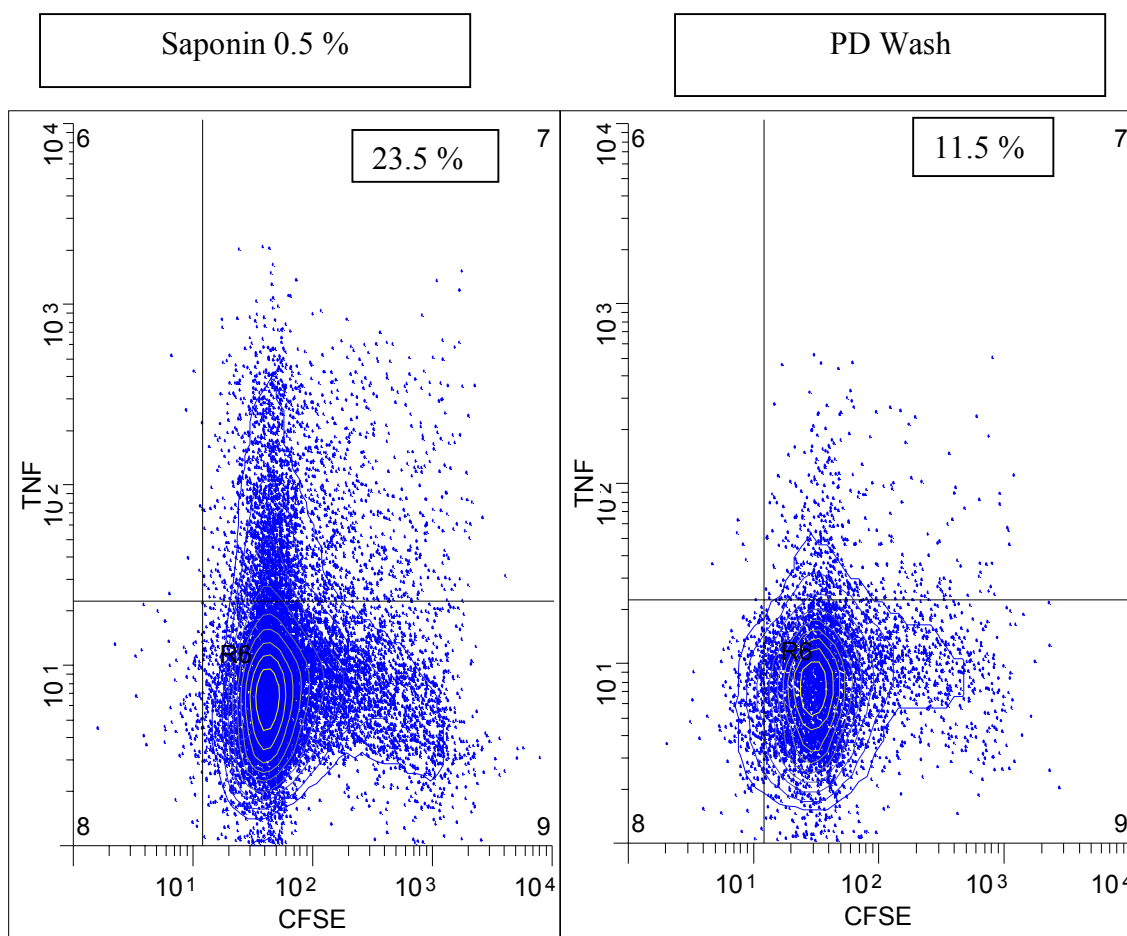
Figure 13: 0.5 % saponin led to superior detection of intracellular TNFα than with PD wash *n=2*

PBMCs were stimulated with PMA/ionomycin for 8 hours with brefeldin, fixed with 2 % paraformaldehyde and then permeabilized with either PD wash or 0.5 % saponin and the

percentage of lymphocytes expressing TNFα, IL-2 and IFNγ was determined. As can be seen from Figure 12, 0.5 % saponin was superior to PD wash in detecting intracellular TNFα. Similar results were obtained for IL-2 and IFNγ.

Thus in summary, in order to optimize our intracellular cytokine staining in alloreactive T-cells, we co-cultured PBMC with brefeldin 1μg/ml, for an incubation period of 8 hours and then after fixing, permeabilized the cells with 0.5 % saponin.

Kinetics of expression of Activation Markers and Cytokines during Allo-MLR

We initially determined expression of surface and cytokine markers in immunomagnetically selected CD3$^+$ lymphocytes cultured with HLA-mismatched mature DC over the course of a 7 day allogeneic MLR flow cytometrically. As controls, unmanipulated PBMCs from the same donors were cultured under identical conditions. The mean purity of CD3+ selected cells was 99.15 %. DCs showed the characteristic morphology and marker profile (mean % CD83- 97.35 %). Co-cultures were sampled for flow cytometric analysis of the CD3+ T cells in the lymphocytes gate on days 0, 1, 3, 5,and 7 of co-culture. Figure 13 summarizes the cumulative data from 5 donor-recipient pairs on up regulation of these markers on donor T-cells with unstimulated controls subtracted. CD25 was up regulated within 24 hours of an allogeneic MLR, peaking on days 3 (mean 47 % of total T-cells) and then plateaued. CD71 showed similar kinetics, peaking on day 3 (mean 39.3 % of total T-cells). This high level of expression was consistent cross all 5 donor-recipient pairs. There was little expression of CD25 or CD71 in the unstimulated T-cells (mean 1.4% and 2.6 % respectively on day 3). CD69 showed rapid up regulation on day 1 (mean 19 % of T-cells) but expression subsequently declined. HLA-DR, ICOS, and CD95 all showed similar patterns of expression, progressively increasing their expression with time during culture and peaking on day 7, but were all expressed on lower proportions of total T-cells. There was a strong up regulation of the Th$_1$ cytokines IL-2 and IFN-γ, with over 20 % of total T-cells secreting these cytokines by day 3-5 of co-culture. In contrast, TNFα secretion was only weakly up regulated throughout the period of co-culture.
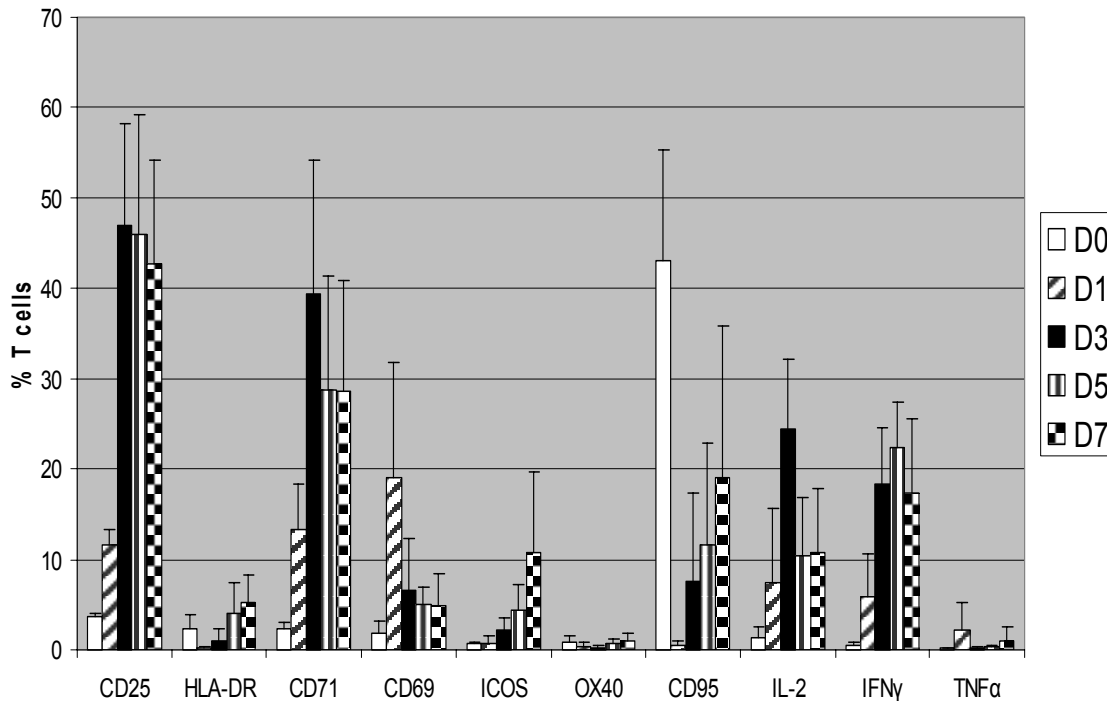
Figure 14: Kinetics of Surface Markers and Cytokine Expression in alloreactive T- cells.
(*n=5*) FACS analysis of expression of surface markers and intracellular cytokines in CFSE labelled T cells co-cultured with HLA- mismatched DCs. Results are mean + SD. Unstimulated control data has been subtracted from the stimulated data. As CCR7 and CD45RA showed high expression in unstimulated PBMCs they are not shown

As outlined above a key hallmark of an alloreactive T-cell is its ability to proliferate in response to alloantigen. In order to identify these alloreactive T-cells we labelled donor T-cells with CFSE and then co-cultured them with or without HLA mismatched dendritic cells. We then quantified the CFSE Dim (the alloreactive proliferating T-cell) population on days 0,1,3,5 and 7 allowing us to determine the precursor frequency and the kinetics of alloreactive T-cell proliferation in a MLR.
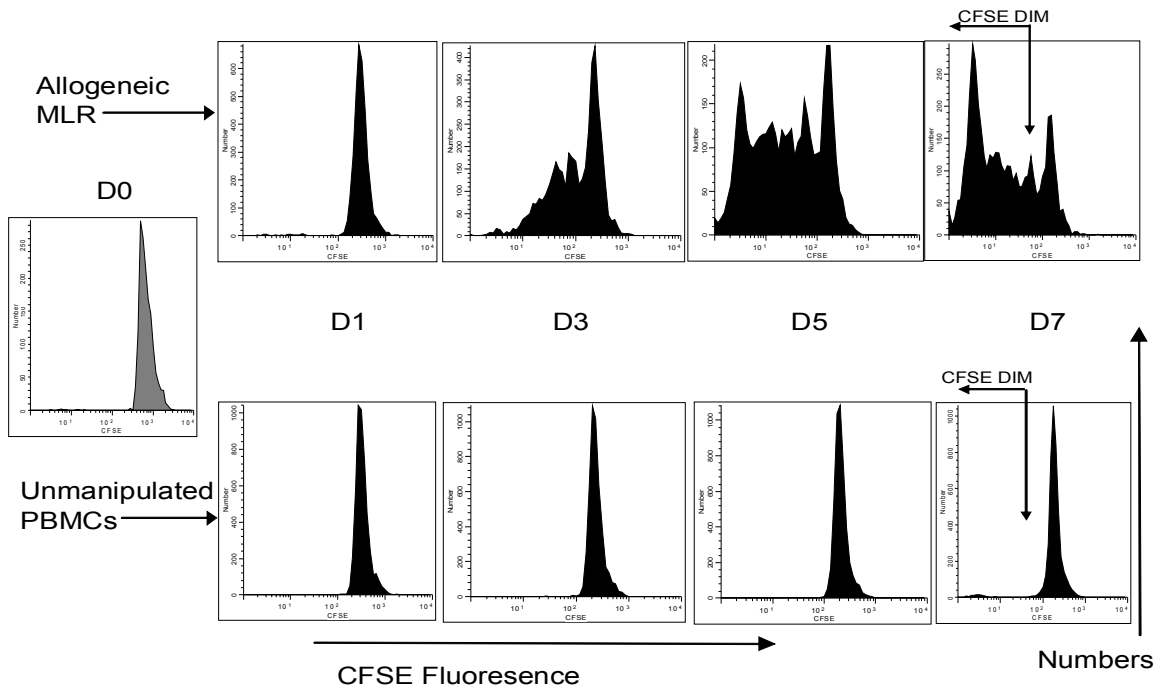
Figure 15: Kinetics of CFSE Florescence in Allogeneic MLR and Unmanipulated T-cells.(*n=5***)** FACS analysis of a representative example of the kinetics of CFSE fluorescence in the allogeneic MLR after CFSE labelled T-cells were cultured with/without HLA-mismatched DCs. Proliferation of alloreactive T-cells results in reduction in CFSE fluorescence intensity. Gating on the CFSE dim region, which selectively identifies the proliferating T cell population is shown on the day 7 FACS plots.

Identification of Proliferating Alloreactive T-cells using CFSE Dye Dilution

In order to characterize the phenotype of proliferating alloreactive T cells population we labelled donor T-cells with CFSE and then co-cultured them with or without HLA-mismatched DC. Gating on the CFSE-dim population enabled us to track the proliferating alloreactive T-cell population. We initially quantified the CFSE-dim population on days 0,1,3,5 and 7. Figure 14 shows the progressive dilution of CFSE fluorescence with time in an allogeneic MLR. By day 7 of the MLR culture, 70 % of the T-cells in the culture were CFSE-dim. The mean alloreactive precursor frequency (calculated using ModFit LT software (Verity software House) was 4.2 % ± 1.5 % and there was a mean of 8 cell divisions. In contrast, there was very little shift in fluorescence in the unmanipulated PBMCs. Figure 14 shows our gating strategy for identifying CFSE-dim cells, taking into account the shift in CFSE fluorescence of unmanipulated PBMC with time. To validate this gating strategy, we compared CFSE dye

dilution in CFSE-labelled unstimulated PBMCs and PBMCs co-cultured with autologous DC in 5 normal donors. (See Fig 15) As previously reported[166,167], T-cells cultured with autologous DC showed minimal proliferation (mean 1.3 % ± 1.19 % of CFSE-dim T-cells, at day 7 of co-culture) and little up regulation of CD25 or CD71. We are thus confident that the CFSE-dim population truly represents proliferating alloreactive T-cells.
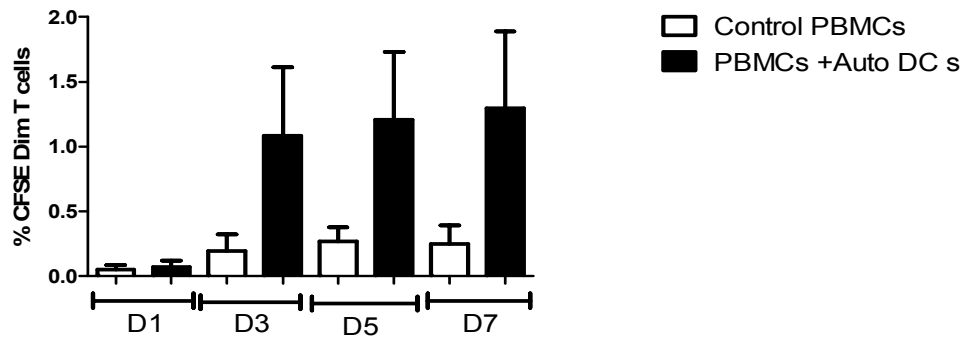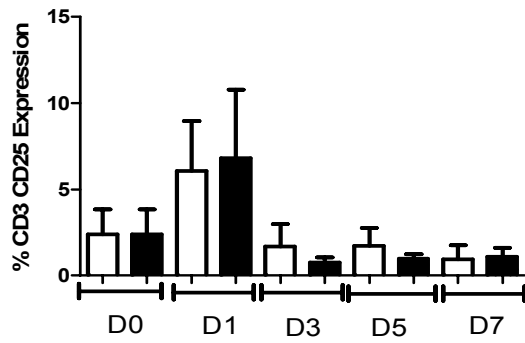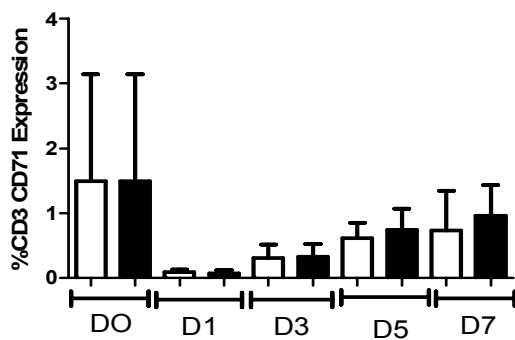
Figure 16: T-cells cultured with autologous DC showed minimal proliferation and little up regulation of CD25 or CD71. Activation marker Expression and Kinetics of CFSE Fluorescence in T cells co cultured with Autologous Dendritic Cells.(a) CFSE Fluorescence (b) CD25 expression (c) CD71 expression. Data is the mean ± SD of cultures from 4 donors

As shown in Fig 16, which illustrates the % of CFSE-dim T-cells in the MLR, proliferation was greatest between Days1-3 of culture, and then plateaued between days 5-7. This suggests that strategies targeting proliferating alloreactive T cells e.g. chemotherapy agents would be most successful between days 1-3 of the MLR.
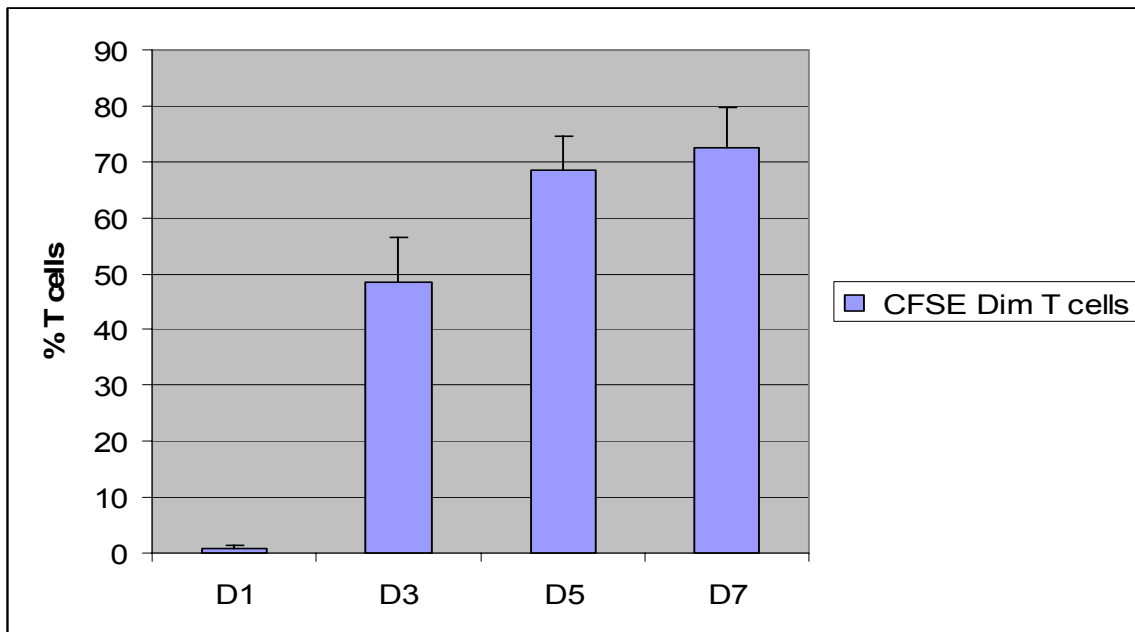
Figure 17: Time course of Proliferation in Allogeneic MLR. *(n=5)* FACS analysis demonstrating the percentage of T cells which are CFSE dim proliferating alloreactive T cells in the MLR, serially assessed over a week. Results are the mean + SD .The percentage of CFSE dim populations in the unstimulated control has been subtracted from the results obtained in the MLR.

Phenotypic Characterization of Proliferating Alloreactive T-cells

To determine the phenotype of alloreactive T-cells, we then analyzed surface marker and cytokine expression in the proliferating alloreactive T cell population by gating on the CFSE-dim population on days 3, 5, and 7 of the MLR. The cumulative data for 5 HLA- mismatched pairs is shown in Fig 17.
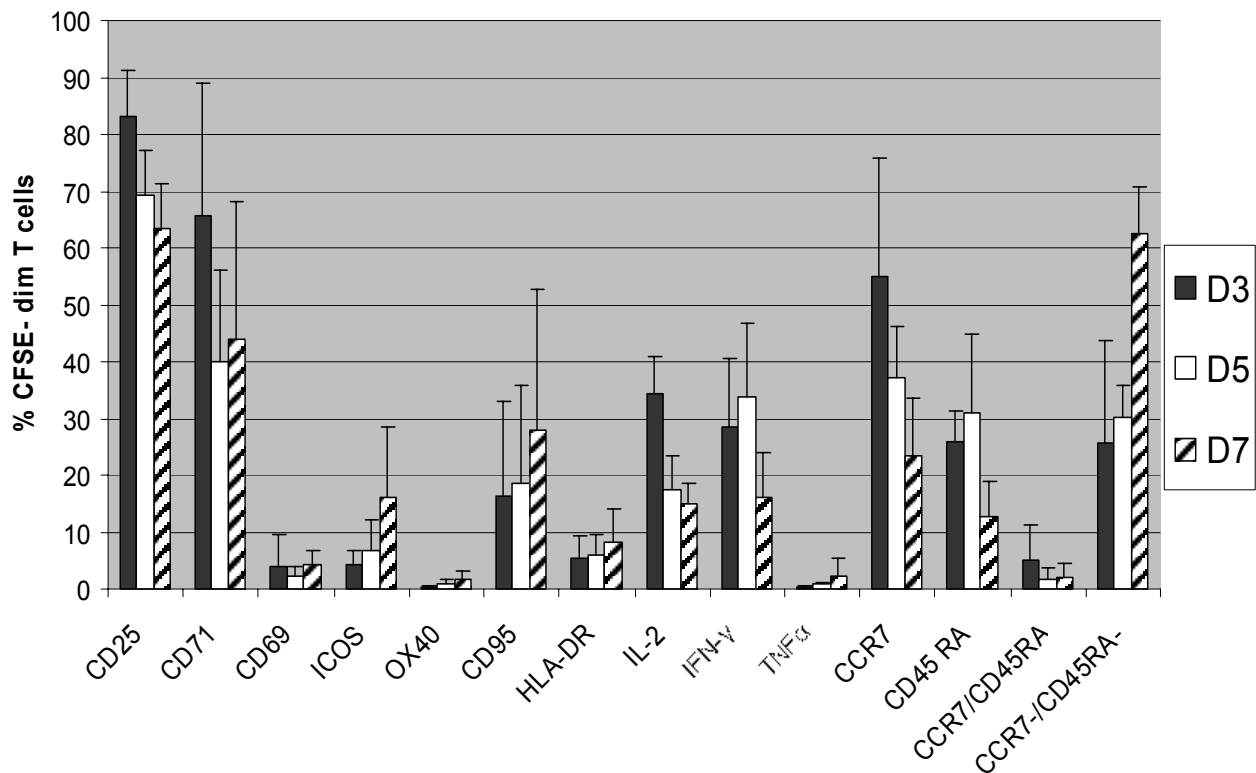
Figure 18: Kinetics of Surface Marker and Cytokine Expression in Proliferating Alloreactive T cells. (*n=5*) Normal donor PBMCs were co-cultured with HLA-mismatched DCs and the expression of surface markers and intracellular cytokines in the proliferating alloreactive T-cell population was determined by gating on the CFSE dim population using FACS analysis. Data shown is mean + SD.

CD25
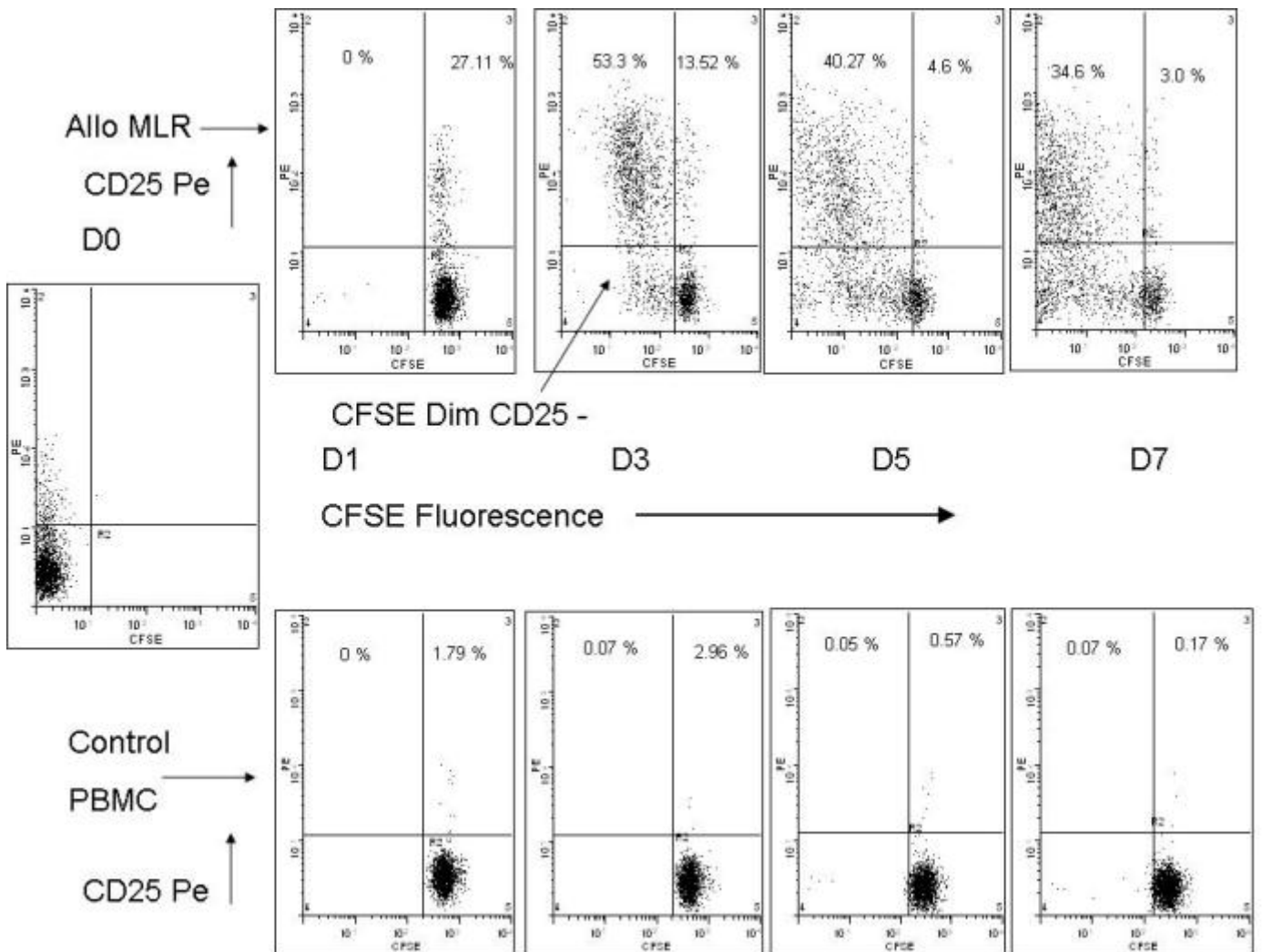
Figure 19: Up regulation of CD25 is seen in the CFSE-dim T cells but a Significant CFSE Dim CD25 negative population is discernible. Data is representative of CD25 expression in all 5 experiments
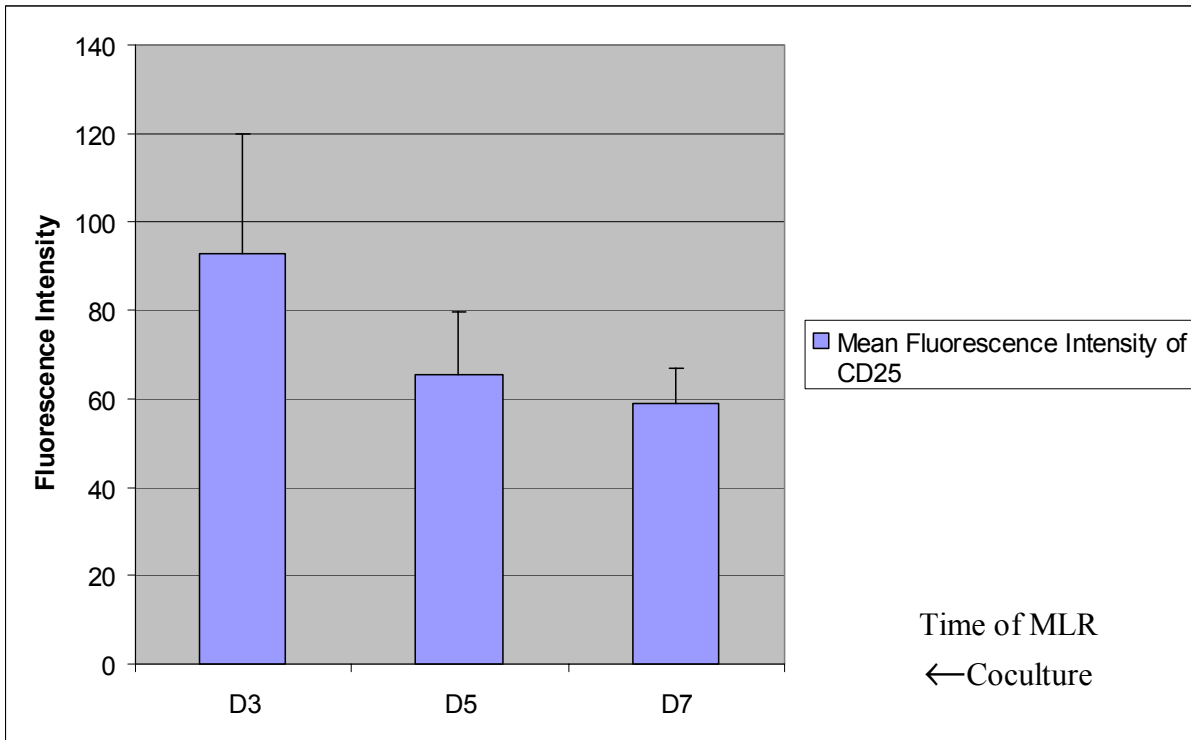
Figure 20: Mean Fluorescence Intensity of CD25 Expression on the Proliferating Alloreactive T cells peaks on day 3. Data is mean ± SD of 5 donor recipient pairs.

As seen in Fig 18, CD25 (IL-2 receptor α) shows little expression in resting PBMCs. However, in PBMCs co cultured with HLA mismatched DCs, it is up regulated within 24 hours, peaks on days 3 and then plateaus. Thus CD25 is strongly and consistently expressed on the proliferating alloreactive T-cells. In 5 donor recipient pairs a mean of 83 % ± 8 % of the proliferating T-cells express CD25 on day 3 (Fig 17). The mean fluorescence intensity of CD25 peaks on day 3 (see Fig 19) thus confirming that the optimal time for CD25 allodepletion is on days 3 of a MLR. This data validates the strategy of using a day 3 CD25 allodepletion. However, as can be seen in the representative donor shown in Figure 18, a significant population of CFSE dim T-cells do not express CD25 .Therefore CD25 based negative selection strategies alone will not deplete a significant proportion (mean 17 % on day 3 see Fig 17) of proliferating alloreactive cells.

CD71

CD71 (transferrin receptor) expression had similar kinetics to CD25, being unregulated on the majority of proliferating T-cells by day 3 of MLR (mean 65 ± 23 % of the CFSE-dim T-cells), with some subsequent down regulation at later time points and somewhat more variability than CD25 (see Fig 17).
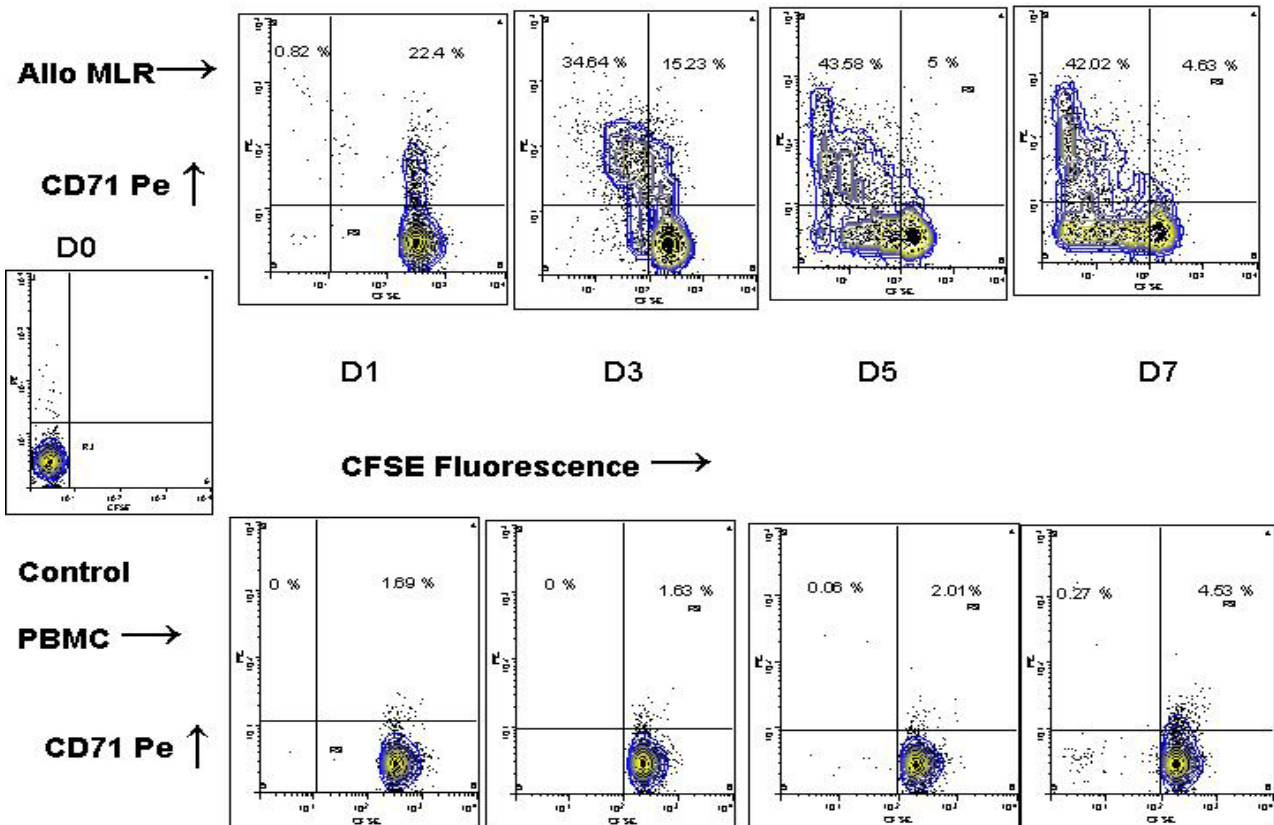
Figure 21: CD71 is strongly up regulated on proliferating alloreactive T cells but shows little expression on resting T cells. CD71 is up regulated on day1 and peaks on day 3 of the MLR. Data is representative of CD71 expression in all 5 experiments

There is very little expression of CD71 in the unstimulated T-cells, and thus allodepletion strategies targeting CD71 should preserve desirable antiviral and anti-leukaemic responses. (See Fig 20). Analysis of the mean fluorescence intensity of CD71 expression demonstrated this to be highest on day 3 of co-culture (Figure 21)
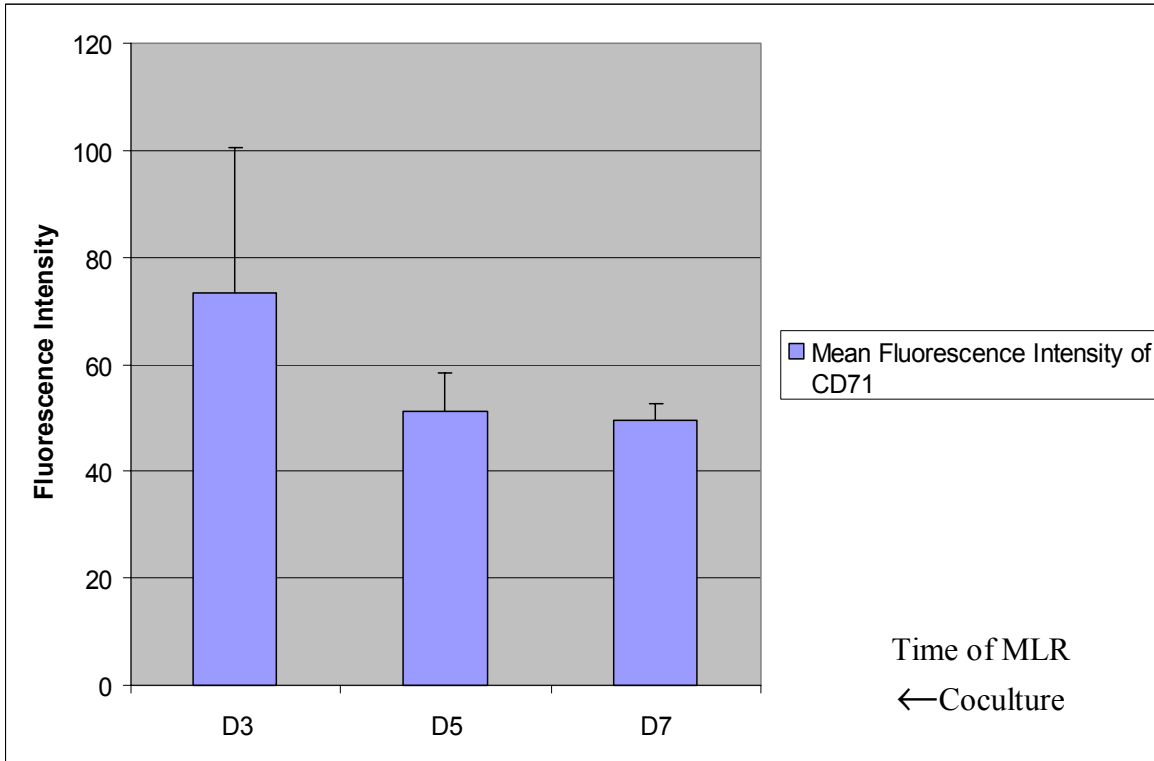
Figure 22: Mean Fluorescence Intensity of CD71 Expression on Proliferating Alloreactive T cells is highest on day 3. Data is mean ± SD for 5 donor recipient pairs

These data identify CD71 as a promising novel target antigen for depleting alloreactive T-cells and demonstrate that the optimal time point for CD71 allodepletion is on day 3 of a MLR. (See Figs 17 & 21)

CD69
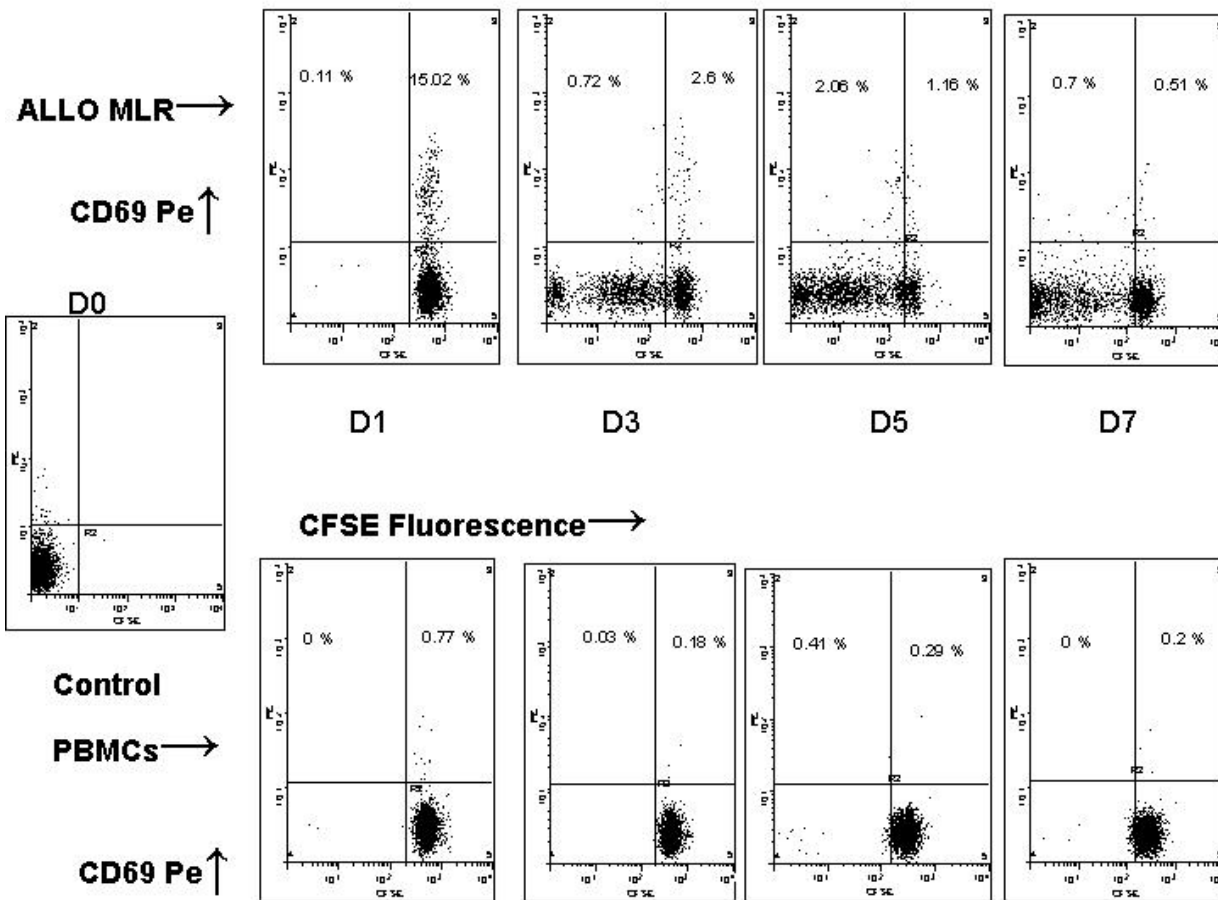
Figure 23: CD69 is the strongly up regulated on day 1 in the allogeneic MLR, but then rapidly declines. Data is one of a representative sample

As shown in Figure 17, the other activation markers analyzed were only expressed on a minority of proliferating CFSE-dim T-cells. CD69 was expressed on only 4 ± 5 % of CFSE-dim cells at day 3 of MLR. As a CFSE-dim population was only discernible from day 3 onwards (Fig 16), it was not possible to determine if the higher expression of CD69 on total T-cells at day 1 of the MLR (Fig 13) represents a truly alloreactive population.

As shown in the representative FACS plot in Fig 22, CD69 is the earliest marker to be up regulated, in an allogeneic MLR demonstrating significant up regulation by day 1. Up to 20 % of CD3+ve lymphocytes express this marker in the alloreactive MLR, with little or no expression in unstimulated control co-cultures. However, expression of CD69 decreases progressively with time of co-culture. In this regard, it is of note that groups who have used CD69 based strategies to deplete alloreactive T-cell on day 3 which may not be the optimal time point. As illustrated in Fig 17, the majority of the CFSE dim population do not express

116

CD69. This is likely to reflect down regulation of CD69 by the time appreciable CFSE dye dilution occurs, so that it is difficult to use the latter to identify alloreactive cells expressing CD69. Furthermore, compared to molecules such as CD25 and CD71, CD69 expression during allo MLR showed significantly more variability (Fig 17) limiting the usefulness of this marker as a target for allodepletion strategies. In this regard, it is of note that groups who have used CD69 to deplete alloreactive T cells have done so on day 3.[126]

Activation Markers

Inducible co stimulator (ICOS) was not expressed on unstimulated T-cells and was progressively up regulated over time in the allo-MLR, but only on a minority of proliferating alloreactive T cells (mean 15 ± 12 % at day 7) (see Figs 17) at day 7 with significant variability. **Fas (CD95)** was expressed on a higher proportion of unstimulated T cells. Like ICOS, CD95 showed a progressive but more variable increase over time in the allo-MLR with expression on proliferating alloreactive T cells peaking on day 7 (a mean of 27 ± 24 % at day 7 Fig 17). This is consistent with groups targeting Fas +ve alloreactive T-cells, who have shown that optimal timing for Fas mediated allodepletion is between days 5-7 of an allo- MLR. [66] However, the significant variability between donor recipient pairs, limits the usefulness of targeting this marker.

CD134 (OX40) was not expressed on unstimulated control, but showed little up regulation on the alloreactive T-cells, with maximal expression on day 7 of the allo-MLR (1.64 ± 1.42 % Fig 17). Our data indicates that targeting OX40 would not be a successful allodepletion strategy. **HLA-DR** showed low levels of baseline expression in the unstimulated control but was only expressed in small subpopulations of proliferating alloreactive T-cells (mean 8% $\pm5.87$of CFSE-dim T-cells at day 7, Fig 17). Unlike ICOS and CD95, HLA-DR expression did not show any significant increase in expression with time during the MLR.

Memory Markers (CD45RA and CCR7)

In light of the recent murine data suggesting that alloreactive T-cells recognising minor histocompatibility antigens[28,29] in murine models, reside predominantly in the naïve T cell compartment, we next examined expression of the CD45RA isoform and the chemokine receptor (CCR7) on proliferating alloreactive T-cells. We observed strong expression of CCR7 (mean 54 ± 20 % on day 3) and CD45RA (mean 25.9 % on day 3) in the CFSE-dim population (Fig 17), but there was also high expression on the unstimulated PBMCs at the same time-points (CCR7 mean of 52.7 % and CD45RA 12.3% of total T-cells on day 3) (see Fig 23 for representative example). The intensity of expression of CD45RA was greatest on day 3 (Fig 24) suggesting this would be the optimal time point for allodepletion. As time progressed, the percentage of CFSE-dim T-cells expressing CCR7 and CD45RA declined, consistent with a progressive increase in T-cells of effector memory phenotype (mean 62 ± 8% by day 7 of co-culture).

(a) CD45 RA

.

118
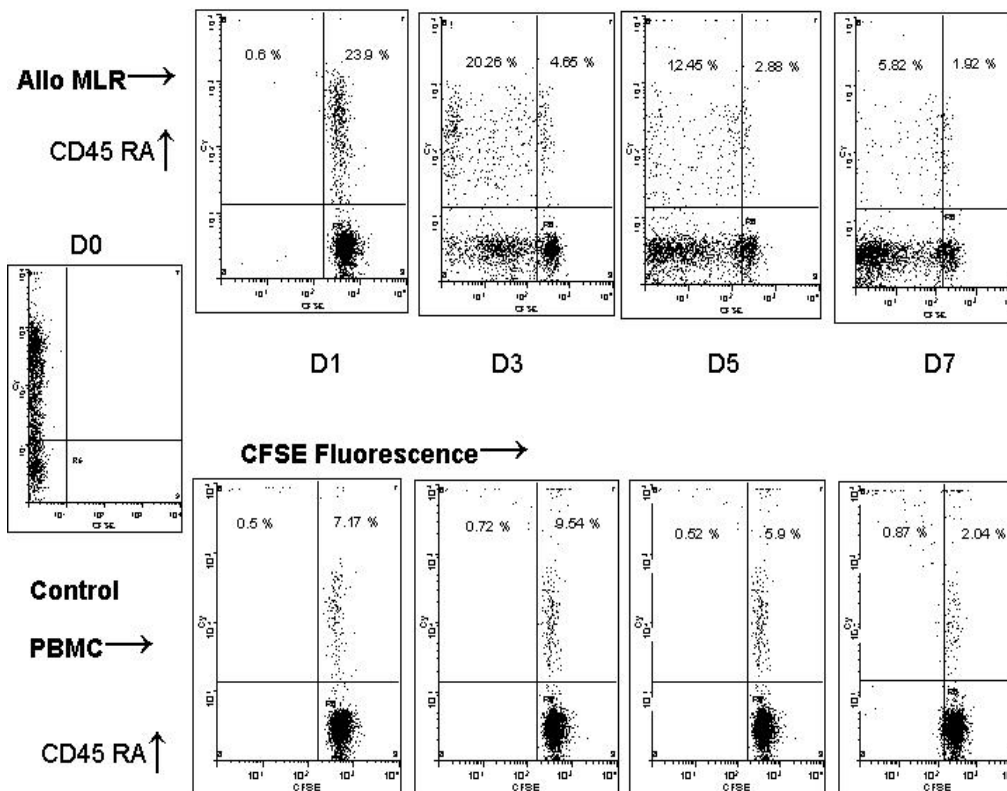
(b) CCR7
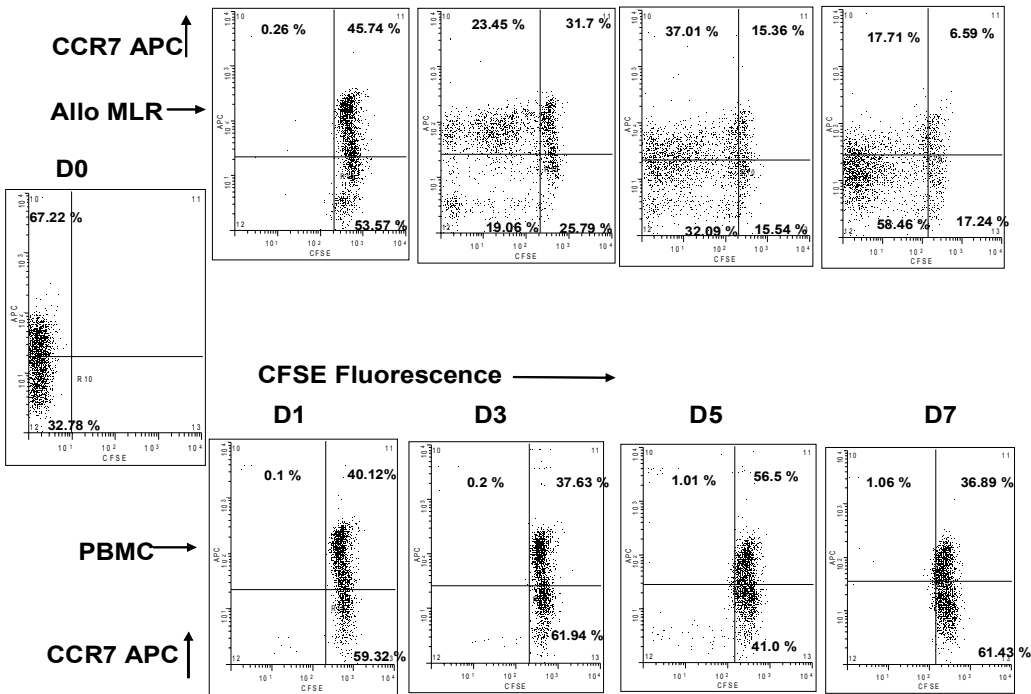
Figure 24: (a) **CD45 RA Expression shows significant expression on resting lymphocytes and progressively decreases during the time course of the MLR co culture.** (b) **Significant Expression of CCR7 on resting lymphocytes limits its usefulness as an Allodepletion Target**. Data is from a representative sample

While CCR-7 is expressed on approximately half of proliferating, alloreactive T-cells, CD45RA is only expressed on a minority of such cells, limiting its usefulness as a target of allodepletion. Further, as shown in Fig 23, these markers are expressed in a significant proportion of unstimulated PBMCs, and thus allodepletion approaches targeting CD45RA or CCR7 would lead to a large cell loss and could adversely affect desirable third party responses, anti-viral and anti leukaemic responses.
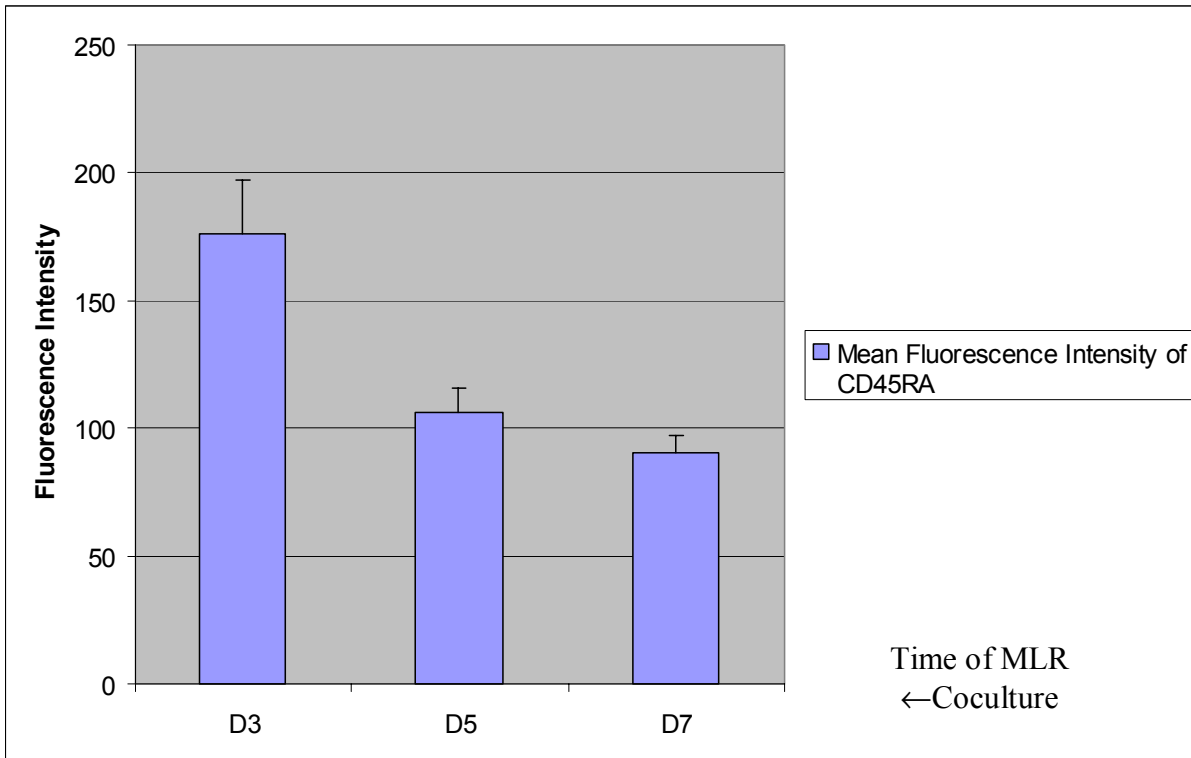
Figure 25: Mean Fluorescence Intensity of CD45RA Expression on Proliferating Alloreactive T cells peaks on day 3 Data is mean ± SD in 5 donor recipient pairs**.**

The intensity of expression in proliferating alloreactive T cells of CD45RA is greatest on day 3, (Fig 24) suggesting that this would be the optimal time point for an allodepletion.

Intracellular Cytokines

The presentation of alloantigen induces a response involving proliferation of donor T cells and secretion of interleukin-2 (IL-2) and interferon γ (IFNγ) leading to the generation of T cytotoxic clones. The central importance of IL-2 signalling in GVHD is illustrated by the use of ciclosporin and FK506, in GVHD prophylaxis, which inhibit IL-2 production. The presumed contribution of tumor necrosis factor-alpha (TNFalpha) to acute GVHD provides the rationale for the use of anti-TNFalpha antibodies (e.g. infliximab) in the treatment of GVHD[43]. We therefore next determined Th_1 cytokine expression on proliferating CFSE-dim T-cells using intracellular cytokine staining. As shown in Figure 17 & 25, there was little baseline expression of IL-2 in the unstimulated control T-cells, whereas IL-2 was up regulated in the alloreactive T cells in the allo- MLR. IL-2 expression peaked on day 3 of co-culture (mean 34 ± 6% of CFSE-dim T-cells), and progressively decreased thereafter.

120

IL-2
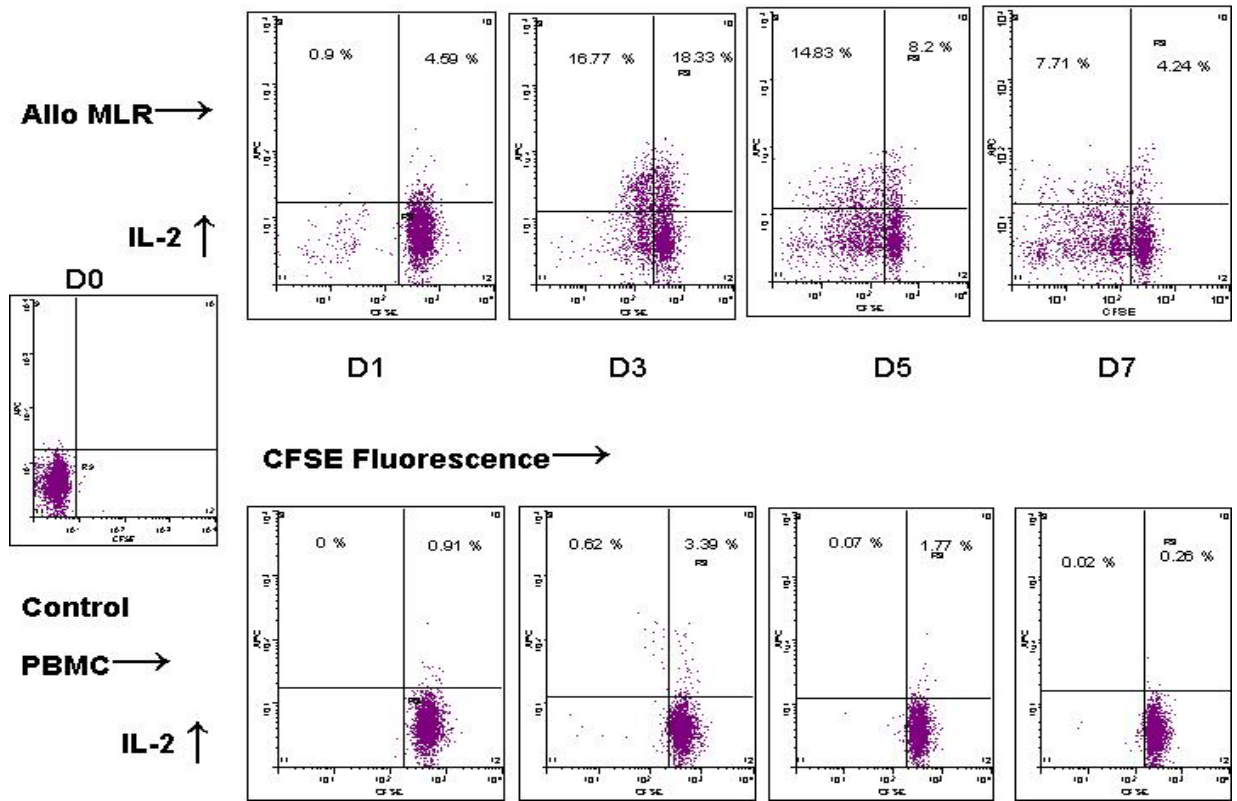
Figure 26: IL-2 is expressed in a minority of proliferating alloreactive T-cells Data is from a representative sample

IFNγ
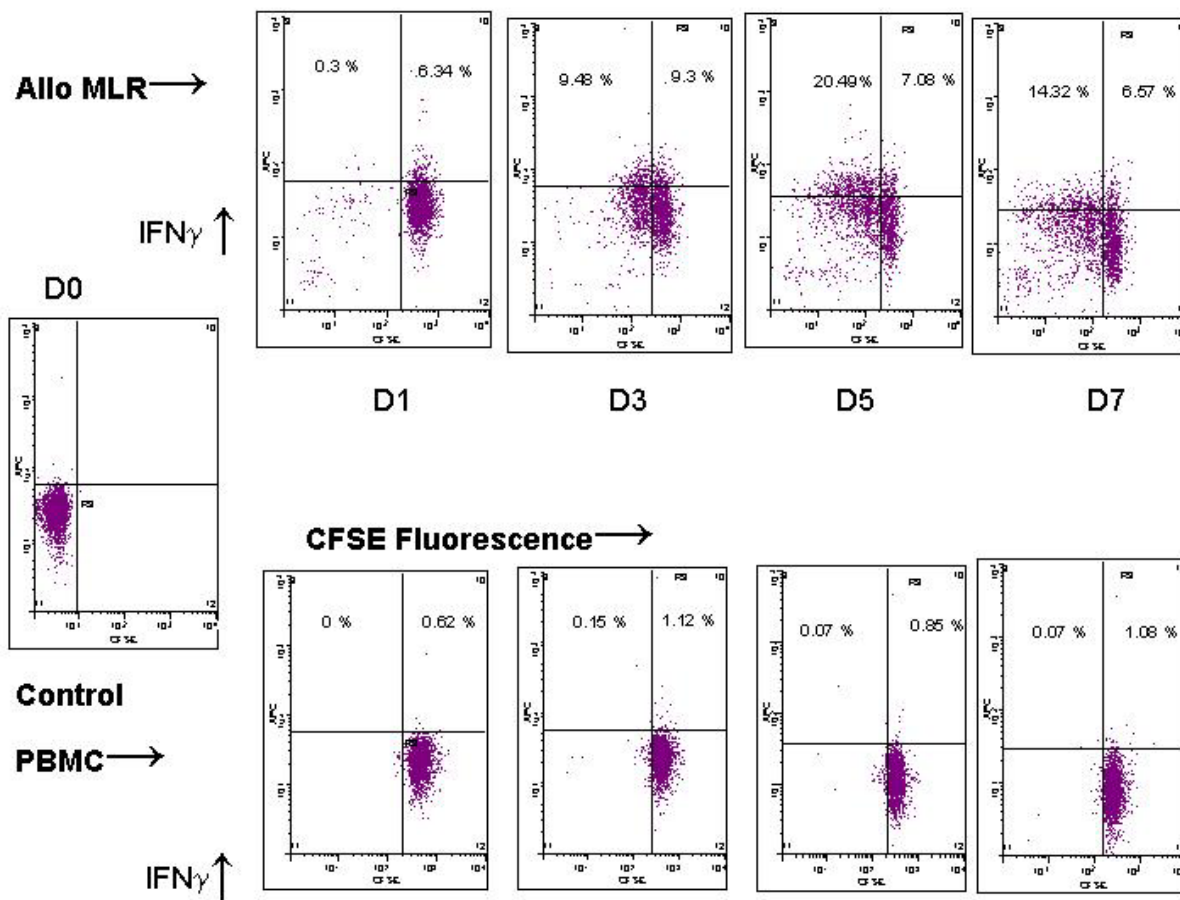
Figure 27: IFNγ is expressed on a minority of proliferating alloreactive T-cells. Data is from a representative sample.

IFN-γ was expressed in a similar proportion of the CFSE-dim population as IL-2. Expression of IFN-γ peaked on day 5 (mean 33 ± 13 %) and then decreased slightly by day 7 (Fig 17 & 26). Thus around a third of proliferating alloreactive T-cells express the Th_1 cytokines.

TNFα

In contrast to IL-2 and IFNγ, we observed little up regulation of TNFα in alloreactive T-cells at any time point during co-culture (mean 1 % on day 7 Fig 17). Thus any strategy targeting TNFα producing T-cells as a single step allodepletion procedure would be unlikely to be successful.

Overall, the majority of phenotypic markers analysed appear to be expressed only in small subpopulations of alloreactive T-cells, limiting their usefulness as potential targets for allodepletion strategies. Only CD25 and CD71 were expressed in the majority of T-cells proliferating in response to alloantigens. These data provides strong support for targeting CD25 in allodepletion strategies, and identify CD71 as a promising novel target for similar approaches.

Characterization of CD25-negative alloreactive T-cells

While the majority of proliferating alloreactive T-cells express CD25, a significant population (17 ± 8 % at day 3 of co-culture, Figure 17) does not and would be retained by strategies targeting this molecule alone. We therefore gated on the CFSE- dim, CD25^{-ve} T-cell population in order to determine phenotypic markers that could be used to target this CD25^{-} population. Data on the expression of surface markers and cytokines by this population are shown in Figure 27. Our studies identified CD71 and CD45RA as the markers expressed on the majority of CD25^{-ve} proliferating alloreactive T- cells (mean 69 ± 21 % and 62 ± 12 % respectively at day 3 of MLR). In 5 donor-recipient pairs, a mean of 94%/93% of proliferating alloreactive T-cells could potentially be deleted by effective depletion of cells expressing either CD71/CD25 or CD45RA/CD25 (Table 4). Other surface markers including CD69, ICOS, OX40, CD95, HLA-DR, CCR7, and the cytokines IL-2, IFN-γ, and TNFα were only expressed on a minority of cells in this population and thus would be of limited value in enhancing the depletion of alloreactive T-cells achieved with CD25-based approaches.
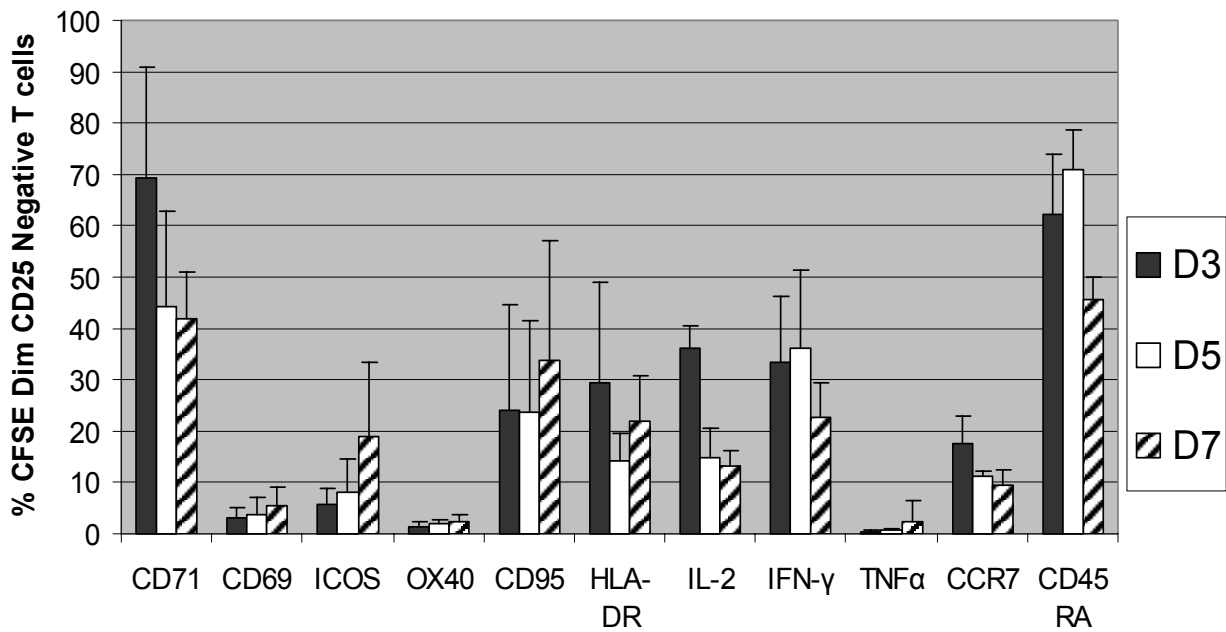
Figure 28: CD71 and CD45RA are highly Expressed in Proliferating CD25 negative Alloreactive T-cells. (*n=5*) CFSE labelled T-cells were co-cultured with HLA mismatched DCs and the kinetics of expression of surface markers and intracellular cytokines in the proliferating alloreactive CD25$^-$ population determined flow cytometrically by gating on the CFSE dim CD25$^-$ cells. Results are mean+ SD.

Table 4: **Percentage of proliferating alloreactive T-cells expressing markers**

	% of Proliferating Alloreactive T Cells Expressing Marker
CD25	83 %
CD25+ CD71	94 %
CD25+ CD45RA	93 %

Percentage of proliferating alloreactive T expressing CD25 alone, CD25 and CD71, and CD25 and CD45RA on day 3 of the MLR was determined by FACS analysis of CFSE Dim CD3 positive cells. *N=5*

Conclusions

We systematically characterized the activation and cytokine marker profile in proliferating alloreactive T-cells. While alloreactive T-cells can be identified by a number of phenotypes, we focused on proliferation in response to alloantigen as the most fundamental. Further, given the numbers of allodepleted T-cells infused in the clinical setting, alloreactive T-cells would need to proliferate in order to cause clinical sequelae. We co-cultured CFSE labelled T-cells with HLA- mismatched DCs, and tracked proliferating alloreactive T-cells by gating on the CFSE dim T-cell population. In our model, there was a mean of 8 cell divisions with up to 70 % of day 7 PBMCs becoming CFSE dim, with a precursor frequency to alloantigen of 4 %. Our data showed that CD25 was expressed in over 80 % and CD71 in 65 % of proliferating alloreactive T-cells on day 3 of MLR. In contrast, CD69, CD45RA, ICOS, OX40, CD95, HLA-DR, IL-2, IFN-γ, and TNF α were all expressed only in a minority of proliferating alloreactive T-cells, indicating that these are poor targets for allodepletion. CD69 is a well-established T-cell activation marker but was poorly expressed in the CFSE-dim population. This may in part reflect down regulation of CD69 by the time appreciable CFSE dye dilution has occurred (day 3 of MLR). Recent studies suggest that alloreactivity may reside predominantly in the CD45RA$^+$ naïve T-cell compartment.[29,33] However, the expression of this marker only on a minority of alloreactive T-cells and on bystander T-cells as well as the progressive maturation of alloreactive T-cells to a memory phenotype during MLR suggest that targeting this molecule as a sole strategy for allodepletion is unlikely to be successful.

In contrast, our data provide strong support for targeting CD25 in allodepletion strategies and identify CD71 as a novel target that is highly expressed on proliferating alloreactive T-cells. While our data confirm CD25 as an excellent target for allodepletion strategies, a mean of 17 % of proliferating alloreactive T-cells do not express CD25. Therefore, we studied the phenotype of proliferating CFSE-dim T-cells not expressing CD25. We have identified CD71 and CD45RA as the markers most highly expressed on proliferating alloreactive T-cells that do not express CD25. CD71 (transferrin receptor) is essential for iron transport into proliferating T-cells but is not expressed on resting lymphocytes, whereas CD45RA was expressed on a higher percentage of unstimulated T cells, and hence has a lower specificity for the alloreactive T-cell population. We found that 70 % of the CFSE-dim CD25^{-ve} population express CD71 and 62 % expressed CD45 RA. Potentially, this enables us to target 2

independent phenotypes of alloreactive T-cells at the same time-point within a single co-culture. We showed that the combination of CD25/CD71 and CD25/45RA is expressed in 94 % and 93 % respectively on proliferating alloreactive T-cells. Based on these data we then went on to compare CD25 based allodepletion strategies with CD25/71 allodepletion, to determine if depletion of CD71+ve T-cells would enhance the reduction of alloreactivity seen with CD25 based allodepletion.

Chapter 4

Enhancing CD25 based allodepletion strategies

Aims

1. To compare residual alloreactivity to host and 3rd party after CD25 based allodepletion with CD25/71 combined allodepletion

2. To compare residual alloreactivity to host and 3rd party after CD25/45RA based allodepletion with CD25/71 combined allodepletion

3. To develop a clinically applicable system for CD25/71 allodepletion under GMP conditions

Introduction

Adoptive immunotherapy with allodepleted donor T-cells generated using CD25 IT has been shown to enhance T-cell and anti-viral immune reconstitution in the haploidentical setting. [138,141] However, this and other studies have demonstrated the need to enhance allodepletion beyond CD25 based existing strategies as leukaemic relapse and infections with pathogens evoking low frequency T-cell responses, such as adenovirus remain problematic. [141] Higher doses of allodepleted donor T-cells need to be infused to enhance anti-viral and anti-leukaemic effects but this would require enhancement of CD25-based allodepletion if GVHD is to be avoided. Our data from Chapter 3 validate the use of CD25 as an excellent target for allodepletion and identified CD71 and CD45RA as the optimal markers to target CD25 negative proliferative alloreactive T-cells. These data suggest that a combined CD25/71 allodepletion might enhance allodepletion compared to existing CD25-based strategies. In this chapter we examined residual alloreactivity between combined CD25/71 immunomagnetic allodepletion against CD25 allodepletion.

Optimization of allodepletion

Prior to comparison of CD25 vs. combined CD25/71 allodepletion we initially optimised conditions for CD25-based depletion and measurement of residual alloreactivity.

Comparison of Dendritic cells, LCLs and PBMCs as APCs

In order to determine the optimum APC, in 4 donor recipient pairs, PBMCs were co-cultured with either HLA mismatched mature DCs, LCLs or cytokine pre-treated PBMCs The APCs were from the same donor for each experiment. The PBMCs were pre-treated with

recombinant human TNFα and IFNγ at 1000 iu/10^6 PBMCs in order to up regulate HLA class I and II expression and hence optimize presentation of alloantigen. Normal donor PBMCs were thus co-cultured with irradiated APCs [DC(R: S 10.1), LCLs(R: S 40.1) or cytokine treated PBMCs (R.S 1.1) from the same recipient]. As can be seen in table 5, flow cytometric analysis showed stronger up regulation of CD25 and CD71 in T cells co-cultured with allogeneic DCs or LCLs compared with cytokine treated PBMCs from the same donor. Similar results were obtained for the MFI of CD25 and CD71 (Table 6). In view of these results and our previous data showing that using LCLs rather than PBMCs as stimulators resulted in more consistent depletion of alloreactivity, LCLs were used as stimulators in subsequent experiments.

	CD3 CD25	CD3 CD71
Unmanipulated	2.44 ± 0.01	1.47 ± 0.24
BC +PBMCs	7.13± 2.32	1.91±1.22
BC + DCs	9.26± 4.72	5.71±4.37
BC + LCLs	10.9± 5.54	4.24±3.43

Table 5: Increased up regulation of CD25 and CD71 in co cultures with allogeneic DCs and LCLs compared with PBMCs. Normal Donor PBMCs were co cultured with irradiated HLA mismatched PBMCs or LCLs or Dendritic cells**.** FACS for % CD3+ CD25+ and CD3+ CD71+ population was performed on day 3 co cultures and unmanipulated control (*N=4*) Mean % ± 1SD

	CD3 CD25	CD3 CD71
Unmanipulated	39.23±0.99	65.30±5.9
BC +PBMCs	104.12±8.97	139.04±40.5
BC + DCs	216.64±99.53	175.87±36.29
BC + LCLs	143.70±28.79	148.2±54.48

Table 6: Increased MFI of CD25 and CD71 in co cultures with allogeneic DCs and LCLs Compared with PBMCs Normal Donor PBMCs were co cultured with irradiated HLA mismatched PBMCs or LCLs or Dendritic cells. FACS for MFI in the CD3+ CD25+ and CD3+ CD71+ populations was performed on day3 co cultures and unmanipulated control (*N=4*) Mean MFI± SD

Optimization of CD25 immunotoxin dose

To determine the optimum dose of our existing CD25 immunotoxin (RFT5-SMFT-dgA) for depletion of CD25+ alloreactive T-cells, donor PBMCs were co cultured with HLA-mismatched LCLs and then on day 3 the co cultures were exposed to varying concentration of CD25 immunotoxin (0 µg/ml, 3µg/ml, 3.5µg/ml, 4µg/ml, 4.5µg/ml, and 5 µg/ml). Co-cultures in the presence and absence of immunotoxin were incubated overnight at 37°C. The next morning co-cultures were washed twice and then resuspended at 2×10^6/mL in AIM V. Aliquots from the different co cultures were then analysed by Trypan blue staining for viability and FACS for the residual CD3+CD25+ (see Table 7) .

	CD3+ CD25 +	Viability
0 µg/ml	10.25	83.25
3 µg/ml	1.34	81.25
3.5 µg/ml	1.1	80.27
4 µg/ml	0.63	77.32
4.5 µg/ml	0.59	61.2
5.0 µg/ml	0.54	52.3

Table 7: Progressive increase in CD25 depletion with increasing concentrations of CD25 IT, but at cost of decreased viability. (*n=1*) Normal donor PBMCs were co cultured with HLA mismatched LCLs and a day 3 CD25 depletion was done with varying concentrations of CD25 immunotoxin. The residual CD3+CD25 + T cells were assessed by flow cytometry on day 4 and viability by trypan staining.

As can be seen in Table 7, progressive increases in CD25IT dose led to an increased depletion of CD3+ CD25 + T cells, but at a cost of decreasing cell viability. A dose of 4 µg/ml was chosen, as this led to an acceptable level of CD25+ depletion with reasonable preservation of cell viability.

Conditions for Resting Allodepleted cells prior to restimulation

Our group has previously shown that proliferative and cytokine responses to cytokine stimulation with host APCs are highly dependent on the timing of 2º stimulation in relation to allodepletion with negligible responses if restimulation was done immediately after depletion.[125] In order to skew our assays to optimize our assays detection of residual anti host responses, we rested cell for 2 days prior to restimulation. We next went on to compare the media in which allodepleted donors T cells should be rested in. Donor PBMCs were co-cultured with HLA mismatched LCLs and a day 3 CD25 immunomagnetic allodepletion was done. The allodepleted T-cells were then rested in AIM V (serum free) medium with or without IL-2 (100 U/ml). On day 5 the allodepleted T cells were then harvested and then restimulated with host or 3rd party LCLs in a 2º IFNγ ELISPOT assay.

	Host LCLs	3rd Party LCLs
AIM V	21.1	61.3
AIMV +IL-2	108.4	162.3

Table 8: Residual alloreactivity to host is enhanced when allodepleted donor T cells are rested in media supplemented with IL-2. Donor PBMCs were co cultured with HLA mismatched LCLs and a day 3 CD25 immunomagnetic allodepletion was done. The allodepleted T cells were then rested in AIMV with or without IL-2. Day 5 allodepleted donor T cells were then co cultured with host/3rd party LCLs in 2º IFNγ ELISPOT assay. Results show the percentage of spot forming cells compared to the response to unmanipulated PBMCs from the same donor. *n=1*

The residual IFNγ response to host was over 5 xs higher when the cells were rested in media supplemented with IL-2. (Table 8) This would imply that the IL-2 promotes the proliferation of alloreactive donor T cells that were not deleted by the allodepletion, and therefore lead to a higher residual alloreactivity. Therefore, for future experiments, the allodepleted donor T-cells were rested in AIM V media without IL-2.

Assaying Residual Alloreactivity

We next compared LCLs vs. PBMCs as secondary stimulators in MLRs and IFNγ ELISPOTS. Donor PBMCs were co-cultured with HLA- mismatched LCLs and a CD25 or CD25/71 immunomagnetic allodepletion was done on day 3. The allodepleted donor T-cells were rested for 2 days in AIM V media and then co cultured with host LCLs/PBMCs or 3rd party

LCLs/PBMCs in a 2º MLR. Results from allodepleted T-cells were compared with thawed unmanipulated PBMC from the same donor after stimulation with LCLs from the original stimulator or a 3rd party. To compensate for the variability in absolute counts, due to the degree of mismatch, results were standardized by calculating the residual proliferation as below:

cpm (donor PBMC+ Host LCL + Allodepletion)- cpm (donor PBMC + Allodepletion)
cpm(donor PBMC alone +LCL; no allodepletion) – cpm (donor PBMCs alone; no allodepletion)

	Host LCLs	3rd Party LCLs	Host PBMCs	3rd party LCLs
CD25	0	71.1	0	68.5
CD25/71	0	78	1.25	84.6

Table 9: Mean residual alloreactivity to host and third party is not significantly affected by using LCLs or PBMCs as 2 º stimulators. Donor PBMCs were co-cultured with HLA mismatched LCLs and a CD25 or CD25/71 immunomagnetic allodepletion was preformed. The rested cells were then restimulated to host or Third party LCLs/PBMCs in a 2º MLR. *n=2*

There was no significant difference in residual alloreactivity to host or 3rd party when either LCLs or PBMCs were used as stimulators in 2º MLRs (see Table 9). In IFNγ ELISPOT assays, the response of unmanipulated PBMCs to primary stimulation with allo LCLs was much greater than with allo PBMCs, so again LCLs were used as secondary stimulators in order to facilitate comparison between allodepleted and unmanipulated PBMCs (data not shown).

Comparison of CD25 Immunomagnetic Beads and CD25 Immunotoxin

To determine an optimum baseline allodepletion method, we first compared residual alloreactivity following allodepletion using CD25 immunomagnetic beads or IT in 6 donor-recipient pairs. In these and subsequent experiments, LCL were used as stimulators. Normal donor PBMCs were co-cultured with HLA-mismatched LCLs and at day 3 of MLR, cultures were split into 2 arms and allodepletion performed using anti-CD25 beads or IT as described in Methods. FACS analysis confirmed very effective depletion of $CD25^+$ cells with both methods (mean $CD3^+CD25^+$ $0.19 \pm 0.17\%$ after bead depletion, $0.97 \pm 0.38\%$ after IT p=NS).

Aliquots were sampled for the primary MLR to determine the median residual response to host calculated using the formula below.

Aliquots of the 1° MLR co-cultures were sampled to determine residual proliferative responses. To compensate for the variability in absolute counts due to the degree of mismatch, results were standardized by calculating the residual proliferation as below:

$$\frac{\text{cpm (donor PBMC+ Host LCL + Allodepletion)- cpm (donor PBMC + Allodepletion)}}{\text{cpm(donor PBMC alone +LCL; no allodepletion)} - \text{cpm (donor PBMCs alone; no allodepletion)}}$$

As shown in Figure 28a, in primary MLR residual proliferative responses to host were undetectable using either anti-CD25 bead or IT (median residual proliferation 0 ± 0 % for both). This reduction in proliferation compared to unmanipulated PBMCs was significant for both methods ($p<0.05$). However, our previous data[125] indicated that this assay may significantly underestimate residual alloreactivity. To skew our experimental system to detect such responses, allodepleted cell fractions were rested for 2 days and restimulated with LCLs from either the original stimulator or a 3rd party and residual responses assessed using IFNγ ELISPOT or 2° proliferation assays. To compensate for variability in responses between donor-recipient pairs, results from allodepleted T-cells were compared with thawed unmanipulated PBMC from the same donor after stimulation with LCLs from the original stimulator or a 3rd party.

In IFN-γ ELISPOT assays (Fig 28b), the median residual number of specific spot forming cells (SFC) after stimulation with host LCL was 38.3 % (range 17.6 – 110.7%) of the response of unmanipulated PBMC after anti-CD25 bead and 54.5 % (range 11.7 – 159.3%) after CD25 IT allodepletion (comparison between beads and IT p=NS). Third party responses were equivalent to unmanipulated PBMCs for both forms of allodepletion. There was no significant difference in the residual response to host following CD25 bead and IT allodepletion in these assays. In the 2° MLR (Fig 28c), the median proliferation to host stimulators was 27.8 % (range 0.05 – 91.5%) of the response of unmanipulated PBMC from the same donor after CD25 bead allodepletion, whilst for CD25 IT it was 16.5 % (range 0.03% - 62.2%) (comparison between beads and IT p=NS). Both depletion methods significantly reduced proliferation to host compared to unmanipulated PBMC s (p<0.05) but again there was no

significant difference in residual proliferative responses to host between CD25 beads and CD25 immunotoxin. Third party responses were equivalent to unmanipulated PBMC for both forms of depletion.

Since there was no significant difference in residual alloreactivity to the original host between the CD25-based methods, we used anti-CD25 immunomagnetic bead depletion in further experiments to minimise cell manipulation in combined depletion methods.
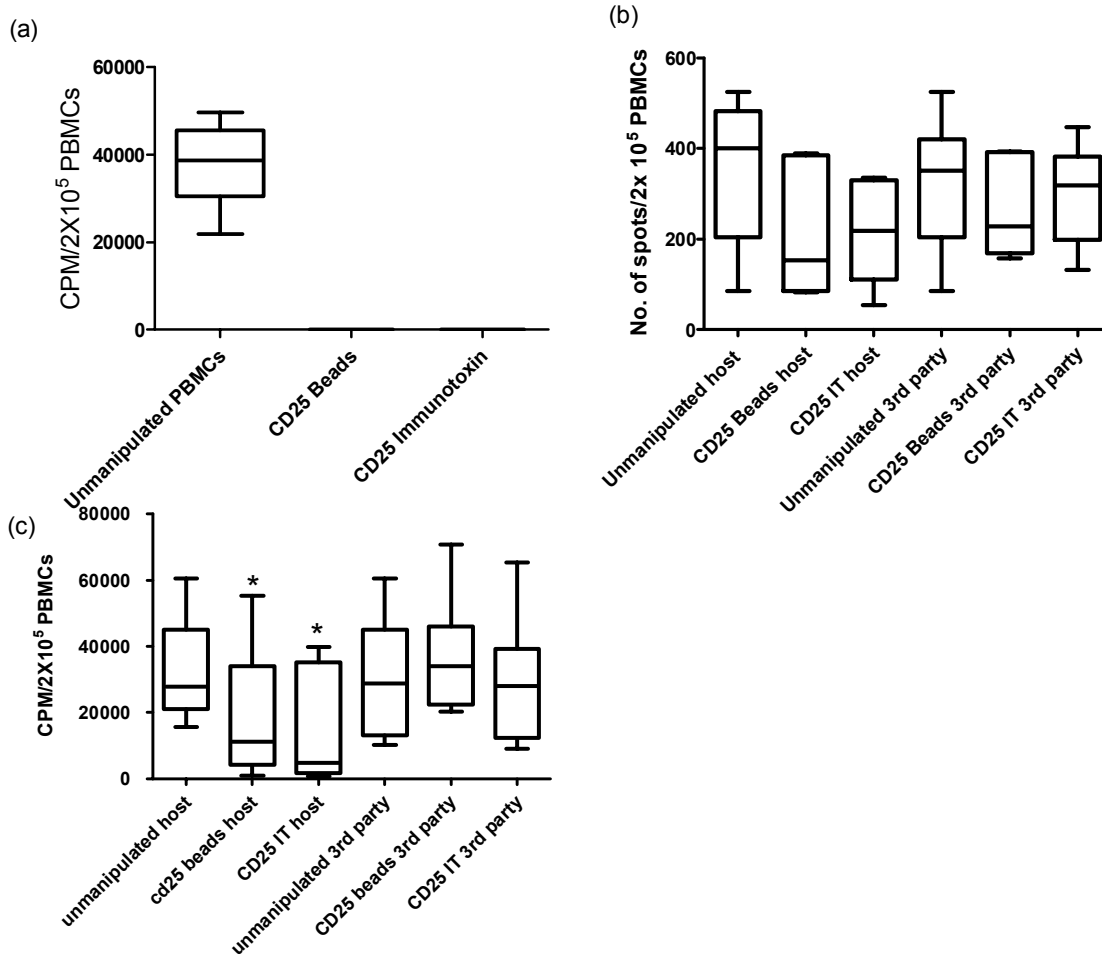
Figure 29:(a) Comparison of Residual Alloreactivity after CD25 Beads *vs.* CD25 Immunotoxin allodepletion in Primary MLR. Residual proliferation to host stimulators in 6 donor-recipient pairs after allodepletion with anti-CD25 beads or immunotoxin following stimulation of donor PBMCs with LCLs (R/S 40:1) Residual proliferation was calculated using the formula on page 142 and was significantly reduced for both forms of CD25 allodepletion compared to unmanipulated PBMCs (p<0.05) Line = median, box =25th-75th centile, error bars = min, max values.

(b) Comparison of Residual Alloreactivity after CD25 bead *vs.* CD25 Immunotoxin Allodepletion in IFNγ ELISPOT *n=6* This figure shows the frequency of cells secreting IFN-ɣ in response to stimulation with original/3rd party LCL in ELISPOT assays. The responses of allodepleted PBMCs generated using CD25 beads or immunotoxin were compared. There was no significant difference in residual response to host between both forms of CD25 allodepletion.

(c) Comparison of Residual Alloreactivity between CD25 beads and CD25 immunotoxin in 2° MLR *n=6* Allodepleted PBMCs generated using CD25 beads or immunotoxin were rested and then stimulated with either original host LCLs or 3rd party in a 2° MLR. Residual proliferation to host was significantly reduced after allodepletion with CD25 beads and CD25 immunotoxin (marked with asterisk) compared to unmanipulated PBMCs but there was no significant difference in residual responses to host between these 2 methods.

Comparison of CD25 vs. CD25/71 Immunomagnetic Depletion

Based on our phenotypic characterisation of CD25 proliferating alloreactive T-cells, we then compared residual alloreactivity and 3rd party responses after CD25 and combined CD25/71 immunomagnetic depletion, to determine if the latter combination enhanced the degree of selective allodepletion achieved with CD25-based methods. Normal donor PBMCs were co-cultured with HLA-mismatched allogeneic LCLs in 8 donor-recipient pairs and at day 3 of the MLR, co-cultures were split into 2 arms and negative selection for $CD25^+$ or $CD25^+$ and $CD71^+$ cells performed (using anti CD71 biotin and anti biotin/antiCD25 beads). Figure 29 shows a representative FACS plot of CD3/CD25 and CD3/CD71 expression in unmanipulated PBMCs, in undepleted co cultures and post CD25 and CD25/71 depletion. Table 10 shows flow cytometric data on the expression of CD25 and CD71 on T-cells before and after CD25 and CD25/71 immunomagnetic depletions. Both CD25 and CD25/71 immunomagnetic depletions effectively deplete $CD3^+$ $CD25^+$ T-cells with no significant difference between the methods (mean < 0.2% after both methods p=NS). However, there was a small $CD3^+$ $CD71^+$ population remaining after CD25 allodepletion (mean 0.62 %), which was effectively removed with combined CD25/CD71 immunomagnetic depletion.

Table 10: Effective depletion of alloreactive T-cells by CD25 and CD25/71 beads

	Unmanipulated PBMCs	Day 3 PBMCs +Allo LCL	Day 3 PBMCs +Allo LCL+25 Beads	Day 3 PBMCs +Allo LCL+25/71 Beads
CD3 CD25	1.51 ± 0.7	10.78 ± 4.11	0.15 ± 0.08	0.06 ± 0.01
CD3 CD71	0.84 ± 0.71	7.43 ± 3.43	0.62 ± 0.31	0.05 ± 0.05

The percentage of T-cells expressing CD25 and CD71 by FACS analysis in the non-depleted and allodepleted co-cultures is shown. Data represent mean ±SD. *N=8*
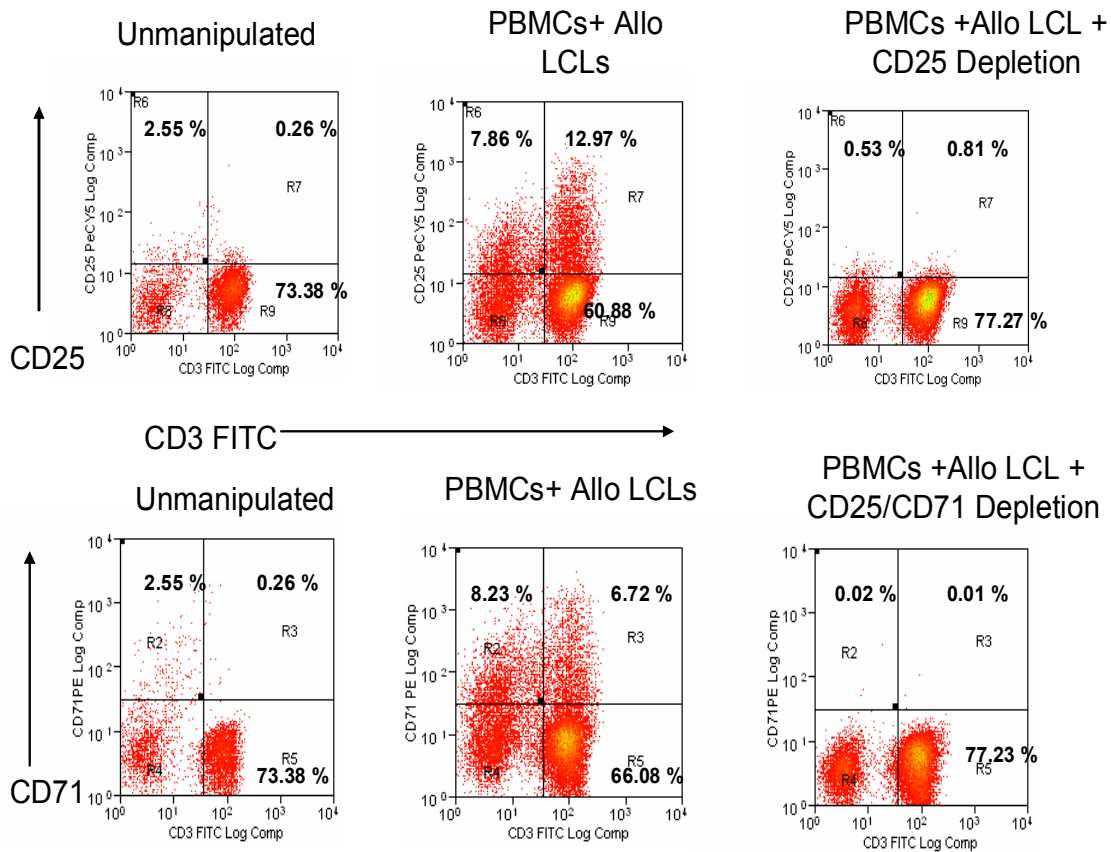
Figure 30: CD25/71 immunomagnetic allodepletion effectively removes CD71+ and C25 + alloreactive T cells. Normal donor PBMCs were co cultured alone or with allo-LCLs. On day 3, aliquots from each sample were stained for CD3 CD25 and CD3 CD71. A CD25 or CD25/71 allodepletion was then done and FACS analysis was then performed. Data shown is of a representative example

In the primary MLR, no residual proliferation to host was detectable after CD25/71 depletion in any donor-recipient pair tested (Figure 32a). To detect residual alloreactivity, allodepleted cells were rested for 2 days and then restimulated with original host/3rd party LCLs in IFN-γ ELISPOT and 2° MLR assays. Results are again expressed as a percentage of the response observed with thawed unmanipulated PBMC from the same donor. In secondary MLRs (Figs 30 & 32b), the median residual proliferation in response to host LCL after combined CD25/71 depletion was significantly lower (4.8% of response of unmanipulated PBMC from the same donor; range 0.98 – 61.3%) than with anti-CD25 beads alone (9.9%; range 5.5 – 76.5%). This difference was highly significant (p < 0.01). Responses to 3rd party LCL were equivalent to unmanipulated PBMC with both methods (median 92.8% response of unmanipulated PBMC for CD25/71 *vs.* 95.01 % for CD25 beads alone). Likewise, in the IFN-γ ELISPOT assay (Figs 31 & Fig 32c), the median response of allodepleted cells to host LCL was over 3 x lower with

combined CD25/71 allodepletion (14.1% of response of unmanipulated PBMC; range 0 – 51.1%) than with anti-CD25 beads alone (54.6 %; range 0.04 – 111.3%), (p < 0.05). Again, 3rd party responses were maintained compared to unmanipulated PBMCs for both methods (median 3rd party response for CD25 beads 69%, CD25/71 beads 76% of response of unmanipulated PBMC). Thus in 2 assays measuring distinct phenotypes, combined CD25/71 depletion led to significantly enhanced and more consistent allodepletion than CD25 alone
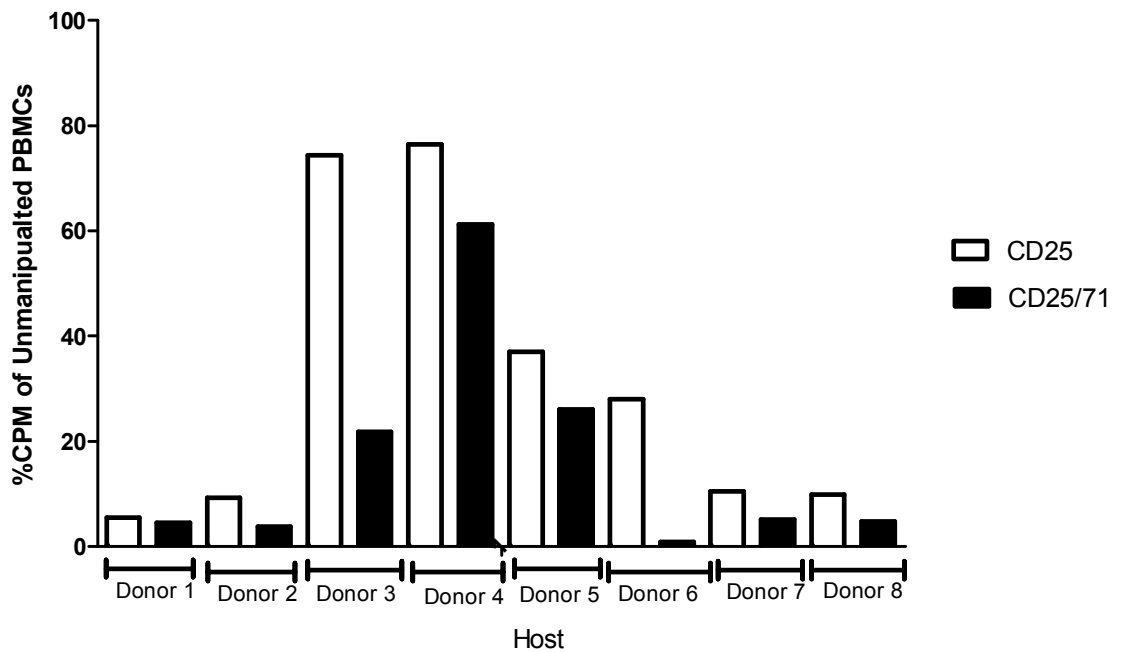
Figure 31: This Figure show the Primary data in the 2° MLR assay for the unmanipulated PBMCs, CD25 depleted and CD25/71 depleted cocultures to host and 3rd party LCLs for the 8 donor recipient pairs. Rested CD25 or CD25/71 allodepleted or unmanipulated PBMCs were restimulated with either host (above) or 3rd party LCLs (below) in a 2° MLR assay. Values are expressed as a % of the response of the unmanipulated PBMCs. i.e. unmanipulated response =100 %
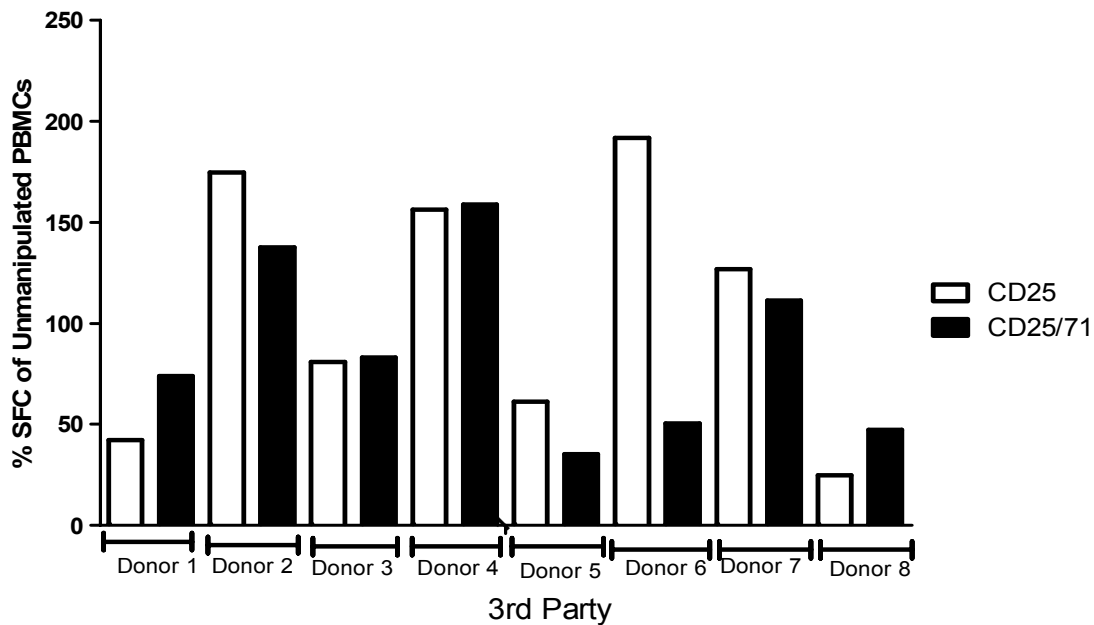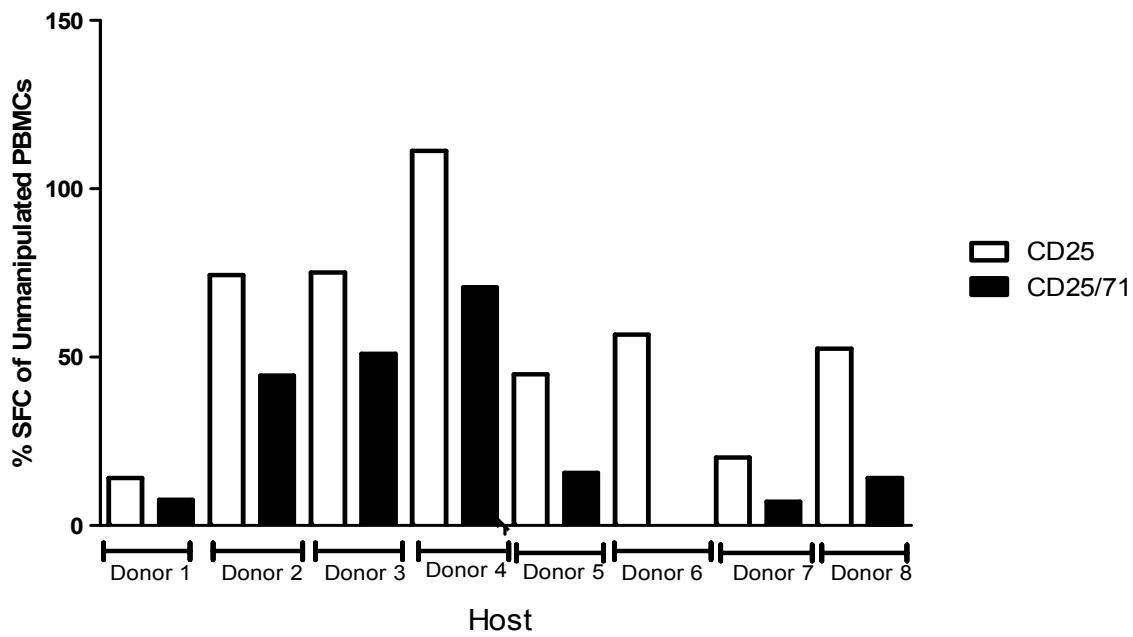
Figure 32: This figure show the Primary data in the IFNγ ELISPOT assay for both the unmanipulated PBMCs, CD25 depleted, CD25/71 depleted to host and 3rd party LCLs for the 8 donor recipient pairs. Rested CD25 or CD25/71 allodepleted or unmanipulated PBMCs were restimulated with either host (above) or 3rd party (below) LCLs in a 2° IFNγ ELIPSOT assay. Values are expressed as a % of the response of the unmanipulated PBMCs i.e. unmanipulated response =100 %

Figure 33 (a). Proliferative responses to host in Primary MLR are undetectable after both CD25 Beads and CD25/71 Immunomagnetic allodepletion. *(n=5)* Residual proliferation after allodepletion with anti-CD25 beads or CD25/71 beads following stimulation of donor PBMCs with LCLs (R/S 40:1) Residual proliferation was calculated using the formula as described on page 142. The median residual proliferation for both CD25 beads and CD25/71 beads was 0 %.Line = median, box =25th-75th centile, error bars = min, max values

(b) Enhanced Depletion of Secondary Proliferative responses to host after CD25/71 Allodepletion compared to CD25 depletion. *n=8.* Rested allodepleted CD25 or CD25/71 PBMCs were restimulated with host or third party LCLs in a 2º proliferation assay. CD25/71 allodepletion led to significantly reduced residual proliferation to host compared to CD25 alone (p <0.01) without affecting third party responses

(c) Residual Alloreactivity to host is lower after CD25/71 allodepletion than CD25 in IFN-γ ELISPOT *n=8* This figure shows the frequency of cells secreting IFN-γ as determined by ELISPOT assays. Rested allodepleted CD25 or CD25/71 PBMCs were restimulated with host or third party LCLs in a 2º IFNγ ELISPOT assay. CD25/71 allodepletion led to significantly reduced residual response to host compared to CD25 beads alone (p<0.05) without affecting third party responses

(a)

(b)

(c)
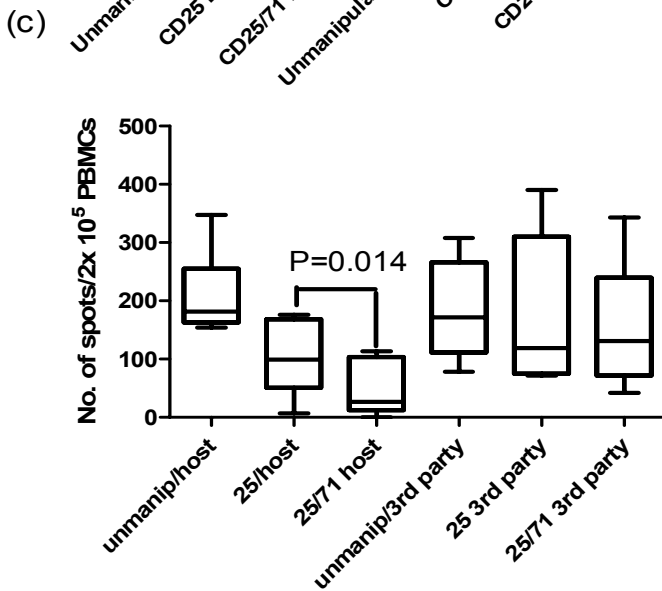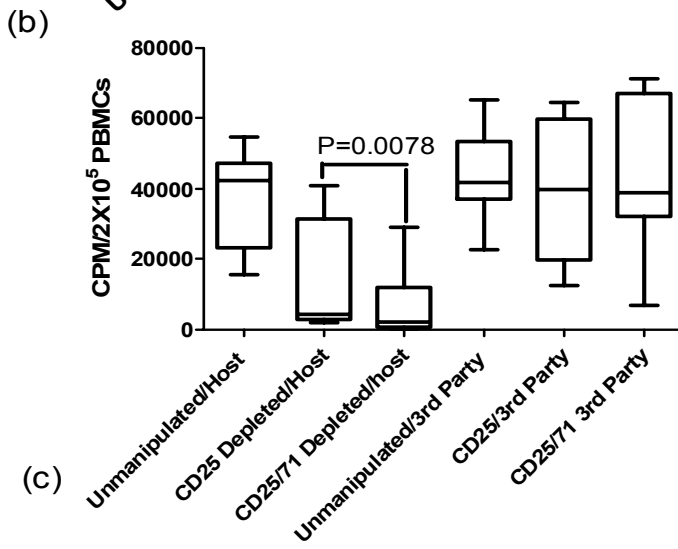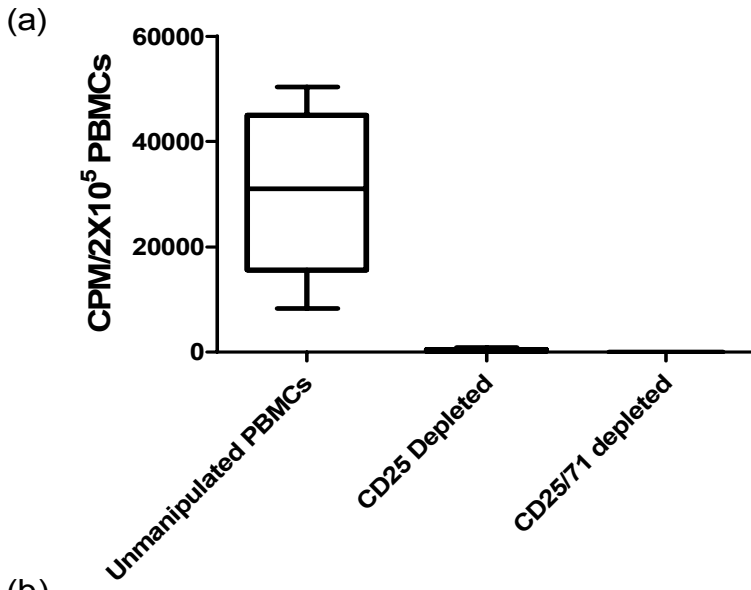
Comparison of CD25/71 vs. CD25/45RA allodepletion

Our data above identified CD71 and CD45RA as the optimal markers to target proliferating alloreactive T-cells that would be retained after CD25-based allodepletion. To determine if combined CD25/71 allodepletion was superior to the combination of CD25/45RA, we co-cultured PBMC with HLA-mismatched LCL for 3 days, split co-cultures into 2 arms and then negatively selected either CD25$^+$ and CD71$^+$ cells or CD25$^+$ and CD45RA$^+$ cells using immunomagnetic beads. (*n=5*) Allodepleted fractions were then rested as above and restimulated with host or 3rd party LCLs in a secondary 2º MLR or IFNγ ELISPOT assays (Table 11). In secondary MLR, the median residual proliferation to host was lower in the CD25/71 arm than in the CD25/45RA fraction (0.02 % *vs*. 9.29 % p=0.08). Similarly, in the IFN-γ ELISPOT assay, the median response to host in the CD25/71 arm was also slightly lower than the CD25/45RA arm (17.5% of response of unmanipulated PBMC *vs.* 23.7% respectively p=NS). Thus combined CD25/71 allodepletion appears non-significantly superior to CD25/45RA allodepletion. Given that CD45RA is also expressed in a higher proportion of unstimulated T-cells than CD71 (so that CD45RA depletion would result in a greater loss of bystander T-cells than CD71), the CD25/71 combination was selected for further studies.

Median Residual Response to Host	2º MLR	2º IFNγ ELISPOT
CD25/71	0.02 % (0-15.9 %)	17.5 % (0-56.1 %)
CD25/45RA	9.29 % (0-30.6 %)	23.7 % (3.3 -63.9 %)

Table 11: **Enhanced Allodepletion with CD25/71 immunomagnetic Depletion compared to CD25/45RA.** Normal donor PBMCs were co-cultured with HLA mismatched LCLs and on day 3 a combined CD25/71 or CD25/45RA immunomagnetic allodepletion was performed. Aliquots from rested fractions were then restimulated to the original host in a 2º MLR or IFNγ ELISPOT assay. Results are expressed as a percentage of the response observed with thawed unmanipulated PBMC from the same donor. *(n=5)*(Median and range)

Scale Up Studies

Prior to scale up runs in the GMP facilities, we initially optimised conditions for a combined CD25/71-based allodepletion.

Determining the Optimal APC

In order to determine the optimum APC for enhancing allodepletion, in 4 donor recipient pairs, PBMCs were co-cultured with either HLA mismatched mature DCs, LCLs or cytokine pre-treated PBMCs .The APCs were from the same donor for each experiment. The PBMCs were pre-treated with recombinant human TNFα and IFNγ at 1000 iu/10^6 PBMCs in order to up regulate HLA class I and II expression and hence optimize presentation of alloantigen. Normal donor PBMCs were thus co cultured with irradiated APCs [DC(R:S 10.1), LCLs(R:S 40.1) or cytokine treated PBMCs(R.S 1.1) from the same recipient] .A combined immunomagnetic CD25/71 allodepletion was then performed on day 3. Due to a shortage of cell numbers, allodepleted donor T cells were rested in CTL media supplemented with IL-2 20u/ml, which would lead to an increase in residual alloreactivity. On day 5 rested allodepleted T cells were co cultured with either host or 3rd party LCLs in a 2º MLR. As can be seen in Fig 33 median residual reactivity to host was 44.5 % for PBMCs, 15.5 % for DCs, and 10.25 % for LCLs. 3rd party responses were maintained with the PBMC and LCL arms (73.3 % and 133.7 % respectively), but were reduced for the DC arm (39.87 %). Though the number of donor recipient pairs was small, this preliminary experiment would suggest that LCL or DCs, are the optimal APC for allodepletion. Given the possibility that allodepletion after PBMC/DC APCs might lead to spuriously higher residual alloreactivity compared to LCLs, due to preserved EBV specific responses restricted by shared HLA antigens, we plan to repeat this experiment using host/3rd party PBMCs as 2º stimulators in the MLR.
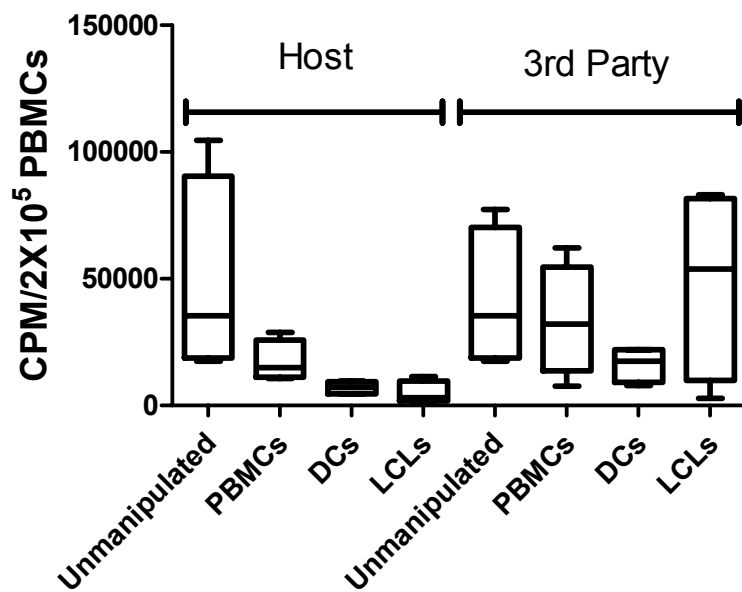
Figure 34: LCLs are the Optimum APC. Donor PBMCs were co cultured with 3 different APCs from the same individual (DC, LCLs, and cytokine stimulated PBMCs). On day 3 a combined CD25/71 immunomagnetic depletion was performed. Rested allodepleted PBMCs were then co cultured with either host LCLs or 3rd party LCLs in a 2º MLR. (*n=4*). Using LCLs as APC led to the lowest median reactivity to host and the best 3rd party activity.

Evaluating the Potential for Transmitting infectious EBV with Irradiated LCLs

Infusion of residual LCLs with the allodepleted PBMCs could potentially result in post transplant lymphoproliferative disease. This is unlikely because (a) LCLs are lethally irradiated (70Gy), and are pre-treated with acyclovir for 2 weeks prior to use and (b) we have previously shown that allodepleted T cells confer EBV specific immunity through T cells restricted by the non- shared haplotype[86]. However, in order to determine the transforming potential of LCLs, 4 LCLs lines were grown in RF10 containing 100µM acyclovir for 2 weeks. After this period the LCLs were spun down and the supernatant was sent for EBV PCR. This showed that all 4 LCL lines were strongly positive for EBV DNA.

The 4 LCLs lines were then irradiated (70 Gy) and co-cultured with PBMCs (R.S 1.1) from 3 donors in RF10 supplemented with 1µg/ml of ciclosporin. After 6 weeks, the number of viable

B cells was determined using FACS (7AA-D and CD19 staining). In none of the 12 donor recipient pairs were any viable B cells elicited. This demonstrates that while EBV DNA is detectable in LCLs supernatants, the risk of transmitting infectious EBV with irradiated acyclovir treated LCLs is very low.

Comparison of co culture in Bags vs. Flasks

We then wanted to determine the optimal conditions for co culturing our cultures (i.e. bags or T75 flasks). In 4 donor recipient pairs, PBMCs were co-cultured with HLA mismatched DCs (R: S 10.1). Co cultures were split from each donor recipient pair into either T75 flasks or cell expansion bags (Miteny Biotec 200-074-301). On day 4, aliquots from each were assessed for CD3+ CD25+ and CD3+ CD71+ by FACS expression. CD3+CD25 +expression was 1.4 xs higher in the co-cultures in the flasks, whilst CD3+CD71+ expression was 3.5 x times higher. (Fig 34) The viability (assessed by trypan staining) was 63.8 % in the flasks, whilst 56.9 % in the bags after 4 days of co culture. This work was done by a post doctoral student Dr. Christoph Mancao. A representative FACS plot in shown in Figure 35.

Mean Fluorescence Intensity

		1	2	3	4	AVRG	STDV
flask	CD3+/CD25+	178	138	164	135	154	20.8
bag	CD3+/CD25+	128	158	101	153	135	26.2
flask	CD3+/CD71+	237	157	242	154	198	48.6
bag	CD3+/CD71+	204	165	158	152	170	23.4
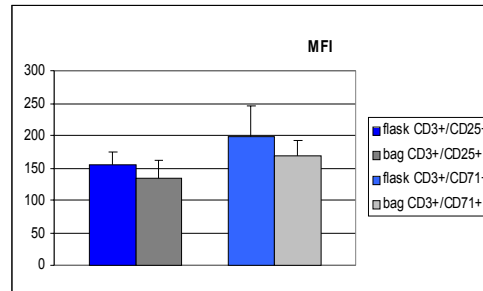
Percentage of activated cells

		1	2	3	4	AVRG	STDV
flask	CD3+/CD25+	8.05	9.42	6.73	8.71	8.23	1.1
bag	CD3+/CD25+	4.88	8.08	3.35	7.9	6.05	2.3
flask	CD3+/CD71+	2.13	6.27	2.13	5.7	4.06	2.2
bag	CD3+/CD71+	0.99	1.54	0.57	1.56	1.17	0.5
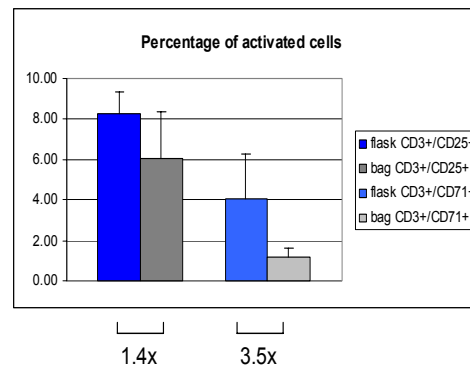
Figure 35: Co cultures in T75 flasks led to superior up regulation of CD25 and CD71 expression and MFI compared to bags. *n=4.* Donor PBMCs were co cultured with HLA mismatched DCs and were then cultured in bags or flasks. On day 4, aliquots were then taken for FACS to determine CD3+CD25+ and CD3+CD71+ expression (shown below) and MFI (shown above).
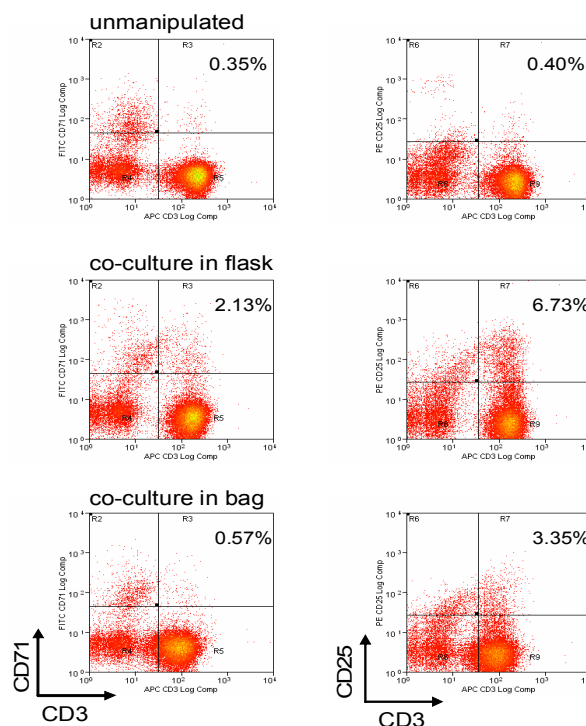
Figure 36: Superior Upregulation of CD3CD25 and CD3CD71 in allogeneic MLRs in flasks compared to bags. Normal donor PBMCs were co-cultured with HLA-mismatched DCs either in cell culture bags or flasks and on day 4 aliquots were taken for FACS to determine expression of CD3+CD71+ and CD3+CD25+. A representative example is shown above.

Comparison of anti CD25 Bead/Anti CD71 Biotin+ anti Biotin Beads vs. Anti CD25 Biotin/ Anti CD71 Biotin+ anti Biotin Bead Immunomagnetic Allodepletion

In order to optimize our scale up studies, we first compared CD25 immunomagnetic beads as part of our CD25/71 immunomagnetic depletion with a biotinylated anti CD25. Data from Necker Hospital in Paris had suggested that the biotinylated CD25 may result in more effective depletion of alloreactivity than the CD25 immunomagnetic beads. Potentially, this could simplify combined CD25/71 allodepletion as both primary antibodies could be added in a single step. To confirm this, in our combined CD25/71 allodepletion, we obtained biotinylated anti CD25 from Dr. Marina Cavazzana-Calvo. Normal donor PBMCs were co-cultured with irradiated HLA mismatched LCLs. On day 3, we split the co-cultures and did a day 3 CD25/71 immunomagnetic depletion using anti CD25 beads +biotinylated CD71 + anti biotin beads as we had previously done vs. biotinylated CD25 (biotinylated CD25+Biotinylated CD71+ antibiotin beads). The percentage of residual CD3+CD25+ and CD3+ CD71+ T cells was determined flow cytometrically. (See Table 12)

148

	Unmanipulated	Co culture pre depletion	CD25 Bead + Biotin CD71	Biotin CD71+ Biotin CD25
CD3 CD25	2.34 %	10.41 %	0.39 %	0.31 %
CD3 CD71	1.23 %	5.67 %	0.1 %	0.09 %

Table 12: Comparison of anti CD25 Bead + anti CD71 biotin/anti Biotin vs. anti CD25+ anti CD71 biotin/biotin bead immunomagnetic depletion. Normal donor PBMCs were co cultured with HLA mismatched LCLs and on day 3 a CD25/71 immunomagnetic depletion was done using either CD25 beads or a biotinylated anti CD25. The % of T cells expressing CD25 and CD71 was determined by flow cytometry (*N=1*)

As shown in Table 12, in a single experiment, both methods of depletion gave effective depletion of CD25 and CD71. The allodepleted PBMCs were rested for 2 days and then restimulated with host/3rd party LCLs in a 2º IFNγ ELISPOT (Fig 36). We chose the 2º IFNγ ELISPOT assay rather than a 2º MLR, because our previous data had suggested that the former assay had higher levels of residual alloreactivity and thus might enhance differences between the forms of allodepletion.
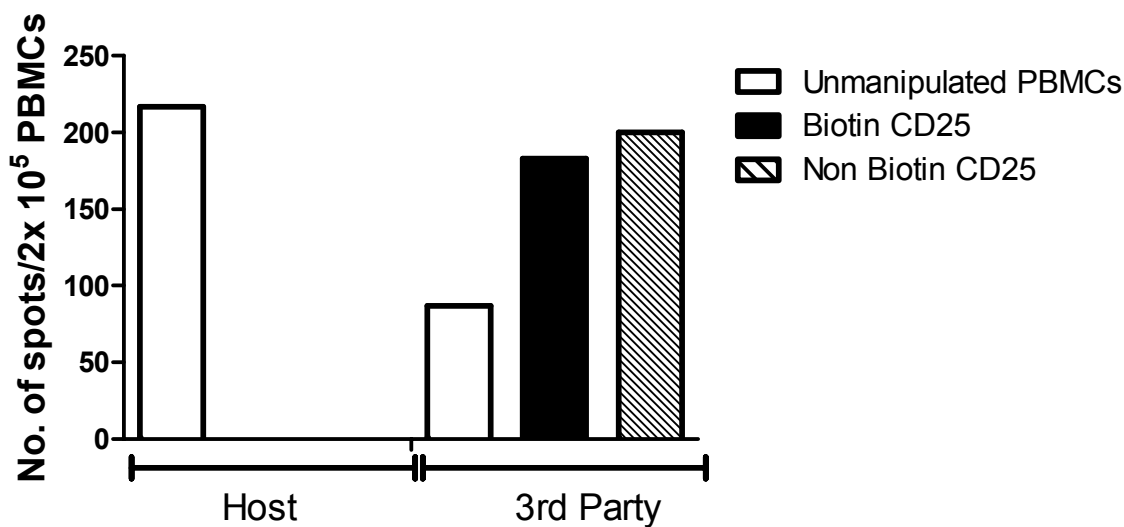
Figure 37: Effective reduction of Residual Alloreactivity with both biotinylated anti CD25 and CD25 microbeads. Normal Donor PBMCs were co cultured with HLA mismatched LCLs. A day 3 CD25/71 depletion was done using an anti biotinylated CD25 or CD25 microbeads. Rested allodepleted PBMCs were then co cultured to host/3rd party LCLs in a 2º IFN gamma ELISPOT (*n=1*).

As can be seen from our preliminary data, both anti CD25 beads and the biotinylated anti CD25/anti biotin beads gave undetectable residual responses to host, with preservation of 3rd party responses. However, further comparisons between the anti biotinylated anti CD25 and CD25 microbeads will need to be done.

Yield

For the 8 donor recipient pairs, yield following a day 3 CD25/71 allodepletion is shown below. (Table 13)

Donor	Day 0 Count (x10^6 PBMCs)	% Day 0 Viability	Day 3 Pre depletion Count (x10^6 PBMCs)	% Day 3 Pre depletion Viability	Day 3 Post depletion Count (x10^6 PBMCs)	% Day 3 Post depletion Viability	% of the Starting Count
1	91.5	100	42	99	13.6	99	14.86
2	101	99	51.5	95	16.6	95	16.4
3	135	99	58	90.6	22.35	77	16.56
4	165	99	62	74	42	93	25.47
5	193.1	100	94	75	28	85	14.51
6	178	99	88	79	31.02	95	17.4
7	67	100	62	79	12.2	95	18.2
8	113	99	71	83	16.49	92	14.59
Mean	138.125	99.375	66.0625	84.325	29.8	91.375	17.2

Table 13: Yield and Viability after a Day 3 CD25/71 allodepletion. Data demonstrating the total number of PBMCs (x 10^6 PBMCs) at start of co culture, pre CD25/71 depletion on day 3 and after CD25/71 depletion on day 3. % Viability was assessed by trypan blue staining. (*n=8*)

The mean % yield ± 1 SD from 8 donor recipient pairs is 17.2 % ± 3.58. We are routinely able to generate 8 x 10^7 allodepleted donor T-cells (ADTs) from a 450 ml blood donation, so that add back of 10^6/kg CD25/71 ADTs is straightforward. The mean viability post a day 3 CD25/71 allodepletion was over 90 %.

CD25/71 Allodepletion using The CliniMACS under GMP conditions

To determine if we could reproduce our in vitro data on a larger scale, we performed scale up studies in our GMP facility, the Cell Therapy laboratories at Great Ormond Street Hospital using the CliniMACS system for depletions. Since we have not as yet obtained regulatory approval from The Medicine and Healthcare Regulatory Agency (MHRA) for use of EBV

transformed LCLs within this facility, DCs were used as stimulators in these experiments. Normal donor PBMCs from buffy coats were co-cultured with HLA mismatched irradiated dendritic cells (R.S.10.1) for 4 days (*n=2*). These experiments were performed before our comparison of bags vs. flasks and co-cultures were performed in cell culture bags, in order to maintain a closed system throughout the experiment. On day 4, co-cultures were harvested and a CD25/71 immunomagnetic labelling was done using biotinylated anti CD25/CD71 and anti biotin beads.

For one of the donor recipient pairs, the depletion on the CLINIMACS was done using a TS tubing set (recommended by Miltenyi Biotec to perform our depletions). For the other donor recipient pair, the depletion was done using a CLINIMACS depletion tubing set. The TS tubing set is used for depletions for up to 60×10^9 total PBMCs and the CLINIMACS depletion tubing set for up to a total of 120×10^9 PBMCs. Samples were taken to determine the efficacy of depletion and for sterility.

	TS Tubing	CLINIMACS depletion Tubing set
CD3 CD25	3.84 %	0.09 %
CD3 CD71	2.14 %	0.04 %

Table 14: CD25/71 depletions using TS Clinimacs set does not give adequate depletions.
Normal donor PBMCs were co cultured with HLA mismatched DCs. (*n=1*). On day 4 a CD25/71 allodepletion was performed using a TS CLINIMACS set in 1 donor recipient pair, and a CLINIMACS depletion tubing set in the other pair. The allodepleted samples were then stained for residual CD3+ CD25+ and CD3+ CD71+ using flow cytometry.

Following allodepletion the samples from the 2 different tubing sets were co stained for CD3 CD25 and CD3 CD71 flow cytometrically. As can be seen in Table 14, depletions using the TS tubing did not lead to acceptable levels of allodepletion. In contrast, depletions performed with the CLINIMACS tubing set, did lead to comparable levels of depletion to that what we achieved using the MIDI MACS. Viability as assessed by 7AA-D staining was greater than 95 % in both arms. The % cell yield as percentage of the starting PBMC count was 8.8% for the CLINIMACS depletion tubing set, and 10.4 % for the TS tubing set. These values are less than the 17 % we achieved using the MIDI MACS, and this may reflect increased cell loss in the bag system. Further comparison between the TS tubing set and the depletion tubing set using

the same donor recipient pairs are needed. The CD25/71 allodepleted PBMCs from the depletion tubing set fraction were rested for 2 days and then restimulated to host/3rd party in a 2ºIFNγ ELISPOT (see Table 15a) or 2ºMLR (Table 15b). Anti CMV responses were assessed by co-culturing unmanipulated PBMC or CD25/71 allodepleted PBMCs from the same donor with irradiated autologous PBMC pulsed with a CMV pp65 peptide mix in an IFN γ ELISPOT (*n=1*). (Table 15a)

(a)

	Host	3rd Party	CMV pp65
Unmanipulated PBMCs	407	302	885
CD25/71 allodepleted	0	20	395

(b)

	Host	3rd Party
Unmanipulated PBMCs	30553	21850
CD25/71 allodepleted	0	13023

Table 15: Loss of 3rd party responses after CD25/71 allodepletion using the CLINIMACS.
(n=1) PBMCs were co cultured with HLA mismatched DCs and then on day 4 a CD25/71 allodepletion was performed using the CliniMACS system. Rested allodepleted PBMCs were then restimulated to host/3rd party LCLs in a 2ºIFNγ ELISPOT (a) (No of spots/$2x10^5$ PBMCs) or 2ºMLR (CPM/$2x10^5$PBMCs) (b)
CMV responses were assessesed in an IFNγ ELISPOT by stimulating unmanipulated PBMC or allodepleted PBMCs with autologous PBMCs pulsed with CMV pp65 pepmix

Our data show that while CD25/71 depletion completely abrogated IFN-γ responses to host, there was extensive loss of 3rd party responses in both assays (6.6 % on the IFNγ ELISPOT), (59.6 % on the 2º MLR). Responses to CMV in the IFNγ ELISPOT assay, showed relative preservation, though somewhat diminished compared to unmanipulated PBMCs were also decreased compared to unmanipulated PBMCs (44.6 %) (Table 15). These results are very preliminary, and the loss of 3rd party responses, may reflect the time taken to perform the depletion using the CLINIMACS due to the lack of familiarity with this system. Our Current Standard Operating Procedure (SOP) for generation of CD25/71 allodepleted donor T cells in shown in the Appendix . We plan to repeat the comparison between the tubing sets and subsequently to perform clinical scale CD25/71 depletions using this system to determine

yield, residual alloreactivity and preservation of antiviral responses in a further 5 donor-recipient pairs.

Conclusions

To optimize our allodepletion strategy, we initially compared the effectiveness of CD25 immunomagnetic depletion to that of CD25 IT. In 2 different assays we found no significant difference in residual alloreactivity to host between these methods. Clinical grade anti-CD25 immunomagnetic beads (Miltenyi Biotec) are now available, overcoming the limited availability of IT. Whilst both methods gave extremely effective depletion of CD25$^+$ T-cells (<1%) and residual proliferation to host in primary MLRs (undetectable with both methods), significant residual alloreactivity to host was observed in secondary MLRs and ELISPOT assays, when CD25 alone was targeted.

We found that 70 % of the CFSE-dim CD25^{-ve} population express CD71. Potentially, this enables us to target 2 independent phenotypes of alloreactive T-cells at the same time-point within a single co-culture. Flow cytometric analysis showed that immunomagnetic depletion deletes CD25$^+$ and CD71$^+$ cells to below background levels. In primary MLRs, residual responses to the stimulator were undetectable after CD25/71 depletion. In 2 separate functional secondary stimulation assays measuring distinct phenotypes, the combination of CD25/71 allodepletion led to significantly enhanced and more consistent allodepletion compared to CD25 alone without compromising third party responses. Furthermore the combination of CD25/71 was superior to the CD25/45RA combination. Our studies indicate that combined CD25/71 depletion results in a 20-fold reduction of proliferating alloreactive T-cells flow cytometrically and in secondary MLRs, with a 1-log depletion of response to host in IFN-γ ELISPOT assays. Hence, combined CD25/71 depletion may enable safe transfer of larger doses of allodepleted donor T-cells than hitherto possible.

Additionally, we showed that LCLs are an excellent APC, for allodepletion strategies, eliciting allodepletion that is equal to mature DCs, and superior to that of cytokine treated PBMCs, with excellent preservation of 3rd party responses. We further showed that in 12 donor recipient

pairs, irradiated LCLs lines were unable to transform co-cultured donor PBMCs. This would suggest that the risk of transmitting infectious EBV by using acyclovir treated irradiated LCLs is extremely low. Finally, we have demonstrated that immunomagnetic CD25/71 allodepletion using The CLINIMACS system is feasible under GMP conditions. Though our results are very provisional, we showed acceptable levels of depletion of CD25+ and CD71+ T cells, undetectable anti host responses and relative preservation of anti CMV responses, using CliniMACS depletion tubing set. Further scale up experiments are planned.

CHAPTER 5

Assessment of Antiviral Immunity after Combined CD25/71 Allodepletion

Aims

1. To determine if T cell responses to CMV, EBV and adenoviral antigens are preserved after combined CD25/71 allodepletion compared to unmanipulated PBMCs

Introduction

The rigorous T- cell depletion required to avoid graft versus host disease (GVHD) after haplo-SCT results in delayed immune reconstitution, resulting in a high mortality/morbidity from viral (chiefly CMV, EBV and adenovirus) and fungal infections[7]. In particular, adenovirus appears to be the single most important cause of infectious death after haplo-SCT.[7,168,169] CD25 based allodepletion strategies preserve *in vitro* CMV and adenoviral immunity, [86] and when LCLs were used as APCs, partial immunity to EBV was preserved through the non shared haplotype. Clinically, patients who received adoptive immunotherapy with doses of 3 x 10^6/kg of allodepleted donor T-cells generated using CD25 IT after haplo-SCT[141] showed accelerated T-cell reconstitution and recovery of CMV and EBV-specific immunity. However, 2 patients in this study died of adenovirus associated complications, and importantly, no patient had detectable T-cell responses to this virus before 9 months post-SCT. These data suggest that larger doses of allodepleted T-cells may be necessary to confer protective responses to pathogens which evoke low frequency T-cell responses in the donor.

In the previous chapter we demonstrated that a combined immunomagnetic CD25/71 allodepletion resulted in significantly lower residual alloreactivity to host compared to CD25 based allodepletion. We next studied whether anti-viral responses are preserved following CD25/71 allodepletion.

Antiviral Responses are Preserved after CD25/71 Allodepletion

To determine the specificity of allodepletion with CD25/71 immunomagnetic negative selection, we studied whether anti-viral T-cell responses were retained following allodepletion. PBMCs from CMV or EBV–positive donors known to have significant populations of virus-specific CD8$^+$ cells detectable by MHC- peptide pentamers were co-cultured with HLA-mismatched LCLs for 3 days and then negatively selected for CD25/71 using immunomagnetic beads as described in Chapter 4. As shown in Table 16 and the representative FACS plot in Fig 37a, in 4 donors there was no significant difference in the frequency of CMV-pp65–specific

CD8^{+} T cells in allodepleted donor T-cell cultures compared to unmanipulated PBMCs from the same donors (median 2.5 % *vs.* 3.3 % respectively p=NS). Similarly (Table 17 and representative FACs plot in Figure 37b), the frequency of EBV-specific pentamer[+] T-cells in CD25/71 allodepleted donor T-cells was equivalent to unmanipulated PBMCs (median of 0.34 % *vs.* 0.37 % respectively p=NS). These results suggest that virus-specific CD8+T-cells are retained following allodepletion.

Table 16: **CD8^{+} T-cells response to CMV are preserved after CD25/71 allodepletion**

	Donor 1 B7 TPR	Donor 2 B7 TPR	Donor 3 B7 TPR	Donor 4 A2 NLV
Unmanipulated	3.01 %	14.23 %	2.05 %	0.46 %
CD25/71	2.52 %	8.45 %	3.98 %	0.29 %

FACS analysis of 4 CMV seropositive donors in unmanipulated PBMCs (top row) or after CD25/71 allodepletion (bottom row) following staining with HLA-A2 CMV pentamer. The percentage of pentamer positive cells in the CD3+/CD8+ lymphocyte gate was expressed as a proportion of the CD8+ cells. *CMV*- pp65-specific HLA-A*0201-NLVPMVATV (A2-NLV), and HLA-B*0702-TPRYTGGGAM (B7-TPR)

Table 17: CD8^{+} T-cell responses to EBV are preserved after CD25/71 allodepletion

	Donor 1 A2-CLG	Donor 2 A2-CLG	Donor 3 A2-CLG	Donor 4 A2 CLG
Unmanipulated	0.19 %	0.11 %	0.49 %	0.55 %
CD25/71	0.09 %	0.12 %	0.62 %	0.72 %

FACS analysis of 4 EBV seropositive donors in unmanipulated PBMCs (top row) or after CD25/71 allodepletion (bottom row) following staining with HLA-A2 EBV pentamer. The percentage of pentamer positive cells in the CD3+/CD8+ lymphocyte gate was expressed as a proportion of the CD8+ cells. *EBV*- LMP-2 specific HLA-A*0201-CLGGLLTMV (A2-CLG).

(a)
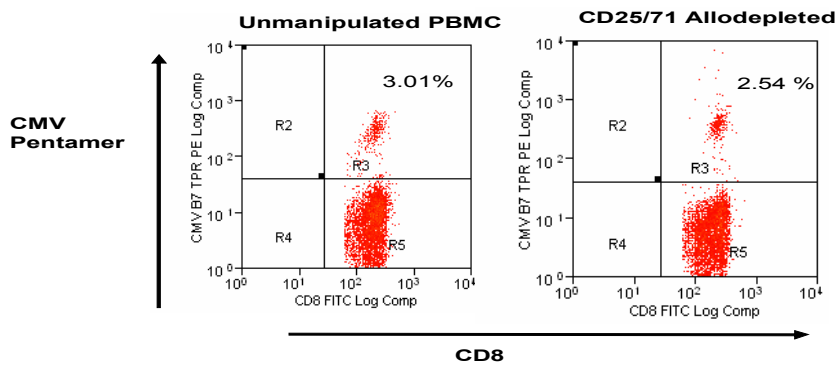
(b)
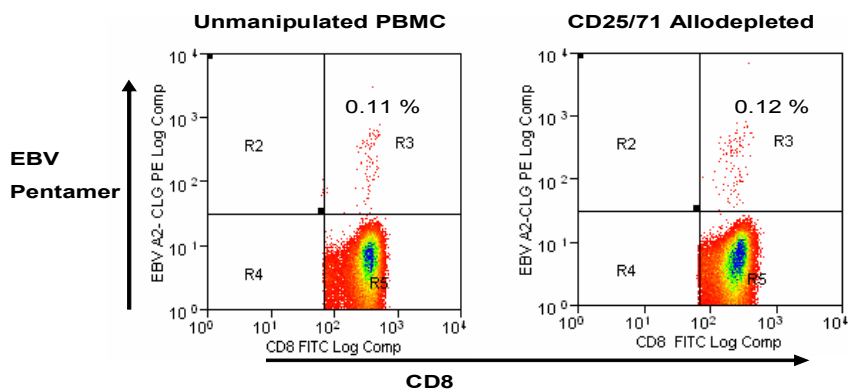
Figure 39: (a) CMV-specific CD8$^+$ T-cells are preserved after CD25/71 allodepletion The figure shows a representative FACS analysis from one of 4 donor-recipient pairs demonstrating staining of either unmanipulated PBMCs (left column) or CD25/71 allodepleted cells (right column) in a HLA-A2–positive, CMV-seropositive donor with a HLA-A2–CMV pp65 pentamer (top right quadrants). The percentages of pentamer-positive cells as a proportion of CD8$^+$ cells with isotype subtracted are shown.

(b) EBV-specific CD8$^+$ T-cells are retained after CD25/71 allodepletion. The figure shows a representative FACS analysis from one of 4 donor-recipient pairs demonstrating staining of either unmanipulated PBMCs (left column) or CD25/71 allodepleted cells (right column) in a HLA-A2–positive, EBV-seropositive donor with a HLA-A2–CLG pentamer (top right quadrants). The percentages of pentamer-positive cells as a proportion of CD8$^+$ cells with isotype subtracted are shown

To study the functionality of anti-viral T-cells, we then performed IFN-γ ELISPOT analyses to determine the frequency of CMV, adenoviral and EBV-specific T-cells before and after allodepletion. Donors were co-cultured with completely HLA antigen mismatched LCLs and CD25/71 allodepletion was performed at day 3 of co-culture. Unmanipulated PBMC or rested

CD25/71 allodepleted T-cells from the same seropositive donors were then restimulated with irradiated autologous PBMC pulsed with a peptide mix from the immunodominant CMV antigen pp65 or transduced with an adenoviral vector (AD5f35-GFP) or with autologous LCLs. As shown in Table 18 (showing the primary data for each donor recipient pair in the IFNγ ELISOT assay for CMV, EBV and adenovirus in unmanipulated and CD25/71 allodepleted co cultures) and Figure 38a and 38b (showing the median and range for SFC to CMV, EBV and adenovirus in unmanipulated and CD25/71 allodepleted co cultures), there was no statistically significant difference (p=NS) in the frequency of cells secreting IFN-γ in response to CMV, EBV or adenoviral antigens in allo-depleted T-cell co-cultures and unmanipulated PBMC, implying that allo-depletion with combined CD25/71 immunomagnetic selection does not affect the function of virus-specific T-cells

(a) Primary Data for Each donor Recipient Pair for CMV Responses in the IFNγ ELISPOT assay

	Unmanipulated	CD25/71
Donor 1	55	109
Donor 2	100	93
Donor 3	332	257
Donor 4	206	92
Donor 5	63	64
Mean ± SD	151.2±117.62	123±76.63876

(b) Primary Data for Each donor Recipient Pair for EBV Responses in the IFNγ ELISPOT assay

	Unmanipulated	CD25/71
Donor 1	100	155
Donor 2	120	165
Donor 3	80	110
Donor 4	90	185
Donor 5	110	95
Mean ± SD	100±15.81	142±38.01316

(c) Primary Data for Each donor Recipient Pair for Adenovirus Responses in the IFNγ ELISPOT assay

	Unmanipulated	CD25/71
Donor 1	135	205
Donor 2	155	150
Donor 3	425	615
Donor 4	115	145
Mean ± SD	207.5±145.91	278.75±225.80

Table 18: Primary data for each virus for every donor recipient pair in the IFNγ ELISPOT assay. The frequency of cells secreting IFNγ (SFC/10^5 PBMCs) in response to stimulation with irradiated autologous PBMC pulsed with a peptide mix from CMV pp65 (a) or autologous EBV LCL (b) was determined by ELISPOT assays. Unmanipulated PBMC or CD25/71 allodepleted T-cells from the same seropositive donors were compared *(n=5)* (c) The frequency of cells secreting IFN-γ in response to stimulation with irradiated autologous PBMC transduced with an adenoviral vector (Ad5f35-GFP) was determined by ELISPOT assay (SFC/10^6 PBMCs). Unmanipulated or CD25/71 allodepleted T cells from the same seropositive donors were compared. *(n=4)*
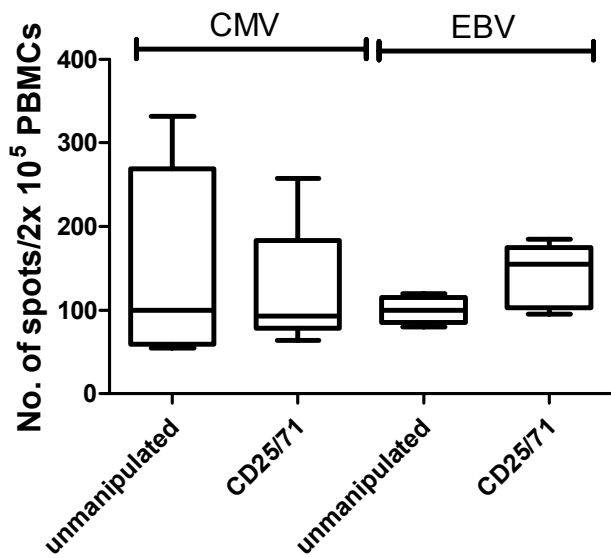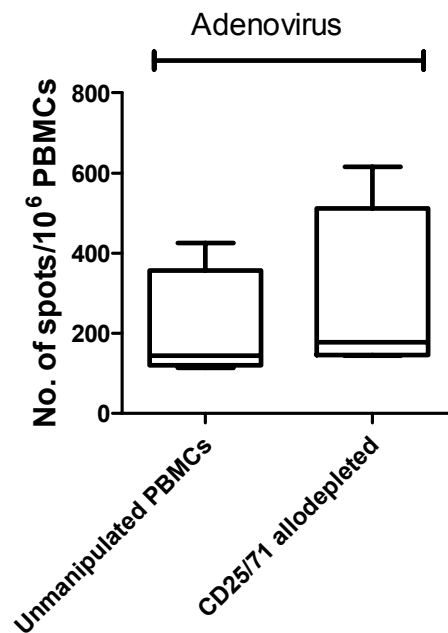
Figure 41 (a) Functional T-cell responses to CMV and EBV are preserved after CD25/71 allodepletion. (*n=5*) The frequency of cells secreting IFNγ in response to stimulation with irradiated autologous PBMC pulsed with a peptide mix from CMV pp65 or autologous EBV LCL was determined by ELISPOT assays. Unmanipulated PBMC or CD25/71 allodepleted T-cells from the same seropositive donors were compared. Line = median, box =25th-75th centile, error bars = min, max.

(b) T cells responses to Adenovirus are preserved after CD25/71 allodepletion *N=4* The frequency of cells secreting IFNγ in response to stimulation with irradiated autologous PBMC transduced with an adenoviral vector (Ad5f35-GFP) was determined by ELISPOT assay. Unmanipulated or CD25/71 allodepleted T cells from the same seropositive donors were compared.

Conclusion

Infections remain a significant cause of morbidity and mortality after CD34 selected haplo-SCT. Adoptive immunotherapy with EBV CTLs [92] and CMV-specific CTLs [94], [98] has been demonstrated to enhance anti-viral immunity after HLA-matched transplant but these approaches are untested in the haploidentical setting . Further, these approaches are limited to individual pathogens, and frequently require either prolonged periods of cell culture, thus limiting broader clinical applicability. In contrast, allodepleted T-cells confer immunity to a wide array of pathogens including all 3 viruses tested, and since the majority of alloreactive T-cells have been deleted, the risk of GVHD is lower in the haploidentical setting. Moreover, the generation of allodepleted T-cells is substantially simpler than many existing protocols for CTL generation.

We have demonstrated in 2 distinct assays that anti-CMV and EBV responses are maintained following combined CD25/71 allodepletion. Furthermore, IFNγ responses to adenoviral antigens are also preserved following CD25/71 allodepletion. We have not assessed whether protective responses to fungal pathogens such as Aspergillus are preserved in allodepleted T-cells because the immunogenic antigens of these organisms are as yet poorly defined. While we would predict such responses would be preserved, as T-cells responding to them should not be activated, and therefore not depleted, this will need to be confirmed in future studies. Given that combined CD25/71 allodepletion is enhanced compared with CD25 depletion suggests that it may be possible to safely transfer larger doses of allodepleted donor T-cells than hitherto possible and our data suggest that, this may confer protective immunity not only to CMV and EBV but also to pathogens which evoke low frequency T-cell responses in the donor, such as adenovirus.

Chapter 6

Enhancement of Anti leukaemic Activity of CD25/71 Allodepleted Donor T cells

Aims

1. To redirect the specificity of CD25/71 allodepleted donor T-cells with a single chain ScFv CD19 chimeric T cells receptor and demonstrate cytotoxicity and cytokine release to CD19+ targets

Introduction

Leukaemic relapse remains a major cause of mortality post T-cell deplete haploidentical transplantation.[8] In clinical studies of adoptive immunotherapy with allodepleted donor T-cells generated using CD25 IT, the major cause of treatment failure was relapse.[139,141] The persistence of anti-leukaemic responses after selective allodepletion is critical if the benefits of adoptive transfer are not to be offset by leukaemic relapse, as in our previous study [141]. Our approach will deplete T-cell responses against the mismatched HLA alleles and ubiquitous minor histocompatibility antigens presented by the shared HLA alleles. Nonetheless, we and others [86,122,142]have shown that anti-leukaemic activity may be retained after allodepletion. In particular, we have demonstrated that T-cell responses to potential myeloid tumour antigens are preserved by virtue of their lack of expression on the LCL used as stimulators. Such T-cell responses could be restricted either through HLA molecules shared by the recipient and donor or allorestricted. However, it is known that the precursor frequency of anti-leukaemic T cells is much lower than against viral antigens. Analogous to the situation with donor lymphocyte infusion, it is possible that infusing higher doses of allodepleted T-cells may confer anti-leukaemic responses in myeloid malignancies. In order to augment the anti-leukaemic activity of allodepleted T-cells in lymphoid malignancies, we have investigated redirecting their specificity to target tumour specific antigens using chimeric T-cell receptors. (ChTCR). We have focused particularly on B lineage acute lymphoblastic leukaemias this is the commonest transplanted malignancy in childhood and relapse is the major cause of treatment failure after SCT in both children and adults.

Existing approaches to enhancing graft versus leukaemia, are limited by the technically difficult nature of the process, the lack of expression of tumour specific antigens or down regulation of the MHC complex. One approach to target B cell malignancies, is based on the combining the effector functions of T-cells with the ability of monoclonal antibodies to

recognize cell surface molecules such as CD19. These chimeric T-cell receptors consist of an extracellular singe chain Fv consisting of the heavy and light chain variable regions of a monoclonal antibody which is attached to the cytoplasmic domain consisting of the CD3ζ chain. As outlined in the introduction, this approach overcomes the lack of immunogenic tumour antigens on ALL blasts, enable us to target tumour cells in a HLA independent fashion, bypasses tumour evasion strategies and should not cause GVHD as we will target CD19 which is highly expressed on B- lineage acute lymphoblastic leukaemias (ALLs), B cell lymphomas, and chronic lymphocytic leukaemias but is not expressed on non hematopoietic cells. The CD19 specific ScFv is derived from a murine IgG2a monoclonal antibody (clone FMC63) fused in frame to an IgG hinge a transmembrane domain and an intracellular CD3ζ. Transduction of PBMCs with retroviral vectors coding for CD19 ChTCRs have shown specific killing of CD19 targets *in vitro* and in mouse studies.[150,153]

 However, the polyclonal stimulation (generally with OKT3/ anti CD28 and IL-2) required to promote T cells for retroviral transduction to occur, significantly alters the phenotype of the transduced cells, promoting differentiation of the transduced T-cells, leading to a reduction in anti-viral immunity.[107,108] Gattinoni *et al* demonstrated that highly differentiated T-cells have potent *in vitro* cytotoxicity but weak *in vivo* activity whilst the converse is true for naïve T-cells. [155] *In vivo* studies with ChTCRs have shown poor efficacy despite strong *in vitro* activity. Potentially, this could be due to neoepitopes in the ChTCRs leading to immune clearance of the receptors, or to poor T-cell proliferation due to a lack of co-stimulatory domains in the TCR or the highly differentiated state of the transduced T-cells.[153,155] Therefore, a transduction regime of allodepleted T-cells which preserves their phenotype, as well as their anti-viral immunity is desirable. Lentiviral vectors have the advantage that they are less dependent than oncoretroiral vectors on cell division for stable transduction to occur so that less intensive stimulation can be used during transduction. Transduction of T-cells with lentiviral constructs stimulated using modest doses of IL-2 or IL-7 only, has been shown to preserve the phenotype of transduced T-cells and preserve anti-viral immunity.[107,170] Moreover, lentiviral transduction is associated with a lower risk of insertional mutagenesis.[161] Thus we investigated if transduction of allodepleted donor T-cells with a lentiviral construct encoding for a CD19 chTCR with minimal stimulation was sufficient to redirect their specificity towards CD19+ leukaemic targets.

Construction of Chimeric TCR and Production of Lentiviral Supernatant

The ScFv CD19ζ plasmid was supplied by Dr. Martin Pule. This consisted of the variable domains derived from the CD19 specific murine monoclonal antibody FMC-63 assembled as a single chain variable fragment (ScFv) molecule, in frame with a sequence encoding the human immunoglobulin IgG G1 hinge CH2-CH3, the CD28 transmembrane domain, and the cytoplasmic signalling domain of the human CD3ζ. The 1946 base pair fragment of the CD19 chTCR was amplified by PCR. Not 1 restriction restriction sites were added to the 5′ end of the reverse primer, and a Bgl II restriction site was added to the 5′ of the forward primer so that the fragment could be sub cloned into the Not 1 and Bam H1 sites of the pHR –SIN-SE lentiviral vector (gift of Dr. Martin Pule). A schematic CD19R/ pHR –SIN-SE is shown in Fig 39. This 2nd generation lentiviral vector has no HIV viral protein expression, but has essential viral *cis* elements for vector infection i.e. LTRs, RRE (rev response element which is required for viral mRNA export from the nucleus to the cytoplasm), and ψ-packaging signal. It is a SIN (self inactivated vector), which is believed to be safer, because of the deletion in the U3 region of the 3′LTR, which results in no viral enhancer and promoter transfer into target cells. There is a cPPT (central polypurine tract) which increases vector titre up to 10 fold, and a WPRE (woodchuck hepatitis post transcriptional element), which increases the stability of the mRNA. The CD19 chTCR is under the control of the SFFV promoter. The SFFV promoter (spleen focus forming virus LTR) has strong promoter activity in most human or mouse cells including primary macrophages, DCs, and lymphocytes.

5′LTR	cPPT	SFFV	S	V_L	L	V_H	Spacer	TM	CD3 Zeta	3′LTRΔU3

Figure 43: Schematic of the CD19R/ pHR –SIN-SE. This consists of a self inactivated (SIN) lentiviral construct, with a HIV central polypurine tract (cPPT), Woodchuck hepatitis virus post transcriptional regulatory element (WPRE), and a spleen focus forming virus (SFFV) promoter. The CD19R transgene consists a human immunoglobulin leader sequence (S), the variable domains of the CD19 specific murine monoclonal antibody FMC-63 assembled as a single chain variable fragment (ScFv) (V_L and V_H), connected by a linker (L), in frame with a sequence encoding the human IgG1 hinge and CH2-CH3 domain (Spacer), the human CD28 transmembrane domain (TM), and the cytoplasmic signalling domain of the human CD3ζ.

A vector map of CD19R/ pHR –SIN-SE is demonstrated in Fig 40a. To confirm that the modified lentiviral vector CD19R/ pHR –SIN-SE contained the CD19 chTCR transgene, the following restriction enzyme digests were performed: EcoR1/Not1, EcoR1/BamH1, and Not1/BamH1. Restriction enzyme digests with EcoR1/Not1, EcoR1/BamH1, Not1/BamH1 produced the predicted size fragments (8.5/2.5 kbp; 9.7/1.3 kbp; 9.86/1.15kbp) respectively indicative of successful sub cloning of the CD19 chTCR transgene into the lentiviral construct. (Fig 40b)

(a)

(b)

1. Vector Alone – 11.1 kbp

2. Vector+ EcoR1/Not1- 8.5 kbp +2.5 kbp

3. Vector +Not1/BamH1- 1.15kbp + 9.86kbp

4. Vector +EcoR1/BamH1 – 1.3 kbp+ 9.7 kbp

5. 1kb+ ladder
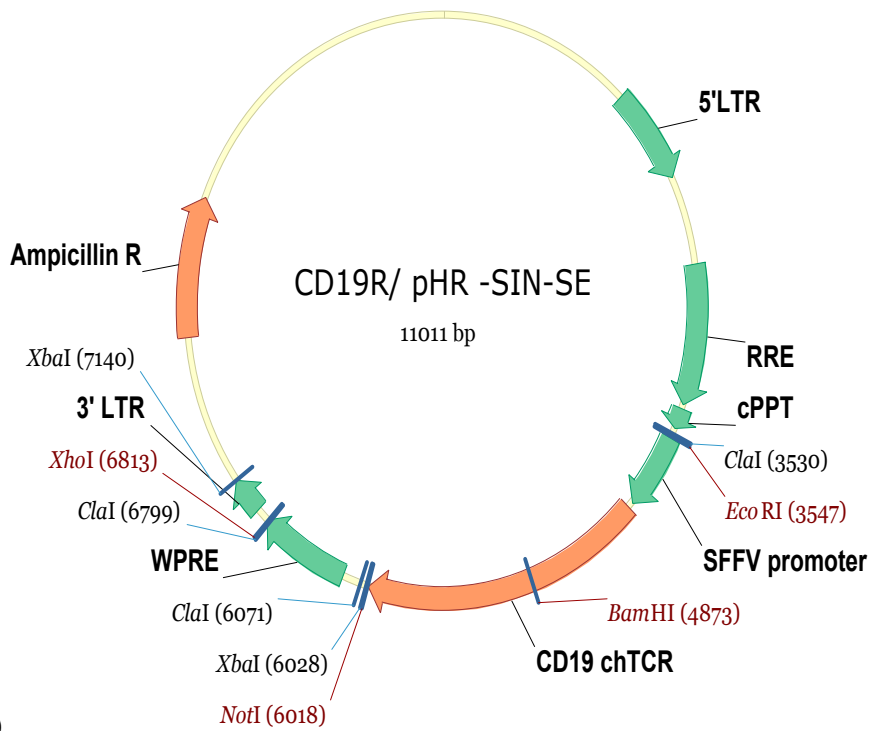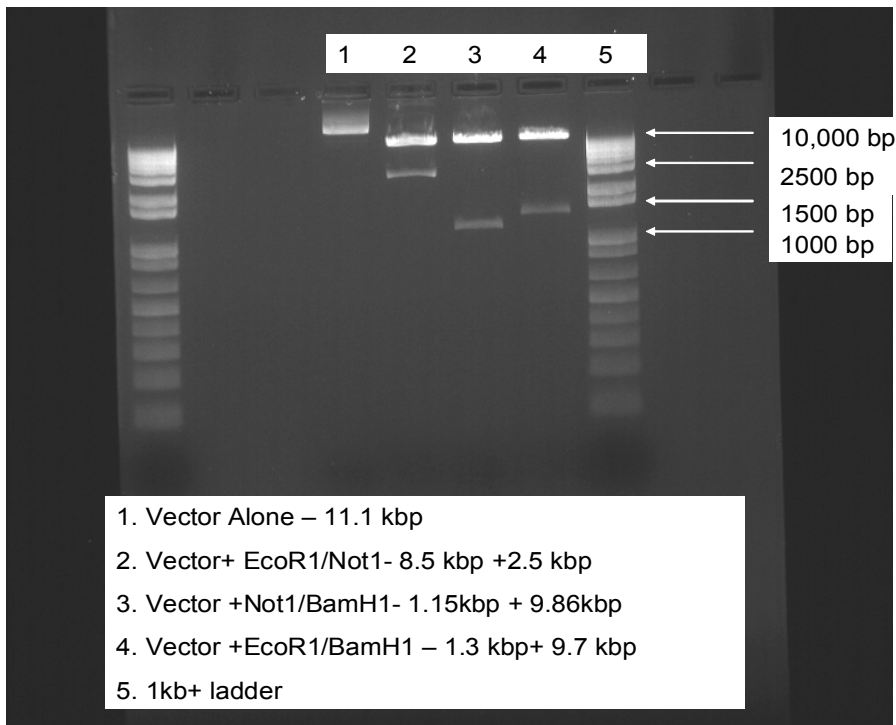
Figure 45: CD19R/ pHR –SIN-SE vector map (b) Agarose gel demonstrating restriction enzyme digestion of CD19R/ pHR –SIN-SE with EcoR1/Not1, EcoR1/BamH1, and Not1/BamH1.

Production and Titration of Lentiviral Construct

The modified lentiviral vector incorporating the CD19 chTCR transgene was prepared by transient co-transfection of three plasmids into 293 T cells: Lentiviral vector DNA (40µg) and packaging plasmids pMDG.2 (VSV-G) (10µg) and pCMVΔ8.74(gag-pol) (30µg) as described in methods. Viral supernatant was harvested at 48 and 72 hours post transfection and concentrated by ultracentrifugation. In order to determine the number of transducing particles, the virus was titred on 293 T cells using serial dilutions of 1:5 and the cells were analysed for anti Fc expression 72 hours later.(Fig 41)
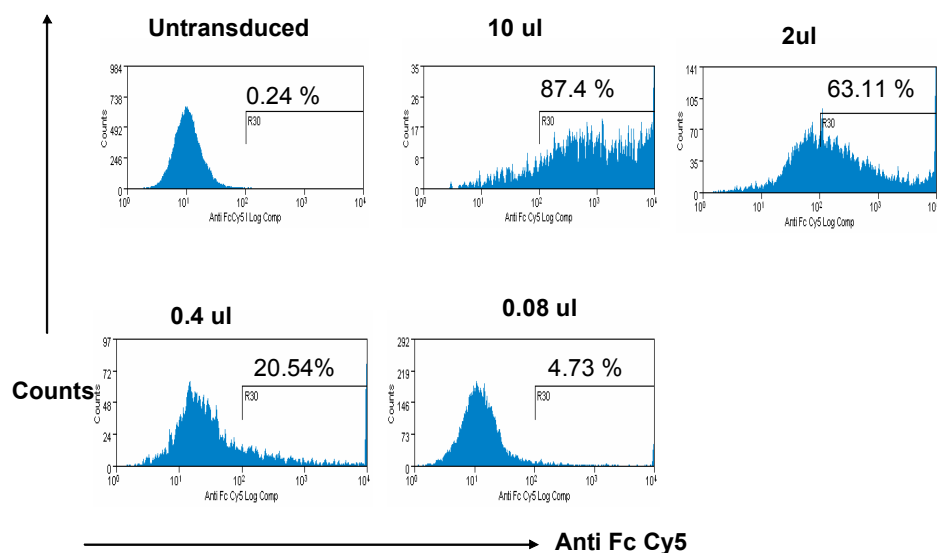
Figure 47: Lentiviral construct (CD19R/ pHR –SIN-SE) was titrated on 293 T cells using serial dilution of viral supernatant. The equivalent volume of concentrated supernatant used per well of 293 T cells is shown. Cells were analysed by flow cytometry 72 hours later for anti Fc expression and the number of transducing particles per ml was determined. Representative flow cytometry plots of anti Fc expression in 293 T cells following exposure to **CD19R/ pHR –SIN-SE**

The number of transducing particles per ml was determined as described in Chapter 2. Two batches were used for subsequent transductions, one of which gave the number of transducing particles as 2.04×10^{10}/ml and the other gave 1.7×10^{10}/ml

Optimization of lentiviral transduction

Transduction of T cells with lentiviral constructs using IL-2 or IL-7 only, has been shown to preserve the phenotype of transduced T cells and preserve anti-viral immunity.[107,170] In order to determine the optimal cytokine conditions for transduction with our lentiviral vector, normal donor PBMCs were stimulated with IL-2 100 u/ml or IL-7 (5 or 10 ng/ml) for 96 hours and then transduced with concentrated lentiviral supernatant at a MOI of 20. On day 8, the cells were harvested and the percentage of transduced T cells was determined by flow cytometry. (Fig 42). As shown in Fig 42, the transduction efficiency with IL-2 prestimulation was more than double that achieved with IL-7 (40.8 % vs. 21.49 %). Thus, for future experiments, prestimulation with IL-2 100U/ml was used.
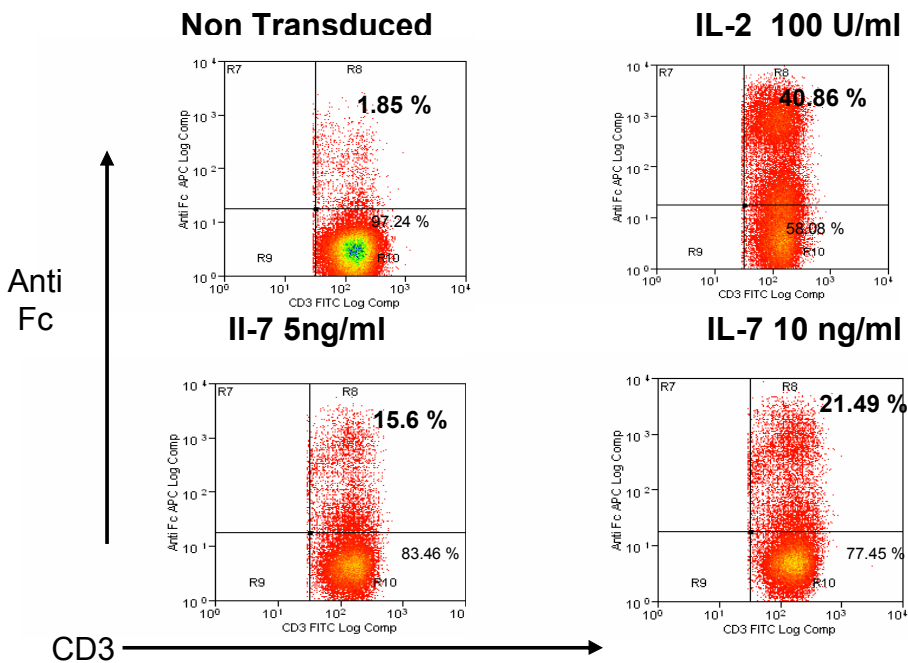
Figure 49: IL-2 promotes superior transduction of T cells than IL-7. Normal donor PBMCs were co cultured in media supplemented with IL-2 (100u/ml) or IL-7 (5 or 10 ng/ml) for 96 hours. **CD19R/ pHR – SIN-SE** Lentiviral supernatant was then added at a MOI of 20. On day 8 the expression of anti Fc in T cells was determined by flow cytometry. (*n=1*) The percentage of transduced T cells is shown in the top right hand quadrant. Data displayed is gated on the CD3 positive population.

Optimization of Multiplicity of Infection
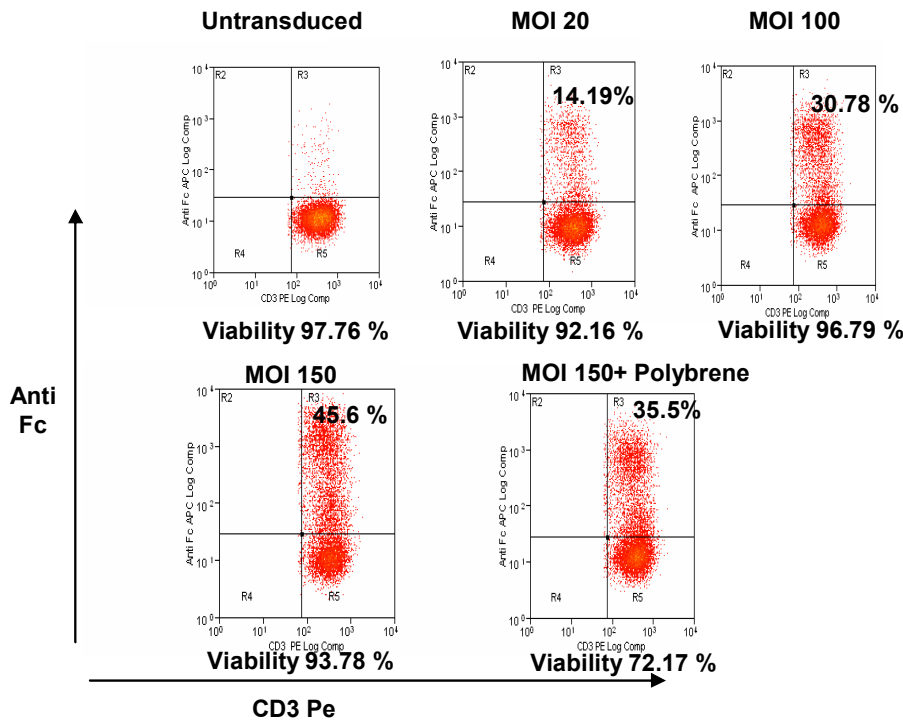
Figure 51: MOI of 150 provides Optimum Transduction. Donor PBMCs were cultured in 1 ml media supplemented with IL-2 100 U/ml. On day 4 concentrated lentiviral supernatant (CD19R/ pHR –SIN-SE) was added at various MOIs. On day 8 the cells were stained for CD3 and anti Fc. Data displayed is gated on the CD3 positive population. The percentage of T cells staining for anti Fc is shown in the top right hand quadrant. NT- non transduced (*n=1*)

A transduction regime using a MOI of 20 with IL-2 of 100 u/ml only has been shown to lead to transduction efficiencies of typically 20-25 %. Higher MOIs have not been reported to increase transduction efficiencies.[107] In order to verify this and determine the optimum MOI to transduce allodepleted T cells, PBMCs were co-cultured in media supplemented with IL-2 100 u/ml. On day 4 concentrated lentiviral supernatant was added at varying MOIs (20,100,150) and with /without polybrene (5μg/ml), a cationic polymer that increases transduction efficiency with retroviral vectors by enhancing receptor independent virus adsorption on target cells. Cells were harvested 4 days later and the percentage of transduced T cells was determined by flow cytometry and the viability was determined by trypan staining. As shown in Fig 43, the transduction efficiency of T-cells increased with increasing MOI and a MOI of 150 provided the best transduction (45.6 %), over 3 x times than that achieved with a

MOI of 20. Adding polybrene to the co-cultures did not increase transduction efficiencies, but decreased viability. Co-cultures to which lentiviral supernatant was added at a MOI of 150 had a viability of 94 % after 8 days. Thus, for future experiments a transduction without polybrene at a MOI of 150 was used.

Detecting Cytotoxicity of CD19+ Targets by Transduced T cells

We then studied whether transduction of PBMCs with our lentiviral CD19chTCR vector redirected their specificity so that they recognized and lysed CD19+ target cells. Normal donor PBMCs were resuspended in CTL media, and prestimulated with IL-2 100 u/ml. On day 4 lentiviral supernatant at a MOI of 150 was added, and on day 8 the cells the cells were used as effectors in a ^{51}Cr assay. A transduction efficiency of 31.2 % of T cells was achieved. Targets included K562 cells, K562 cells stably transduced with a CD19+GFP+ transgene, K562 cells transduced with GFP alone, autologous LCLs and HLA mismatched allogeneic LCLs. Effector: target ratios of 1:1, 5:1, and 30:1 were plated. In this assay, no specific lysis was detected to any of the CD19+ targets in the ^{51}Cr assay (*n=1*). This may represent the predominantly naïve phenotype of the transduced population, [107] (though the CCR7/CD45RA expression of transduced cells was not determined) with the percentage of transduced CD8+ CTL being too small to elicit any detectable cytotoxicity.
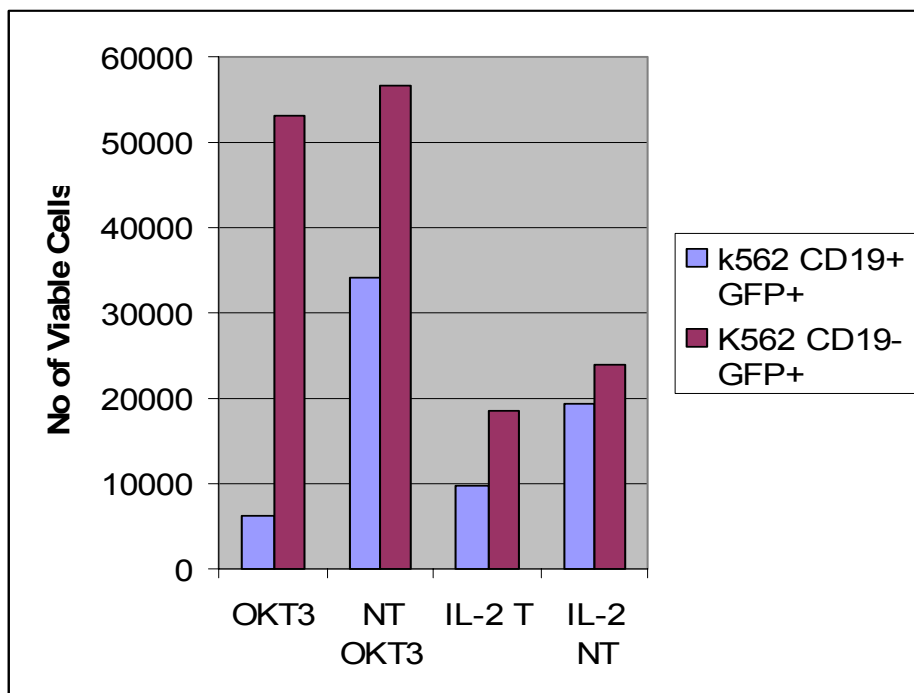
Figure 53: OKT3 transduced ζCD19 PBMCs show significant cytotoxicity against CD19+ Targets. Normal donor PBMCs were stimulated with OKT3, CD28, IL-2 or IL-2 100u/ml only. *(n=1)* Mock transduced or PBMCs transduced with **CD19R/PHR-SIN-SE** were co cultured with K562 GPP+ CD19+/- cells. After a week, cells were stained with 7AA-D, and trucount beads. A fixed number of counting beads was acquired and the number of 7AA-D negative, GFP+ cells was determined. NT-non transduced T-transduced

To investigate this further, in our next experiments we included a positive control consisting of PBMCs transduced after polyclonal stimulation with OKT3/αCD28 and IL-2 which promotes with high efficiency transduction and differentiation of naïve T cells into effector CTLs. Additionally, we developed a more sensitive flow cytometric cytotoxicity assay based on assessment of cell viability. As we assessed cytotoxicity after a week of co-culture between effectors and targets, it enabled an assessment of cytotoxicity over a longer period, and thus was felt to be more sensitive than the ^{51}Cr based cytotoxicity assay.

Normal donor PBMCs were transduced/mock transduced in media supplemented with OKT3 (1µg/ml) anti CD28 (1µg/ml) and IL-2 (100U/ml) to serve as s positive control, or IL-2 100 u/ml only as described. Transduced/mock transduced PBMCs were co-cultured with an equal number of K562 GFP+CD19+ or K562GFP+CD19- cells. (Fig 44) After a week, the number of viable GFP+ cells was determined by staining with 7-AAD. A fixed number of trucount beads was acquired to ensure that comparison could be made between the different co- cultures. As shown in Fig 45, PBMCs stimulated with OKT3/αCD28 demonstrated 82 %

cytotoxicity against K562-CD19+GFP+ cells but there was no significant cytotoxicity against CD19 negative K562-GFP+ targets. (cytotoxicity = 1-number of viable K562+CD19+/number of viable K562+CD19- x 100). PBMCs stimulated with IL-2 100 u/ml only demonstrated a 48 % cytotoxicity at an E:T ratio of 1:1 against K562-CD19+GFP+ targets again with minimal cytolysis of CD19-ve K562+GFP+ cells. Though this assay, demonstrated that our transduction regime can lead to detectable cytotoxicity of CD19+ targets, the level of cytotoxicity was disappointing. In order to maximize cytotoxicity in the transduced fractions, in future experiments we plan to flow cytometrically sort the CD3+/antiFc+ cells to enrich for transduced PBMCs and repeat the assay.

An alternative assay to detect cytotoxicity is the granzyme B ELISPOT, which has been reported to be more sensitive than the ^{51}Cr assay.[171] Thus, CD19/pHR-SIN-SE transduced and mock transduced PBMCs were co cultured with CD19+/- targets and cytotoxicity was detected using the ELISPOT assay, as described in Methods. The transduction efficiency was 34.3 %
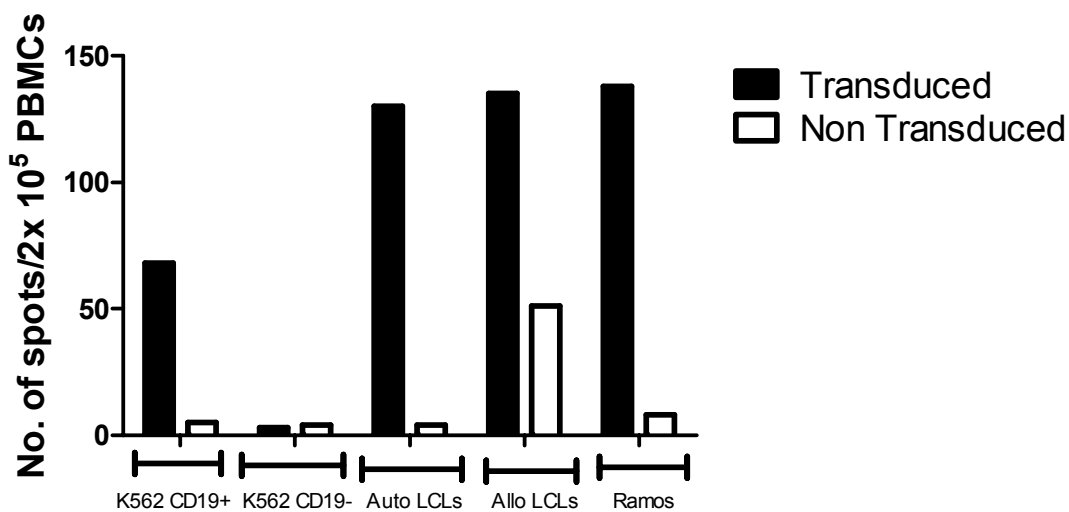
Figure 55: **CD19ζ Transduced PBMCs secrete Granzyme B in Response to CD19+ Targets.**
Normal donor PBMCs were cultured in media supplemented with IL-2 100 u/ml and on day 4 concentrated **CD19/pHR-SIN-SE** lentiviral supernatant was added. On day 8, the transduced and mock transduced PBMCs were cultured with CD19+/- targets in a granzyme B ELISPOT. (*n=1*)

As shown in Fig 45, transduced PBMCs demonstrated granzyme B release in response to K562 CD19+ (68 SFC/2 x 10^5 PBMCs) but only background levels in response to K562 CD19- ve targets (3 SFC/2 x 10^5 PBMCs). Similarly, tranduced PBMCs showed enhanced granzyme B responses compared with mock transduced PBMCs to autologous LCL (130 vs. 4 SFC respectively for transduced and mock transduced) and HLA mismatched allogeneic LCLs

(138 vs. 51 SFC for transduced and mock transduced respectively). The response of mock transduced PBMCs to HLA mismatched LCLs is likely to reflect an alloreactive T cell responses, which is augmented in transduced PBMCs through recognition of CD19. Transduced PBMCs also demonstrated enhanced granzyme B response to Ramos cells, an EBV- CD19+ Burkitt's lymphoma cell line (138 vs. 8 SFC for transduced and mock transduced respectively). Thus, as the granzyme B ELISPOT was able to specifically and sensitively detect cytotoxicity elicited by transduced T cells, we then used this assay and the IFNγ ELISPOT assay to detect anti tumour responses elicited by transduced allodepleted PBMCs.

Enhancement of Anti-leukaemic Activity of Allodepleted PBMCs

In order to enhance the anti leukemic activity of allodepleted PBMCs against B-cell malignancies, CD25/71 allodepleted donor T cells from 6 HLA- mismatched normal donor-recipient pairs were transduced with the CD19R/ pHR –SIN-SE lentivirus (MOI 150) using a method (prestimulation with IL-2 100 u/ml) which has been shown to preserve the phenotype and anti-viral immunity of transduced T-cells.[107,170] The mean transduction efficiency was 19.6 %± 6.4. A representative FACS plot of transduced and mock transduced allodepleted T-cells is shown in Fig 46. Unselected transduced CD25/71 allodepleted donor T-cells were then used as effectors in IFNγ and granzyme B ELISPOT assays. We observed that CD19/pHR-SIN-SE transduced allodepleted T-cells compared to mock transduced cells, secreted significantly more IFN-γ (Figure 47a) and granzyme B (Figure 47b) following stimulation with CD19$^+$ tumor cell lines (K562-CD19 and Ramos, both $p < 0.05$), autologous and allogeneic LCL ($p < 0.05$) and 1° blasts from 4 patients with ALL ($p < 0.05$). Transduced CD25/71 allodepleted donor T-cells showed no significant IFN-γ and granzyme B secretion to CD19^{-ve} targets (K562 or K562-GFP). These results indicate that allodepleted T cells can be redirected to recognise leukaemic targets through lentiviral chimeric TCR transfer.
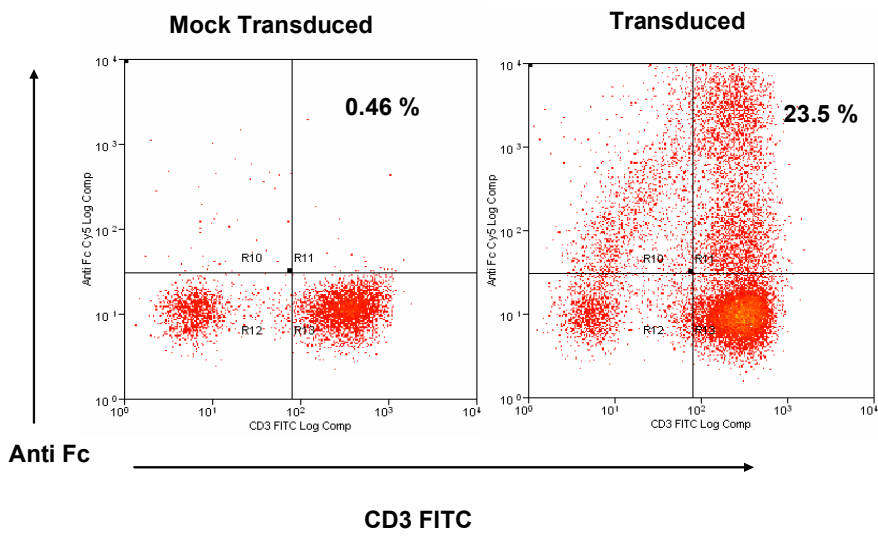
Figure 57: Representative FACS plot demonstrating transduction of allodepleted T-cells with CD19R/ pHR –SIN-SE lentiviral construct

(a)
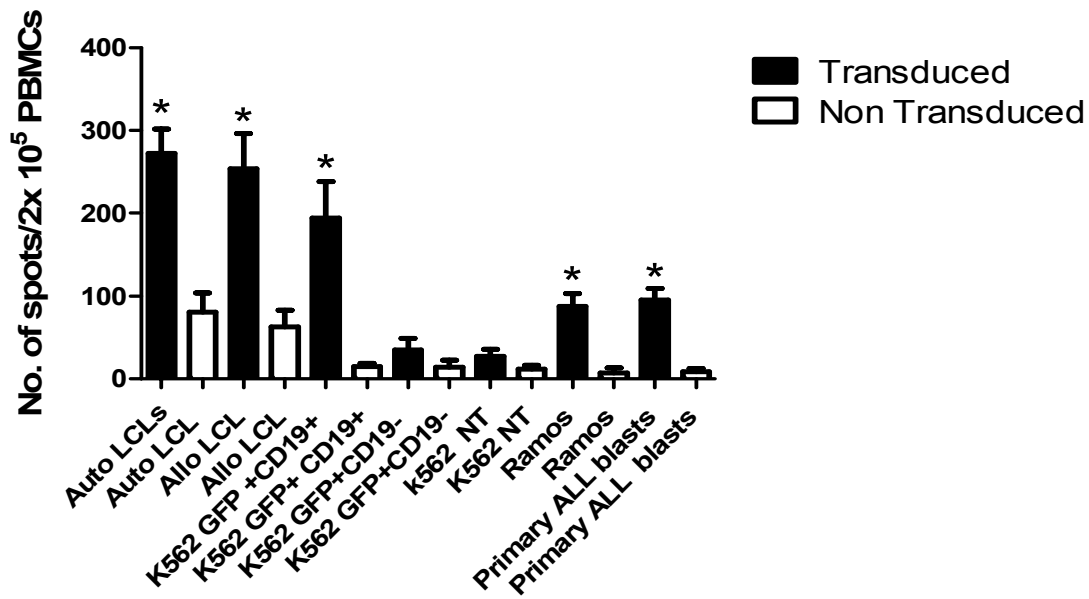
(b)
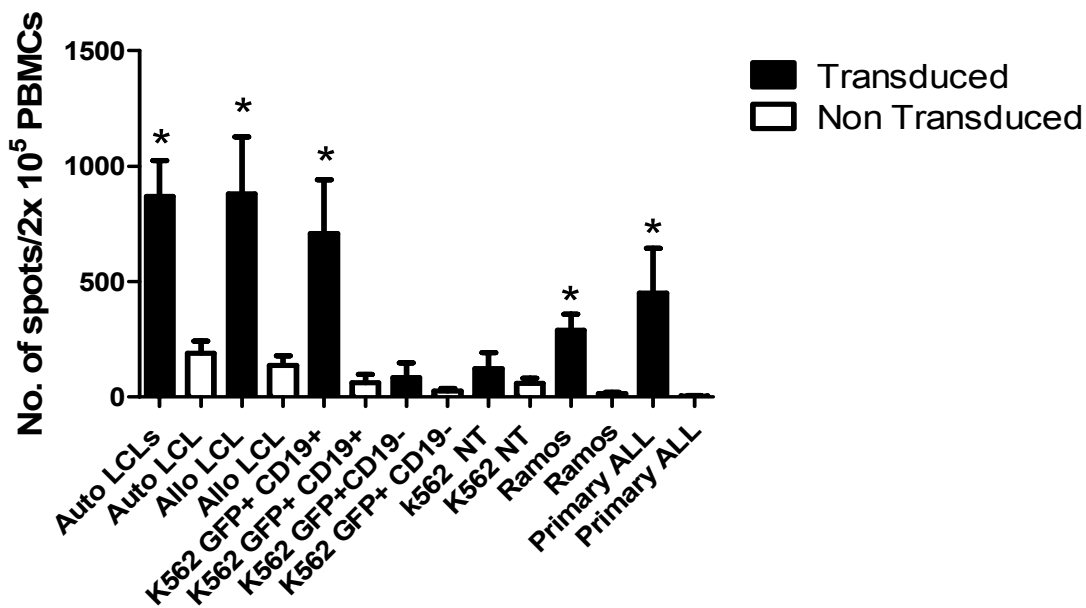
Figure 59 : Transduced Allodepleted PBMCs demonstrate CD19 specific cytotoxicity and cytokine release.(a) CD19R/ pHR –SIN-SE Transduced CD25/71 allodepleted PBMCs demonstrate significantly enhanced Granzyme B secretion to CD19+ targets*(n=6)* CD19R transduced or mock transduced CD25/71 allodepleted PBMCs were cultured with CD19+/- targets in a granzyme B ELISPOT assay. Transduced allodepleted PBMCs showed significantly enhanced granzyme B secretion to autologous and allogeneic LCLs, K562 cells stably transduced with a GFP-CD19+ transgene, Ramos, and 1° ALL blasts (* p<0.05) compared to mock transduced (Mean ± SEM) **(b) CD19R/ pHR –SIN-SE** transduced CD25/71 allodepleted PBMCs demonstrate significantly enhanced IFNγ secretion to CD19+ targets. *(n=6)* CD19R transduced or mock transduced CD25/71 allodepleted PBMCs were cultured with CD19+/- targets in an IFNγ ELISPOT assay. Transduced allodepleted PBMCs showed significantly enhanced IFNγ secretion to autologous and allogeneic LCLs, K562 cells stably transduced with a GFP-CD19+ transgene, Ramos, and 1° ALL blasts (* p<0.05) compared to mock transduced (Mean ± SEM)

Conclusion

Leukaemic relapse is a major cause of mortality post haploidentical transplantation.[8,141] While unmanipulated donor lymphocyte infusion (DLI), may have some role in preventing relapse in patients who become mixed chimeras post-SCT,[172] this approach is ineffective in frank relapse.[173] To enhance anti-leukaemic responses of DLI, some investigators have repeatedly stimulated donor PBMCs with host ALL blasts to generate tumour CTLs which have been infused post haploidentical transplantation. These CTLs however, still show significant host reactivity. [174]

The advantages of our approach are that over 90 % of alloreactive T-cells have been deleted, thus minimizing the risk of GVHD, and that a much higher proportion of transferred T-cells will recognize leukaemic targets following chimeric TCR transfer. We have demonstrated efficient transduction of allodepleted T-cells with a lentiviral CD19 chTCR construct using a regime incorporating IL-2 100 u/ml only. Furthermore, we then demonstrated that these transduced allodepleted T-cells have significantly enhanced granzyme B release and IFNγ release to CD19+ targets compared to mock transduced allodepleted donor T-cells. The degree of cytokine secretion to LCLs was considerably higher than to primary ALL blasts and Ramos cells. This may reflect the high expression of CD80 and CD86 on the LCLs, and the corresponding lack of co-stimulatory ligands on Ramos and primary ALL blasts. [175,176] The levels of cytotoxicity we observed after transduction of the CD25/71 allodepleted donor T-cells, is somewhat lower than that reported after retroviral transduction of normal donor T-cells with CD19ζ constructs using OT3/CD28 stimulation.[150,153] This may reflect that our transduction regime resulted in predominantly naïve T-cells being transduced, which lack cytolytic activity. However, lentiviral transduction may be associated with a lower risk of insertional mutagenesis,[161] and our transduction regimen has been shown to maintain the phenotype of T-cells, as well as preserve anti-viral immunity and third party activity.[107,170] Clearly, it will be important to confirm this is true of CD19 chTCR transduced allodepleted T-cells in future studies.

Furthermore, though highly differentiated tumours CTLs demonstrate potent *in vitro* cytotoxicity, they have considerably less *in vivo* efficacy. [93,155] It is unclear how many

allodepleted donor T-cells would be required to confer clinically relevant anti-leukaemic responses in the haploidentical setting: because of the possibility of allorestricted responses, this may in fact be considerably lower than the numbers of DLI required to induce GVL after HLA-matched SCT. Because of the limitations of experimental models, it may be that this question can only be answered in a clinical study.

Chapter 7

Discussion

Discussion

Interest in BMT from haploidentical donors arises from the immediate availability of a one haplotype-mismatched donor for virtually all patients, particularly those who urgently need transplantation. In the international registries the probability of finding a matched unrelated donor (MUD) ranges from 10% in poorly represented ethnic groups to 60% in Caucasians,[1] so that the only feasible option in many cases is a haploidentical transplant. However, to avoid GVHD, grafts are T-cells depleted leading to a profound delay in T-cell reconstition. This delay in T-cell reconstition is exacerbated by thymic dysfunction induced by chemo radiotherapy and the occurrence of GVHD and its treatment. This predisposes to a high risk of mortality from relapse and infection especially from adenovirus. There have been numerous approaches to improving immune reconstitution after haplo-SCT such as allodepletion, RIC after CD3/19 depletion, transduction of donor T-cells with a suicide gene and *ex vivo* induction of anergy to alloantigens with CTLA-4 Ig. Allodepletion strategies selectively deplete alloreactive T-cells after *ex vivo* stimulation of donor lymphocytes in an allogeneic mixed lymphocyte reaction (MLR) and to adoptively transfer the residual allodepleted donor T-cells. Strategies for depletion of activated alloreactive donor T cells include negative selection of donor T cells expressing activation markers (e.g. CD25, CD69, CD134, CD137, CD147, HLA-DR) [114-118] [59,122,177], using immunotoxins or immunomagnetic selection, fluorescence activated cell sorting[18,24], killing activated T-cells by photodynamic purging[52,140,178] chemotherapy agents,[120] or inducing Fas mediated apoptosis.[119]

Allodepletion has numerous advantages compared to other techniques used to enhance immune reconstitution after haplo-SCT. Compared to transfer of antigen-specific CTL [88,94,98,100], it augments cellular immunity to multiple pathogens and reduces the risk of GVHD in the HLA-mismatched setting. Adoptive transfer of donor T-cells transduced with a suicide gene in the haplo-SCT requires transduction with integrating viral vectors with the attendant complex regulatory requirements. Suicide genes may be 'leaky' with basal toxicity (e.g. icaspase 9[110], and truncated CD20[179]) and immunogenic (e.g. HSV-TK[104,180]). Most importantly, *in vitro s*tudies suggest that the transduction process affects desirable anti-viral reponses.[27,107,132]

Ex vivo induction of anergy may have negative regulatory effects on desirable T-cell responses; indeed, in the only clinical study of this approach to date[91], no data was provided on recovery of immune responses to pathogens and a high rate of infection related mortality was observed. Further until very recently, clinical grade antibodies to induce co-stimulatory blockade were not available. Chen *et al* [10]have observed more rapid immune reconstitution after reduced intensity conditioning and CD3/19 negative selection of the graft compared with conventional intensity CD34 positively-selected haplo-SCT. In this approach larger doses of residual T-cell doses (~10^5 CD3/kg) are infused with the graft as well as a multitude of accessory cells. However, this was associated with a 36% incidence of significant aGVHD. Further, while T-cell reconstitution appears improved compared to CD34-selected haplo-SCT, it remained slower than that observed with our previous study of adoptive transfer of allodepleted T-cells at 3 x 10^5/kg (median circulating T-cells at 3 months post-SCT 350/μL *vs.* 616 /μL) and there was no assessment of anti-viral immunity. The increased T-cell reconstitution after RIC CD3/19 haplo-SCT may reflect increased proliferation of alloreactive T-cells in the patients who developed GVHD. No assessment of T-cell reconstitution in patients without GVHD was done. Thus, allodepletion offers a safer, more effective way of improving T-cell immunity after haplo-SCT.

At present clinical trials of immunotherapy with allodepleted donor T-cells have focused on CD25-based approaches. We and others[86,122] have demonstrated that allodepletion using a CD25 immunotoxin RFT5-SMPT-dgA (IT) specifically deletes host-reactive T-cell responses with preservation of anti-viral and anti-leukemic responses. Andre-Schmutz *et al* first showed that adoptive transfer of allodepleted donor T-cells generated using this IT after HLA-mismatched SCT was safe and feasible[138]. Only 2/15 patients developed significant aGVHD and this correlated with the residual proliferative response to host in 1° MLR. Solomon *et al.* have adoptively transferred larger doses of allodepleted donor T-cells generated using a similar approach in 16 adults transplanted from HLA-matched sibling donors[139]. Despite allodepletion, grade II-IV GVHD was observed in 46 % of patients and was inversely associated with the efficacy of allodepletion. These patients showed good recovery of T-regulatory cells[140], so that GVHD is likely to reflect insufficient removal of alloreactive T cells, rather than deletion of T-regs.

With our collaborators, our group compared immune reconstitution after adoptive immunotherapy with 2 doses of allodepleted donor T-cells generated using CD25 IT in 16 paediatric patients after haplo-SCT[141]. We demonstrated that transfer of allodepleted donor T-cells at a dose of 3 x 10^5/kg accelerated T-cell reconstitution and recovery of CMV and EBV-specific immunity. However, the rate of leukemic relapse rate was high, resulting in an overall survival of only 5/16. This may be explained by the high-risk nature of this patient group and the low precursor frequency of leukaemia-reactive T-cells within the infused allodepleted donor T-cells. Additionally, 2 patients died of adenovirus associated complications, including 1 who had persistent adenoviraemia despite 3 infusions of allodepleted donor T cells at 10^5/kg, which cleared after a single infusion at 2.5 x 10^6/kg. Importantly, no patient had detectable T-cell responses to this virus before 9 months post-SCT. These data suggest that larger doses of allodepleted donor T-cells may be necessary to confer protective responses to pathogens such as adenovirus which evoke low frequency T-cell responses in the donor [102] and for a graft-versus-leukaemia (GVL) effect. While the incidence of significant acute and chronic GVHD was low in our clinical study, this was nonetheless observed in 2 cases, raising concerns about the safety of administering larger doses of allodepleted donor T cells in the haploidentical setting, particularly as our *in vitro* data indicates that significant residual alloreactivity persists after CD25 IT mediated allodepletion[125]. There is thus a pressing need to enhance the degree of depletion of alloreactive T-cells, to enable add back of sufficient T-cells for protective anti-leukemic and anti-infective responses without causing GVHD.

Activated T-cells express a variety of surface markers, including CD25, CD69, CD71, CD95, CD137, CD147, OX40, ICOS, HLA-DR, and secrete Th_1 cytokines IL-2 and IFN-γ. As such, a plethora of potential targets and methods now exist for allodepletion strategies[59,114-118] but there is no data on the relative expression of these targets on alloreactive T-cells to enable identification of the optimal targets for allodepletion. The data above imply that a significant minority of alloreactive T-cells are retained after CD25-based allodepletion, suggesting that CD25 may be expressed only in a subset of alloreactive T-cells and raising the possibility that targeting other molecules, perhaps in combination with CD25, could enhance allodepletion. In order to rationally design strategies for enhanced allodepletion, we systematically characterized the phenotype of alloreactive T-cells. While alloreactive T-cells have multiple phenotypes, proliferation in response to alloantigens is their most basic hallmark. We have identified proliferating alloreactive T-cells using carboxyfluorescein diacetate succinmidyl ester (CFSE) dye dilution. Godfrey *et al*[24] have shown that flow cytometric depletion of

CFSE-dim T-cells almost completely abrogates *in vitro* alloreactivity in secondary MLRs and markedly reduces GVHD in an MHC Class II disparate murine model. While it is not practical to use this approach clinically, we used this method to systematically characterize the expression of cytokines, effector molecules and activation markers on proliferating alloreactive T cells. CFSE labelling is easier than alternative measurements of T-cell proliferation e.g. Ki67, BrDU which require cell fixation, and enables elucidation of the precursor frequency of dividing cells to be determined.

Measurement of Alloreactivity

Following depletion of alloreactive T-cells, there are currently a multitude of assays to determine residual alloreactivity. The lack of a standardised read out for measuring residual alloreactivity in this setting remains a significant problem, hindering comparisons between different methods of allodepletion. We have used a number of assays including flow cytometry to assess residual percentages of CD3+25/71+cells, primary MLRs, and delayed restimulation with host/3rd party antigen presenting cells in IFNγ ELISPOT and bulk 2º MLR assays.

Each of these assays has its own strengths and weaknesses. If allodepletion involves deleting cells expressing given surface markers(s), then while flow cytometry on the allodepleted fraction gives information as to the efficacy of depletion, it tells nothing about the persistence or otherwise of alloreactive T cells that do not express this marker(s). Thus, as such assays, examine only a single targeted phenotypic marker at a single time-point, as a general principle, it is preferable to assay an alternative phenotype to the method used for depletion. Further, the assay, used to estimate residual alloreactivity should detect as broad an array of alloreactive T-cells as possible, rather than focussing on phenotypes expressed by only a minority of alloreactive-T-cells. Thus, we believe that multiple assays, are necessary and that, for instance, given that proliferation in response to alloantigen is almost a universal hallmark of alloreactivity, proliferation based assays are preferable to those assaying narrower alloreactive T-cells subsets, including LDA- based cytotoxicity[114] and granzyme assays[135],assays measuring the expression of individual markers or release of cytokines e.g. IFNγ[59,125] in response to alloantigeneic stimuli, which may significantly underestimate residual alloreactivity.

2º IFNγ ELISPOTS are easy to perform, reproducible, but only examine a subset of the alloreactive response (CD4 TH_1/ CD8 memory + effector). Our own data also showed that only a minority of proliferating alloreactive T-cells secrete IFNγ. Using this assay, Amrolia *et al* demonstrated that allodepletion using CD25IT led to a median 17 fold reduction in residual alloreactivity[125], whilst Wehler *et al* showed a 10 fold reduction after CD137 allodepletion compared to unmanipulated PBMCs[59]. The IFNγ ELISPOT is quantitative, and our data suggests that it correlates with the 2º MLR, though the levels of depletion are less. However, unmanipulated PBMCs show a poor response to allo PBMCs, thus they require allo LCLs as secondary stimulators. Furthermore, if PBMC/DCs are used as 1º stimulators, the allodepleted PBMCs will secrete IFNγ in response to the shared haplotype upon secondary restimulation leading to a spuriously elevated response.

Proliferation remains the key hallmark of an alloreactive T-cell, and for alloreactive T-cell to cause clinical sequelae would probably require them to proliferate. MLRs examine both CD4 and CD8 proliferative responses to host, but are highly variable. Residual proliferation greater than 1 % in the 1º MLR correlated with GVHD in the Paris allodepletion study[138], but they are insensitive. The 2º MLR is more sensitive, but we have found results to be highly dependent on the timing of the 2º stimulation in relation to allodepletion with negligible responses if restimulation was done immediately after depletion. We found bulk 2º MLRs are more sensitive if the cells are rested after allodepletion and therefore used a delayed 2ºMLR to skew our assays in order to detect residual alloreactivity.. There have also been a variety of controls used in 2º MLRs: we believe thawed unmanipulated PBMCs to be the most appropriate control to compare allodepleted fractions with. In contrast, others have used sensitized activated PBMCs.[115,178] Clearly, however, the 2º MLR is not quantitative and there is no data correlating this assay following allodepletion with GVHD after transfer of allodepleted T-cells clinically.

An alternative assay of residual alloreactivity is the limiting dilutional assay (LDA). This assay determines the frequency at limiting dilution of T-cell progenitors capable of generating an interleukin-2 (IL-2)–producing (T-helper) clone (HTLp assays), cytotoxic responses (CTLp) or colony formation in response to a given stimulator. LDAs are sensitive, but they are time consuming to perform, subjective to analyse, semi quantitative and are complicated by wide confidence intervals, which may obscure significant changes. Furthermore, cytotoxic responses (CD8 CTL)[60] and IL-2 secretion (CD4 Helper) (as evidenced by our own data Fig

17) are expressed only in a minority of alloreactive T cells. Cavazzana-Calvo *et al* demonstrated a 1-1.6 log reduction in CTLp[114] frequencies and Solomon *et al*[139] demonstrated a median 5 fold reduction in HTLp frequencies after CD25IT based allodepletion. The HTLp assay showed poor correlation with the onset of GVHD in their clinical study in the matched sibling setting. Nevertheless, failure of CD25IT allodepletion as indicated by their HTLp assay led to severe visceral GVHD.

Clearly, then we need standardised methodology to measure residual alloreactivity and hence facilitate comparison between allodepletion methods. None of the existing techniques has been proven to correlate clinically with GVHD. We chose to use a combination of primary MLRs (which whilst insensitive, were associated with GVHD in the study of Andre- Schmutz *et al* [138]) delayed bulk 2° proliferation assays, which probably capture most residual alloreactive T-cells, and IFNγ ELISPOTS assays, to assay a different phenotype of residual alloreactive T-cells, acknowledging this is only expressed on a subset of such cells. We accept that such *in vitro* assays have not been shown to correlate clinically with GVHD. However, it could be argued that the absolute level of allodepletion may not be important if techniques show enhanced level of allodepletion compared to existing strategies (e.g. CD25 based) , which have been shown to be safe clinically, is not clear.

Due to the lack of time, we were unable to assess the efficacy of our allodepletion approach in an animal model. The relevance of mouse models in assessing the potential for GVHD in humans after allodepletion is not clear. Animal models have been used by several groups to determine the potential for GVHD after adoptive immunotherapy.[52,112] Chen *et al* demonstrated that despite showing significant *in vitro* alloreactivity after PDP treatment, adoptive immunotherapy with T-cell depleted bone marrow and PDP treated T-cells into a mismatched mouse model, did not lead to GVHD and prolonged survival compared to bone marrow alone.[52] Hartwig et al, used a parent P→F1 model to examine CD95 based allodepletion (H-2^k→H-2^{kd})[119]. Using this model, they demonstrated that adoptive transfer of CD95 deleted T-cells did not induce lethal GVHD, in contrast to primed T-cells, whereas significant *in vitro* alloreactivity persisted when this method used *in vitro* using human PBMCs. [128] Furthermore, animal models of allodepletion do have limitations reflecting differences between mouse and human T-cells. This is exemplified by the finding in mice that

alloreactive T-cells causing GVHD predominantly reside in the naïve T-cell compartment.[28] Our data, demonstrating *in vitro* alloreactivity in both CD45RA+ ve and –ve fractions, indicates that such clear delineation in humans does not seem likely. This highlights the need to use multiple read outs of residual alloreactivity. We have found that proliferative and cytokine responses to 2° stimulation with host antigen presenting cells were highly dependent on the timing of 2° stimulation in relation to immunodepletion, with negligible responses if restimulation was done immediately after depletion. [125] We also examined proliferation in a 1° MLR and assessed the residual % of CD3+CD25+CD71+ after allodepletion by flow cytometry.

In future studies, one potential approach to assess the *in vivo* efficacy of our CD25/71 allodepletion approach in an animal model would be to use a parent→F1 T- cell transfer in H2 mismatched mice: BALB/cJ mice (H-2^d) would be bred with another strain C3H/HeJ (H-2^k) to create H2 hybrid mice. The F1 recipient mice can thus not evoke a host versus graft effect against parental T-cells, whereas parental T-cells will cause lethal GVHD in F1 mice. Donor (parent) mouse PBMCs would initially be co-cultured with the hybrid F1 mouse DCs. Clearly, we would need to confirm that CD25 and CD71 are up regulated on alloreactive mouse T-cells, as what is seen with human T-cells. This being the case, an immunomagnetic CD25/71 allodepletion could be performed. The hybrid mouse could be lethally irradiated and T-cell depleted donor bone marrow with or without residual allodepleted donor mouse PBMCs could then be infused into the hybrid recipient. This mouse model would then assess GVHD across a MHC haplotype mismatch. Weight and signs of acute GVHD (diarrhoea, fur ruffling, and hunchback) in the mice could be assessed in the recipient mice over a 30-100 day period and if GVHD occurred, this could be confirmed histologically. Additionally, if donor T-cells were generated from transgenic mice constitutively expressing firefly luciferase, this would allow tracking of donor T-cells in the recipient mouse. To assess the efficacy of antiviral immunity, the recipient mouse could be injected with lymphocytic choriomeningitis virus (LCMV) as a surrogate for CMV infection or mouse adenovirus strain 1, to assess adenoviral immunity.[181] This model is limited by the fact that activation marker up regulation in mice may differ to that of humans. More importantly, the monoclonal antibody clones used to delete CD25+ and CD71+ mouse T-cells will have different avidity to that used against human CD25+/CD71+ T-cells, thus making it difficult to extrapolate results from a mouse model to that of humans even if transfer of CD25/71 allodepleted T-cells does not cause GVHD in mice. Thus, in

summary there are a multitude of assays used to determine residual alloreactivity. We have chosen to use several assays so that we can assay the diverse phenotypes of alloreactive T cells i.e. 1° +2° MLRs, FACS of activation markers and the 2° IFNγ ELISPOT assays.

Phenotypic Characterisation of Proliferating Alloreactive T cells

In order to design rational strategies to refine allodepletion, we systematically characterized the activation and cytokine marker profile in proliferating alloreactive T-cells. While alloreactive T-cells can be identified by a number of phenotypes, we focused on proliferation in response to alloantigen as the most fundamental. Thus, we co-cultured CFSE labelled T-cells with HLA mismatched DCs, and then tracked the proliferating CFSE dim T-cells using flow cytometry. Our data showed that CD25 was expressed in over 80 % and CD71 in 65 % of proliferating alloreactive T-cells on day 3 of MLR. In contrast, CD69, CD45RA, ICOS, OX40, CD95, HLA-DR, IL-2, IFN-γ, and TNF α were all expressed only in a minority of proliferating alloreactive T-cells, indicating that these are poor targets for allodepletion. Thus this validates our previous strategy of using CD25 as an allodepletion targets and identifies CD71 as a novel target.

Whilst our data provide strong support for targeting CD25 in allodepletion strategies, in order to optimize our allodepletion strategy, we initially compared the effectiveness of CD25 immunomagnetic depletion to that of CD25 IT. In 2 different assays we found no significant difference in residual alloreactivity to host between these methods, confirming similar findings found by other investigators.[124] Clinical grade anti-CD25 immunomagnetic beads (Miltenyi Biotec) are now available, overcoming the limited availability of IT. Whilst our methods gave extremely effective depletion of CD25^{+} (<1%) and residual proliferation to host in primary MLRs was undetectable, significant residual alloreactivity to host was observed in secondary MLRs and ELISPOT assays. These residual responses are comparable to those seen by some investigators [59,115,118,139] but somewhat higher than reported by others using CD25 based [114,122,177] and other allodepletion strategies[178]. However, as highlighted above, comparing residual alloreactivity between such studies is very difficult because of methodological differences. A number of these studies are complicated by reliance in primary MLR[122], the wide confidence intervals for CTLp and HTLp assays[122,177,178] and by comparison of

alloreactivity with donor PBMC previously sensitized to the recipient rather than unmanipulated donor PBMC[18,115,178]. As noted previously, we have found that proliferative and cytokine responses to secondary stimulation with host antigen presenting cells were highly dependent on the timing of secondary stimulation in relation to immunodepletion, with negligible responses if restimulation was done immediately after depletion [125]. In order to skew our assays to optimise detection of residual anti-host responses, in our assays, cells were rested for 2 days after allodepletion before restimulation. By resting the allodepleted PBMCs, alloreactive PBMCs that were not removed by CD25 allodepletion continue to proliferate over the course of the 2 days, so that they constitute a greater percentage at the time of the secondary assays. Nevertheless, down regulation of CD25 before allodepletion, up regulation of this marker after day 3, and the presence of CD25-ve alloreactive T cells may also explain why there was significant residual alloreactivity.

Our studies identified CD71 as strongly expressed on proliferating alloreactive T cells. CD71 is homodimeric receptor (180 kDa) which allows internalization of iron-bound transferrin in clathrin-coated pits. In endosomal vesicles, iron is then released by compartment acidification (pH 5–5.5), while apo-transferrin and CD71 are recycled into the blood or to the cell surface, respectively. CD71 is expressed on marrow stromal cells from bone marrow, on activated T and B lymphocytes, macrophages, and all proliferating cells. It is upregulated on lymphocytes during proliferative responses to antigens or mitogens but is not expressed on resting lymphocytes. CD71 is present on reticulocytes and erythroid progenitors in fetal liver, cord blood, and peripheral blood, yet it is lost as these differentiate to mature erythrocytes. CD71 expression is strictly regulated by intracellular iron level: CD71 mRNA is stabilized and abundant in iron-deficient cells to increase extracellular iron uptake. This post-transcriptional regulation is allowed by the presence of iron responsive elements (IRE) in the 3'-untranslated region of the CD71 transcript that is recognized by two iron regulatory proteins. Our data is consistent with that of Nguyen et al. who demonstrated[69] that there was strong up regulation of CD71 and CD25 on human alloreactive T-cells in an allo MLR, peaking on days 7-9, and that CD71 expression correlated with alloreactive T-cell proliferation. [70] The low level of CD71 expression on resting lymphocytes and its up regulation in a MLR identify CD71 as an excellent novel target for allodepletion. Consistent with this, administration of anti CD71 antibodies have been shown in mismatched mouse model to significantly prolong pancreatic islet graft survival[71]. Administration of gallium (which binds to transferrin, depriving

proliferating cells of iron) potently inhibited MLRs and prolonged survival in a mouse model of GVHD.[72]Additionally, stimulation of CD71 also promotes phosphorylation of the TCRζ complex and thus may amplify TCR signalling .[182] The internal cycling of CD71 may allow this receptor to be targeted with a CD71 immuntoxin, like CD25. However, for simplicity in our studies, the combination of anti- CD71 biotin and anti-biotin magnetic beads was used to delete CD71+T cells. Furthermore, the peak of CD71 expression and the mean fluorescent intensity coincided with that of CD25 (Day 3 of allo MLR) enabling us to target both markers at the same time.

CD69 is a well-established T-cell activation marker but was poorly expressed in the CFSE-dim population. This may in part reflect down regulation of CD69 by the time appreciable CFSE dye dilution has occurred (day 3 of MLR). However, CD69 showed more variable expression compared to CD25 and CD71 and has been noted to be upregulated in bystander cells [116,183], limiting the usefulness of this marker as a target for allodepletion. Recent studies suggest that alloreactivity may reside predominantly in the CD45RA$^+$ naïve T-cell compartment[29,33], at least in mouse studies. The expression of this marker in our studies, on a minority of alloreactive T-cells and on bystander T-cells, as well as the progressive maturation of alloreactive T-cells to a memory phenotype during MLR suggest that targeting this molecule as a sole strategy for allodepletion is unlikely to be successful. Our data also showed that a minority of alloreactive T-cells (<5 %) express OX40 and TNFα, which is comparable to that seen by some investigators[135], but lower than others.[18,53] However, both the latter groups performed their MLRs in media supplemented in serum and/or used thawed cryopreserved stimulators, and therefore up regulation of these markers may reflect responses to bovine peptides presented in the context of human HLA molecules. Activation induced cell death is an important part of controlling T-cells responses, and this pathway has been targeted as a means of enhancing allodepletion.[128] CD95 expression progressively increased over time, but on a minority of proliferating alloreactive T cells (mean of 27 % at day 7), again suggesting that simply targeting CD95 is likely to result in ineffective allodepletion, even if performed at late time points in the MLR.

Rationally Enhancing CD25 based Allodepletion

While our data confirm CD25 as an excellent target for allodepletion strategies, a mean of 17 % of proliferating alloreactive T-cells do not express CD25. Therefore, we studied the

phenotype of proliferating CFSE-dim T-cells not expressing CD25. We have identified CD71 as a novel target that is highly expressed on proliferating alloreactive T-cells, including those not expressing CD25. We found that 70 % of the CFSE-dim CD25^{-ve} population express CD71. Potentially, this enables us to target 2 independent phenotypes of alloreactive T-cells at the same time-point within a single co-culture. Flow cytometric analysis showed that immunomagnetic depletion deletes CD25$^+$ and CD71$^+$ cells to below background levels. In primary MLRs, residual responses to the stimulator were undetectable after CD25/71 depletion. In 2 separate functional secondary stimulation assays measuring distinct phenotypes, the combination of CD25/71 allodepletion led to significantly enhanced and more consistent allodepletion compared to CD25 alone without compromising third party or anti-viral responses. Furthermore, the combination of CD25/71allodepletion was superior to the immunomagentic depletion of CD25/45RA+ T cells. Our studies indicate that combined CD25/71 depletion results in a 20-fold reduction of proliferating alloreactive T-cells flow cytometrically and in secondary MLRs, with a 1-log depletion of response to host in IFN-γ ELISPOT assays. While extrapolation of these results to an *in vivo* setting requires an animal model [127,184], the fact that allodepletion appears enhanced compared with CD25 depletion suggests that it may be possible to safely transfer larger doses of allodepleted donor T-cells than hitherto possible using combined CD25/71 depletion

Our approach led to a 1-1.5 log reduction in residual alloreactivity in our assays without compromising third party responses. Comparison with other clinically applicable allodepletion strategies is difficult because of different methods used. To assay residual alloreactivity after photodynamic purging, Meilke *et al* observed a 4 log reduction in residual alloreactivity compared to unmanipulated PBMCs, but a 1 log reduction compared to untreated sensitized control co cultures in 2º MLRs.[131] This appears counterintuitive and contrasts with our own experience, where untreated sensitized co-cultures demonstrate considerably enhanced responses upon secondary restimulation to the original host. The authors postulate however, that prior sensitization to host induces regulatory T-cells, thus diminishing anti host responses. Despite this, in their HTLp assays, the reduction in alloreactivity was less impressive with a median 20 fold reduction in responses to host. In contrast, Chen *et al* using a similar photodyamic purging method rested their allodepleted PBMCs, and observed over a 6 fold higher IFNγ secretion to host in 2º IFNγ ELISPOT assays compared to unmanipulated PBMCs.[52] This discrepancy highlights the difficulty of comparing similar methods of

allodepletion with different read outs of alloreactivity. Further, the need for specialized equipment for photodynamic purging of alloreactive T-cells limits the applicability of this technique, and the optimal protocol in terms of concentration of the sensitizing agent and timing of light exposure to achieve <u>selective</u> allodepletion are not fully established. Perhaps, most importantly, *in vitro* data suggests that photodynamic purging significantly reduces anti-viral/antifungal activity of the allodepleted donor T-cells. [132] This was reflected in a clinical study of adoptive immunotherapy of PDP treated donor T cells in the haplo-SCT setting in which 3/11 patients developed chronic GVHD and there was a high incidence of infectious related mortality[133]. The lack of systematic examination of anti-viral immunity after PDP treatment highlights a possible flaw in this process.

 There have been numerous other approaches to targeting alloreactive T-cells e.g. CD137, CD69, CD95, naïve T-cells, and chemotherapeutic methods. Wehler *et al* generated CD8+ CTLs against leukaemia/tumour antigens in the haplo setting using CD137 mediated allodepletion.[59] They generated CTL cultures, by stimulating CD8+lymphocytes with single HLA mismatched AML blasts or renal carcinoma cells. [59] On Day 21 of the culture, the lymphocytes were stimulated with HLA-negative K562 cells transfected with the disparate HLA-Class 1 cDNAs. After 24 hours, the CD137 positive alloreactive T-cells were depleted using an immunomagnetic approach.   This study was limited by only showing data on 1 donor recipient pair in the haplo-setting and the complexity and time required for cell culture. Furthermore, there was still significant anti host reactivity after allodepletion in single antigen mismatched donor-recipient pairs allodepletion (1 log reduction in IFNγ ELISPOT compared to sensitized non depleted co-cultures), thus suggesting that this could lead to GVHD in the haplo-SCT setting. Our data demonstrate that CD69 is expressed only in a minority of alloreactive T-cells and that up regulation is much more variable than CD25 or CD71, which could potentially lead to inconsistent allodepletion. Moreover, allodepletion targeting CD69-positive T-cells has been associated with significant reductions in EBV + /WT1+ T cells, suggesting that this marker shows significant bystander up regulation and is thus not specific to alloreactive T cells. [116] CD95 allodepletion was associated with 25-45 % residual alloreactivity compared to sensitized PBMCs on $2°$ IFN gamma ELISPOTs thus suggesting that this method of allodepletion leads to very high levels of residual alloreactivity,[128] particularly given our data showing that sensitized PBMCs show considerably enhanced responses compared to host, compared to thawed unmanipulated PBMCs. Furthermore, CD95 agonistic antibodies have led to haemorrhagic hepatitis in animal models and a GMP grade

CD95 antibody does not exist [129]. There are no published data targeting naïve T-cell markers such as CD45RA in human T-cells. However, our data show that CD45RA is expressed on a much higher proportion of inactivated T-cells than CD25/71, so that targeting naive T-cells may lead to significant depletion of desirable naïve T-cells. Further, CD25/71 allodepletion was non significantly superior to CD25/45RA allodepletion,

A number of groups investigating the use of chemotherapy during ex vivo MLR to selectively delete alloreactive T-cells.[55,120,121,134-136] However, such an approach requires careful dose titration to prevent deleterious effects to bystander T-cells. Indeed, in preliminary dose titration experiments we performed (shown in Appendix), with fludarabine and trimetrexate, we found that the optimum dose for deleting proliferating T cells whilst preserving non proliferating T cells for trimetrexate was, 1μm and for fludarabine was 50ng/ml (Fig 48). At doses above these concentrations, there was significant loss of non proliferating T cells. Due to the expense of bortezomib, we chose the dose for bortezomib for our co cultures based on previous published data.[55] We then co-cultured CFSE labelled T-cells with HLA mismatched DCs, and added trimetrexate 1μm on day 0, fludarabine at day 2 (50 ng/ml) and bortezomib (500nM) on days 1-3 of the co-culture (Fig 49). Our controls included CFSE + T-cells alone and a co-culture set up at the same time but without chemotherapy drugs. The cells were harvested on day 3 and stained by flow cytometry for CD3 and CD25. The percentage reduction in CFSE Dim alloreactive population (including CD25 negative) was then calculated using Trucount beads. The addition of Trucount beads enabled comparison between the different co-cultures as a fixed number of beads were acquired. Our data showed that at this concentration of bortezomib, there was a complete loss of CFSE bright bystander cells as well as the CFSE Dim alloreactive T- cells, thus highlighting the non selectivity of this drug at this concentration. The fludarabine did not significantly reduce the CFSE Dim alloreactive T-cell population compared to control (8.25 % reduction in the CFSE Dim population), thus highlighting the limited efficacy of this drug at this concentration. The trimetrexate produced over 84 % reduction in CFSE Dim population, a 61 % reduction in the CFSE Dim CD25 negative population and a 26 % reduction in the CFSE bright bystander population. Further careful timing of administration of chemotherapeutic agents would be necessary as delayed administration of bortezmib (5 days after BMT) has exacerbated GVHD in a murine model[137]. Likewise, bortezomib exposure in an ex vivo MLR, led to major decreases in third party

activity, thus suggesting that this reagent may not <u>selectively</u> delete alloreactive T-cells. [55].
There are no published data on preservation of antiviral-T cell responses following allodepletion with chemotherapeutic agents. Our data using chemotherapeutic agents to target proliferating alloreactive T-cells is preliminary and will require further work.

Thus, whilst a host of potential methods, for allodepletion now exist, many of these target only a minority of alloreactive T-cells are difficult to scale-up for clinical use or have not been shown to preserve desirable T-cell responses. In contrast, combined CD25/71 immunomagnetic depletion fulfils all these criteria. Ideally, however, comparisons between different methods of allodepletion require assessment of residual alloreactivity and preservation of anti-viral responses in the same donor-recipient pairs. We plan to perform such a comparison between CD25/71 allodepletion with photodynamic purging in collaboration with The Necker group in future studies.

Graft versus host disease represents a fine balance between alloreactive effectors and T regulatory cells. CD25 based allodepletion strategies target T regulatory cells, but this has not led to enhanced rates of GVHD. This probably reflects concomitant effective depletion of alloreactive T-cells. CD69 and CD95 allodepletion do not affect FoxP3 levels, and thus targeting these markers may preserve T regulatory T-cells.[116,128] CD45RA is expressed on human T regulatory cells [185]and thus targeting CD45RA is likely to delete T regulatory cells and could thus potentially exacerbate GVHD if significant numbers of alloreactive effectors persist. Similarly, photodynamic purging also led to a reduction in FoxP3 + T-cells. [131] It is currently not known if CD71 is expressed on human T regulatory T-cells, and we will need to assess this in future studies.

Antiviral Immunity after Allodepletion

If adoptive immunotherapy with allodepleted donor T-cells is to be useful in restoring anti-viral and anti-leukemic T-cell responses clinically, it is critical to demonstrate the specificity of depletion. A number of *in vitro* studies on allodepletion have not adequately addressed the issue of whether anti-viral responses are preserved following allodepletion. Often such studies have relied on demonstration of preserved proliferative or CTLp responses to third party stimulators as surrogate markers for anti-viral T-cell responses in allodepleted donor T-

cells.[117,118] In other studies, where anti-viral responses have been examined, these assays have frequently not been systematic, e.g. responses to key pathogens have not been assessed such as adenovirus [122], or analyses have been non-quantitative without comparison to unmanipulated PBMCs, or limited to a small numbers of donor recipient pairs,[131] or using a single technique.[128]. There remain doubts over whether functionally useful anti-viral immunity is preserved after allodepletion using photodynamic purging or after transducing donor T-cells with suicide genes. Similarly, anti-viral immune reconstitution has not been assessed after RIC haplo-SCT. [27,107,132] The persistence of anti-viral responses after selective allodepletion is vital to confer enhanced immune reconstitution to viral pathogens post haplo-SCT. We have used IFNγ ELISPOT and pentamer assays to compare the frequency of T-cells recognising all 3 major viral pathogens post-SCT (CMV,EBV, and adenovirus), before and after allodepletion. These assays have the advantage that they enable direct quantification of the frequency of viral-specific T cells. In 2 distinct assays, we showed preservation of T-cell responses to CMV/EBV and adenoviral antigens after CD25/71 allodepletion in numerous donor-recipient pairs. The persistence of EBV immunity after CD25/71 allodepletion, despite using EBV derived LCLs as APCs, was due to the use of donor-recipient pairs who were completely HLA mismatched, so that donor T-cells cannot recognise the preserved EBV epitopes. In the haploidentical setting, we have previously shown that T-cell responses to EBV epitopes presented through the non shared haplotype are preserved following CD25-based allodepletion[86] and we anticipate this will also be so with our refined methods.

We did not examine the cytotoxicity of allodepleted T-cells against virally infected cells, as this would either require repetitive stimulation to generate CTL lines or limiting dilutional CTLp assays which are complicated by wide confidence intervals. In our previous clinical study[141], we showed that transfer of 3×10^5/kg allodepleted T-cell was sufficient to confer detectable T-cell responses to EBV and CMV after haplo-SCT, particularly in the context of viral reactivation. This is line with the dose of unmanipulated DLI required to confer cell-mediated immunity to CMV and EBV after HLA-matched SCT.[186,187] Our refined CD25/71 may now allow us to adoptively transfer sufficient T-cells to confer protective immunity to pathogens which evoke low frequency T-cell responses in the donor, such as adenovirus, which is the single most important cause of infectious death after haplo-SCT [168,169]. Based on the precursor frequency of adenovirus-specific CTL in normal donor PBMC [188]and limited clinical data from DLI[141,189], we anticipate that cell doses of between 1- 5×10^6/kg T-cells may be sufficient for such immunity. Since CD25/71 allodepleted T-cells show significantly lower

residual alloreactivity than after CD25 based allodepletion, it may well now be possible to transfer such doses of allodepleted T-cells without causing GVHD. However, because of the limitations of *in vitro* assays and murine models outlines above, this can only be really assessed in the context of a further clinical study.

There are numerous other approaches to improving anti-viral reconstitution post haplo-SCT. IFNγ capture can be used to generate either CMV or adenovirus specific CTLs *in vitro*, but this approach still leaves significant alloreactivity to host (1/2 log reduction in residual alloreactivity following generation of adenovirus specific CTLs).[99] Even though the number of virus-specific T-cells generated is very low (typically 10^6 T-cell from 500 mls of blood) the occurrence of GVHD in MSD/MUD patients who received CMV and adenovirus specific CTLs generated by IFNγ capture at doses of only 10^3-10^4/kg, suggests that this technique is not completely specific [190], and there remains the possibility of significant GVHD in the haploidentical setting. Where MHC Class I restricted immunodominant epitopes have been determined (e.g. for CMV), it has been possible to isolate and adoptively transfer CD8+ virus-specific T-cells using MHC-peptide multimers.[98] However, it is unclear how durable such responses are in the absence of CD4 help (as would be the case early after haplo-SCT). Further, it is only applicable if the donor has one of the common Caucasian HLA Class I alleles (e.g. A2 or B7) and is of limited value for pathogens such as EBV which have a broader array of immunodominant antigens than CMV. Other approaches e.g. generation of virus/Aspergillus-specific CTL lines and clones[101] by repetitive antigenic stimulation remain too complex and time consuming to broadly applicable, even when multiple viruses are targeted [102]. By contrast, our allodepletion approach is simple, applicable to all HLA backgrounds, preserves CD4 and CD8 T-cell responses and show preserved T cell responses to pathogens recognised by the donor.

One limitation of our approach was the lack of assessment to fungal pathogens e.g. Aspergillus. The immune response to Aspergillus involves both the innate immune system and TH_1 responses[191] and Aspergillus remains a major pathogen post haplo-SCT.[101] The T-cell response to Aspergillus and Candidal antigens has so far been poorly defined and the immunodominant antigens are unknown, so that existing techniques for assessing cell-mediated immunity with LDAs [132] ELISAs and proliferation assays.[135,191] have utilised poorly characterised crude lysates from the conidial/hyphal stages. This limits our ability to test quantitatively how completely T-cell responses to fungal pathogens are preserved after

CD25/71 allodepletion but nonetheless we plan to assess proliferative responses to fungal and control lysates in future experiments.

Enhancing Anti-leukaemia Activity of Allodepleted Donor T cells

The persistence of anti-leukaemic responses after selective allodepletion is critical if the benefits of adoptive transfer are not to be offset by leukaemic relapse, as in our previous study [141] and other studies using CD25 immunotoxin based allodepletion: in our study, 6 out of 11 patients with haematological malignancies relapsed, 3/5 in The Necker study[138], and 4/10 in The NIH study[139]. Similarly, relapse remains a significant problem after CD3/19 RIC haplo-SCT (12/29 patients), despite the infusion of higher numbers of alloreactive NK cells [9]. Our approach will deplete T-cell responses against the mismatched HLA alleles and ubiquitous minor histocompatibility antigens presented by the shared HLA alleles. Nonetheless, we and others [86,122,142] have shown that anti-leukaemic activity may be retained after allodepletion. In particular, we have demonstrated that T-cell responses to potential myeloid tumour antigens are preserved by virtue of their lack of expression on the LCL used as stimulators. Potentially, such T-cell responses could be augmented after transfer of allodepleted donor T-cells into HLA-A2+ve patients with myeloid malignancies by vaccination with WT-1 or PR-1 peptides[85] Likewise, allorestricted responses against tumour- associated antigens presented through the non-shared haplotype[143-146] should also be retained. It is unclear how many allodepleted donor T-cells would be required to confer clinically relevant anti-leukaemic responses in the haploidentical setting: because of the extreme lymphodepleted environment after haplo-SCT and the possibility of allorestricted responses, this may in fact be considerably lower than the numbers of DLI required to induce GVL after HLA-matched SCT, where T-cell doses of 10^7-10^8/kg are required.[78,192,193] Because of the limitations of experimental models, it may be that this question can only be answered in a clinical study. The close association of GVHD and GVL, nevertheless, suggests that these are intrinsically related, and by deleting the alloreactive T-cells that cause GVHD, we may abrogate responses to leukaemia. Further, it remains possible that even with the enhanced depletion of alloreactivity achieved with CD25/71 allodepletion, it may still not possible to safely transfer enough T-cells for anti-leukaemic responses without causing GVHD. In order to overcome these limitations and to enable allodepleted T-cells to confer anti-leukaemic responses even in ALL, which is a frequent indication for haplo-SCT, we investigated whether it was possible to redirect the specificity of the allodepleted donor T-cells using a chimeric T-cell receptor transfer. We have shown that

the anti-leukaemic activity of allodepleted T-cells can be augmented, by transfer of a chimeric TCR recognising the surface molecule CD19.

The approach of chTCR transfer has a number of advantages in targeting residual leukaemic cells after SCT;

1) It overcomes the lack of immunogenic tumour antigens on ALL blasts by redirecting T cells to surface molecules expressed by malignant cells. This has been the major obstacle to immunotherapy for ALL.

2) It should not cause GVHD, as CD19 is highly expressed on B-ALLs, but is not expressed on non-haematopoietic cells.

3) It enables targeting of tumour cells in a HLA-independent fashion, so that a single vector system can be used to treat all patients expressing this surface molecule

4) It overcomes mechanisms by which leukaemic cells escape from T-cell recognition, including down regulation of HLA class I molecules or defects in antigen processing which may hinder alternative methods of enhancing anti-leukaemic activity of T cells e.g. TCR gene transfer.

Redirected T-cells can also home to tumour sites, proliferate locally, and penetrate solid tumours.

We investigated if lentiviral transfer of a chimeric TCR directed against CD19 could be used to redirect the specificity of allodepleted donor T-cells so that they recognize this malignancy. Previous studies have demonstrated that human T-cells expressing similar CD19 chimeric TCRs mediate regression of B-lineage malignancies in murine models [152,156]. To preserve their phenotype and anti-viral specificity, we used a lentiviral vector and transduced allodepleted T-cells after stimulation with IL-2 alone rather than with mitogens. This regimen has been demonstrated to preserve the phenotype of naïve PBMCs, and not compromise anti-viral/third party activity.[107,170] Due to a shortage of time we were unable to assess formally if anti-viral immunity is maintained with our CD19R/pHR-SIN-SE lentiviral vector. Potentially, this could be done using pentamer and IFNγ ELISPOT assays. As the transduced allodepleted donor T-cells recognize CD19 expressing B-cells, CMV (pp65) peptide mix-pulsed or adenovirally transduced autologous CD14+ monocytes rather than PBMCs could be used for such experiments. Further experiments to determine the phenotype of the transduced allodepleted

donor T cells (CD4/CD8, CD45RA, CD45RO, CD62L) after transduction will also be required.

The levels of cytotoxicity we observed with CD19-redirected allodepleted donor T-cells, was lower than that reported with retrovirally transduced T-cells expressing the CD19ζ transgene. [150,153] This may reflect the fact that our transduction regimen resulted in predominantly naïve T- cells being transduced, which lack cytolytic activity. However, as outlined above, our transduction may be associated with a lower risk of activation induced cell death ,[161] and may preserve anti-viral responses of our transduced cells [107,170], whereas, transduction regimens using OKT3/CD28 promote differentiation and activation of naïve PBMCs into terminally differentiated CTLs.[156,194] T-cells transduced after polyclonal stimulation, despite demonstrating potent *in vitro* activity, may have reduced *in vivo* activity because of cytokine dependence, activation induced cell death, and reduced capacity to home to tumour sites, proliferate and resist apoptosis. In contrast, naïve PBMCs have shown superior anti tumour activity than terminally differentiated CTLs in mouse models, partly due to higher co-expression of CD62L.[155] Furthermore, transfer of low doses of antigen specific T-cells has led to their dramatic expansion post haplo-SCT in response to their cognate antigen.[101,141] Thus, it is possible CD19-redirected allodepleted donor T- cells could rapidly expand *in vivo* when administered to lymphopenic patients not receiving immunosuppressive medication for GvHD prophylaxis. The addition of a CD28 co-stimulatory domain to the CD19ζ construct would increase IL-2 secretion, and enhance persistence, but there is no evidence to suggest that such a domain would enhance cytotoxicity.[194] However, the addition of a CD28 co-stimulatory domain enhances the risk of autonomous proliferation and the addition of a suicide gene to such constructs may be required by the regulatory agencies as an additional safety measure.

Potentially we could compare CD19ζ constructs with and without CD28 co-stimulatory domains by assessing IFNγ, granzyme B secretion and proliferation of CD19ζ transduced and CD19/CD28ζ transduced allodepleted T cells to CD19+ targets, to determine if using constructs which have CD28 co-stimulatory enhance proliferation and cytokine release. Further comparisons between different CD19 chTCRs would be also useful: e.g. comparison between a fully humanised anti CD19 chTCR[195] and the existing FMC63 ScFv receptor. A fully humanised CD19chTCR (in contrast to FMC63 where the ScFv is murine), would have

the advantage of being less immunogenic and may therefore enhance the survival of transduced allodepleted T-cells *in vivo*.

There are numerous potential approaches to measuring anti-leukaemic activity of allodepleted donor T cells. Although the ^{51}Cr cytotoxicity remains the gold standard, various alternative assays have been developed, such as the granzyme B ELISPOT assay.[171] In direct comparisons of the granzyme B ELISPOT, and the ^{51}Cr cytotoxicity, the results have correlated well between the two.[171] Furthermore, the granzyme B ELISPOT assay is non radioactive and granzyme B is a molecule that is released upon CTL mediated killing.[196] While granzyme B activity may underestimate cytotoxicity, as it does not measure FasL mediated apoptosis, this is equally true of the ^{51}Cr release assays. Furthermore, it is well established that only a small fraction of antigen-specific T-cells secrete cytokines in response to their cognate antigen at a given time point. This explains why despite 19 % of allodepleted T-cells being transduced with the CD19ζ transgene, only 0.2 % of transduced allodepleted cells secreted IFNγ in response to primary ALL blasts, and 0.4 % secreted IFNγ in response to autologous LCLs. On the other hand, CTLs may degranulate normally, but certain targets may be inherently resistant to their effects. Thus, to be certain that degranulation is inducing target cell death, cytotoxicity assays should be performed alongside the granzyme B ELISPOT.

We plan to FACS sort our transduced allodepleted donor T-cells and assess cytotoxicity against CD19+ targets either in a conventional ^{51}Cr release assay or with a FACS based cytotoxicity assay. In the latter approach, FACS cell sorted (CD3+, α Fc+) transduced and mock transduced allodepleted T-cells would be co-cultured with an equal number of CD19+/- targets such as Daudi, SUP-B15 and HSB-2 using a target: effector ratio of 1:1. After a week cells would be stained with 7 AA-D, CD19 and trucount counting beads added. A fixed number of beads would be acquired and the number of viable CD19+/- targets would then be determined in each co-culture. We anticipate that CD19ζ transduced allodepleted T-cells would lyse CD19+ ve but not –ve targets. Irradiated donor LCLs could also be added to co-cultures to promote co-stimulation

An alternative means of assessing anti-leukaemic activity is with MHC-peptide multimers to detect T-cells recognising leukaemia-associated antigens. Amrolia *et al* demonstrated that

proteinase 1 (PR1 +) T cells were maintained after allodepletion with CD25 IT whilst using LCLs as APCs using tetramer studies [86] Likewise, it is possible to detect CD8+ ve T cells recognising the PR1 epitope of proteinase 3 in patients with myeloid malignancies.[83,85] Unfortunately, while we predict T-cell directed against myeloid leukaemia associated antigens should similarly be preserved following CD25/71 allodepletion by virtue of their lack of expression on LCL stimulators, we were unable to obtain patient samples with detectable WT1+ or PR1+ T-cells to determine if our refined allodepletion method affects the frequency of such T-cells. Potentially, we could co-culture PBMCs from HLA-A 0201+ve donors who have detectable CD8+ WT1+/PR1+ T cells, with HLA- A2+ mismatched LCLs and compare the frequency of CD8+ WT1+/PR1+ T cells in unmanipulated PBMCs and CD25/71 allodepleted T-cells flow cytometrically with WT1/PR-1 specific multimers.

Whilst tetramer studies, enable the size of a particular population to be determined and are restricted to defined antigens, they do not give functional data. A number of groups have used LDA based assays to assess functional responses to leukaemic blasts. For example, Mavroudis *et al* [142] demonstrated preserved HTLp responses to myeloid tumours after allodepletion using a CD25 immunotoxin, and Montagna *et al* [122] showed preserved CTLp responses to leukaemic blasts after a similar approach. As highlighted previously, these data are complicated by the indirect nature of the CTLp and HTLp assays, which involve *in vitro* restimulation, as well as by the wide confidence intervals for these assays, which may obscure significant changes. The rationale for using the HTLp assay (which depends on donor CD4$^+$ T cells) to measure a GVL effect rests on accumulating evidence implicating CD4$^+$ T cells in the alloresponse to CML.[142] In experimental studies, CD4$^+$ cells inhibit leukaemia growth and are cytotoxic to myeloid leukaemia cells. Although HTLp assays may correlate with GVL reactivity, they do not directly measure a cytotoxic effector function against leukaemia. Similarly, our IFNγ ELISPOT assays with CD19ζ transduced allodepleted T-cells give us information primarily about CD4+ GVL responses, but not directly about cytotoxicity. Nevertheless, *in vivo* studies suggest that CD4+ T cells may play a critical role in tumor rejection via cross presentation of tumor antigens, and such T helper assistance may be of vital assistance in eradicating malignancies.[197]

The anti-leukaemic effects of CD19ζ transduced allodepleted donor T-cells needs to be tested in an animal model prior to scale up studies. One possibility is to test this approach using allodepleted mouse T-cells in a MHC mismatched mouse with a subcutaneous B-cell lymphoma, as has been done following administration of donor T cells and T regs [112], administration of PDP treated T cells[52] and fludarabine treated DLI[120]. However, the relevance of such models is unclear, as responses in such models are frequently more impressive than clinical responses e.g. to DLI. Further, such experiments would require the use of anti-mouse CD25/71 mouse antibodies which may well have different avidities to their anti-human counterparts, so that it is very difficult to extrapolate from such models. An alternative model is to use a xenogeneic approach in an immunodeficient mouse models e.g. NOD/SCID mouse which has been irradiated and then injected subcutaneously with a human lymphoma/leukaemia cell line. Such models have previously been used successfully to demonstrate the anti-leukaemic effect of T-cells retrovirally transduced with a chimeric TCR against CD19.[152,176] Large doses of transduced donor T-cells expanded *ex vivo* in the presence of IL-15 were required to mediate regression of Raji cell tumors and data on long term persistence of transduced T-cells is lacking. Further, clearly such models do not mimic the localization of leukaemia to the bone marrow. Despite these limitations, this model remains the best available to evaluate anti tumor adoptive immunotherapy *in vivo*. In future studies, we will evaluate the anti- leukaemic activity of CD19ζ transduced allodepleted donor T cells in a Rag I/II deficient, γ chain deficient, C5 deficient NOD/SCID mouse model. Mice would be irradiated and then be subcutaneously injected with a variety of human tumours e.g. primary human ALL blasts, Ramos cells, and LCLs. Human CD19 ζ transduced and mock transduced allodepleted T cells could then be infused and survival and tumour growth could be documented. Tumour cells could also be labelled with luciferase using a retroviral vector and *in vivo* imaging performed following administration of ζCD19 transduced/mock transduced allodepleted donor T cells to determine if transduced allodepleted T-cells mediate tumour regression.

CD19R/ pHR –SIN-SE transduced allodepleted donor T cells efficiently secreted IFN-γ and the cytolytic effector molecule granzyme B in response to CD19 tumor cell lines and primary ALL blasts. Our data suggest that potentially CD19-redirected allodepleted donor T-cells could be used to augment both anti-viral and anti-leukemic T-cell responses after haplo-SCT. Since CD19 is expressed only on the B- cell lineage, and the T-cells are allodepleted, such an

approach should not cause toxicity to extra-haematopoietic tissues. However, targeting of normal B-cells is likely to induce B-cell lymphopoenia, and impair humoral immunity necessitating, immunoglobulin replacement. At worst, the clinical consequences of this are likely to resemble X-linked (Bruton's) agammaglobulinaemia, a genetic disorder of B-cell development. When receiving regular immunoglobulin replacements, such patients have a normal life expectancy. In patients at high risk of leukaemic relapse, this may be an acceptable price to pay for the potential benefit in reducing relapse risk.

One of the concerns of retroviral transduction of human PBMCs is the risk of insertional mutagenesis. Acute leukaemia has developed in 4 of 9 children treated with gene therapy for X- linked SCID in France and 1 out of 10 in the UK. This adverse event was attributed to the integration of the retrovirus into the LMO2 locus in 3 patients, a key transcription factor in T-lymphoid progenitors, resulting in aberrant expression of this gene and uncontrolled proliferation of T lymphoid blasts. In above study, CD34 selected haematopoietic progenitors were transduced. However, there have been no reported cases of insertional mutagenesis in over 100 patients followed up over an extended period following treatment with retrovirally transduced mature T-cells. Likewise, there have also been no reported cases of insertional mutagenesis, in patients treated with T cells retrovirally transduced with a chimeric TCR to treat HIV or neuroblastoma. Thus, the risks of insertional mutagenesis following transduction of mature PBMCs is very low. Furthermore, our use of a self inactivating (SIN) lentiviral construct further reduces the risks of insertional mutagenesis [161], arising in transduced allodepleted T-cells.

Scale Up

Our studies suggest that for strategies targeting surface markers or cytokines secreted by alloreactive T-cells, CD25/71 is the optimal combination for effective depletion of alloreactive T-cells. Further studies comparing residual alloreactivity and preservation of anti-viral responses after CD25/71 depletion and other methodologies such as photodynamic purging[52,140,178] using standardised assays will be required. However, these methodologies have not yet been tested clinically and their complexity/need for specialised equipment may limit their applicability. In contrast, combined CD25/71 depletion is simple and feasible

clinically. Keeping the period of co-culture short (3 days), and manipulations simple would be a major advantage in terms of clinical feasibility. The separation methodology used is already in widespread clinical use for CD34 selection in centres performing haplo-SCT and easily performed under Good Manufacturing Practice (GMP) conditions. Clinical grade anti-CD25 and anti-biotin immunomagnetic beads are available and we are currently generating a GMP grade biotinylated anti-CD71. The mean yield of PBMCs using combined CD25/71 depletion was 17.1 % of the initial PBMC dose (range 14.5-25.4 %). We are routinely able to generate 8 x 10^7 allodepleted T-cells from a 450 ml blood donation, so that add back of 10^6/kg CD25/71 allodepleted donor T-cells would be feasible from a blood draw without the need for leucapheresis.

The optimum APC for further clinical studies is a key issue, as optimizing antigen presentation is critical to allodepletion. PBMCs are easily accessible, but the phenotype is variable and they may not be available in aplastic patients. We have previously demonstrated that LCL stimulation resulted in a more consistent depletion of *in vitro* alloreactivity than stimulation with host PBMCs using anti CD25IT based allodepletion.[86] Because of the difficulty in obtaining sufficient PBMCs, particularly in children, some groups have investigated using PBMCs from the non-donor parent as stimulators for allodepletion. However, we believe this approach is flawed, as it will not activate (and hence deplete) T-cell responses against minor histocompatibility antigens that differ between the donor and the recipient presented through the shared HLA molecules or against allorestricted minor histocompatibility antigens which differ between the recipient and the non-donor parent presented through the non-shared haplotype. Hence, we believe that the APC used for stimulation must be recipient derived.

Our current studies suggest that allodepletion with anti CD25/71 beads following stimulation with host LCLs or DC, appears more consistently effective in depleting *in vitro* alloreactivity than after stimulation with cytokine stimulated host PBMCs The levels of CD25 and CD71 up regulation appear equivalent with DC and LCL, as do residual responses to host after CD25/71 allodepletion in 2° MLRs. PBMCs are easily accessible, though they have a variable phenotype, and are less effective as APCs compared to LCLs. In our comparison of DCs, cytokine stimulated PBMCs, and LCLs as APCs, the use of host/3rd party LCLs as secondary stimulators in the 2° MLR, may have led to higher responses in the DC/PBMC arm, due to preserved EBV responses through shared HLA antigens. We plan to repeat these experiments, but use host/3rd party PBMCs as secondary stimulators. LCLs are an excellent APC, as they

strongly express co-stimulatory molecules, have a standard phenotype, can be generated even in aplastics and do not express potential myeloid leukaemia antigens, and thus may retain a GVL response to myeloid leukaemias. However, they take 6 weeks to generate, significantly complicate generation of allodepleted cells under GMP conditions, and results in deletion of EBV-specific T-cells restricted through the shared haplotype. Though there were high levels of EBV PCR detected in aciclovir treated LCL co-cultures, co-culture of irradiated LCLs with donor PBMCs did not lead to their transformation and no EBV-associated lymphoproliferation was seen in our previous studies. On the other hand, DCs are excellent APCs, which can be generated much more rapidly (7 days), but will require much larger volumes of recipient blood. We estimate that while allodepleted T-cell doses of 10^6/kg could be generated from a blood draw, larger doses will require a leucapheresis. Because of the potential preservation of anti-leukaemic responses, use of LCL stimulators may be advantageous in clinical studies in myeloid malignancies such as AML. However, in view of the regulatory complexity of using LCLs under GMP conditions, for further studies in non-malignant patients we plan to use DCs as stimulators.

Prior to a proposed clinical study, we will need to scale up our experiments in the cell/gene therapy laboratories at Great Ormond Street Hospital, to determine if we can reproduce our in vitro data on a large scale. One concern, from studies from The Necker Hospital group[198], was the failure to reproduce their in small scale *in vitro* data using CD25 beads, when scaled up on the CliniMACS system. After co-culturing donor PBMCs with HLA-mismatched irradiated PBMCs, they performed 3 CD25 immunomagnetic depletion using a CliniMACs system. Initially, allodepleted cells were infused without prior assessment of residual alloreactivity and 2 of 4 such patients developed severe GVHD. Subsequently, residual responses were tested in a HTLp-based LDA assay and CD25 bead allodepleted T-cells only met the release criteria of 1.2 log depletion on host reactivity in half the cases. One limitation of their approach was the use of the PBMCs from the non-donor parent as APCs. As noted above, PBMCs may be sub-optimal APCs for allodepletion, particularly when derived from the non-donor parent. Clearly, however, we need to demonstrate effective depletion of T-cell responses directed against the host in large scale experiments using our system. We hope that our use of host DCs as APCs, combined with targeting an additional marker expressed on CD25 negative alloreactive T-cells will enable us to achieve effective allodepletion even in the clinical setting. Our initial scale-up in 1 donor-recipient pair, suggested that this may well be possible although clearly this will need to be replicated in other donor-recipient pairs.

Our group is currently optimising conditions for CD25/71 immunomagnetic allodepletion under GMP conditions using the CliniMACS system. We have shown that use of upright T-175 flasks rather than cell culture bags results in both better activation of donor T-cells and improved cell recovery. Our group is now testing whether the combination of αCD71 + anti-CD25 beads/anti-biotin beads or αCD25 + αCD71 biotin and anti-biotin beads gives more effective depletion of alloreactivity. Additionally, we will perform further experiments to determine, whether as suggested from our previous large scale run, CLINIMACs depletion tubing sets (which are designed for depletion of larger number of cells) result in improved allodepletion compared to TS tubing sets.

Once we have optimised our large-scale CD25/71 allodepletion under GMP conditions, we plan to collaborate with Prof. Cavazzana-Calvo's group in Paris to systematically compare CD25/71 allodepletion with photodynamic purging using the Kiadis method[131], in the same donor-recipient pairs, using standardised assays to assess residual alloreactivity. We plan to co-culture normal donor PBMC from buffy coats of 4 donors with HLA-mismatched DC (at a ratio of 10:1) under serum-free conditions in T-175 flasks for 3 days. Co-cultures will be split into 2 arms and allodepletion performed under GMP conditions using either immunomagnetic CD25/71 negative selection or photodynamic purging. Allodepleted T-cells from each arm will be plated out in a LDA proliferation assay, or rested in serum-free, cytokine-free media for 2 days and then restimulated to host/3rd party PBMCs in a delayed 2ºMLRs. The residual responses to host and 3rd party using both allodepletion methods will be compared to those of unmanipulated PBMCs from the same donor. The preservation of anti-viral responses after both methods will also be compared. These experiments will involve collaboration between several European groups and will determine the optimum method for selective allodepletion, as a prelude to a further collaborative study of allodepletion after non malignant haplo-SCTs.

Proposed Clinical Trial

While functional assays can provide useful comparisons of the efficiency of allodepletion, they cannot fully predict clinical outcome. We therefore now plan a further clinical study of immunotherapy with allodepleted donor T-cells to determine if CD25/71 allodepleted T-cells can safely augment anti-viral and anti-leukaemic responses after paediatric haplo-SCT.

If our scale-up experiments above our successful, we will assess the safety and biological effects of adoptive transfer of allodepleted donor T-cells generated using negative immunomagnetic selection for CD25/71 in a multi-centre, phaseI/II study children with non-malignant disorders undergoing haploidentical or multiply HLA-mismatched unrelated donor SCT with a CD34-selected graft. We have chosen to focus, on patients with non-malignant diseases in this study in order to determine the impact of this intervention without the confounding factor of relapse of malignant disease. Eligibility criteria are shown below;

Inclusion Criteria:

Patients with the following non-malignant conditions who lack an HLA matched (10/10 or 9/10 allelic) donor and are planned for SCT from a haploidentical or mismatched (≤ 8/10 allelic) family/unrelated donor:

Haemophagocytic lymphohistiocytosis

Combined immunodeficiency state or SCID

Severe aplastic anaemia unresponsive to immunosuppression

Transfusion-dependent Fanconi's anaemia

Osteopetrosis

Hurler's syndrome

Exclusion Criteria:

1.   Patients with a life expectancy (≤ 6 weeks) limited by diseases other than the primary indication for transplant

2.   Patients with pre-existing severe restrictive lung disease (FVC or FEV1< 50% predicted)

3.   Patients with severe hepatic disease (bilirubin greater than 50 uM or ALT>500IU/ml)

4.   Acute graft-versus-host disease ≥ grade 2 (Seattle criteria) at time of infusion of allodepleted cells

5.   Pulmonary disease requiring > 28% O_2 supplementation or active pulmonary infiltrates on chest X-ray at time of infusion of allo-depleted cells

6.   Presence of severe intercurrent infection at time of infusion of allo-depleted T-cells (if present consult with principle investigator)

7.   Patients in whom allodepleted donor T-cells do not meet release criteria will be excluded

Four weeks prior to SCT, the patient will undergo an unstimulated leucapheresis for generation of recipient DCs. Prior to receiving G-CSF for CD34 mobilisation, 500 ml blood will be taken from the SCT donor for generation of allodepleted donor T-cells. For parental donors who consent to entry on the study this should be straightforward and for mismatched unrelated donors in previous studies we have routinely obtained this volume of blood for adoptive immunotherapy from unrelated donor registries. Mononuclear cells from the stem cell donor will be co-cultured with recipient DCs and allo-depletion performed using anti-CD71 biotin + anti-CD25/anti-biotin immunomagnetic beads under GMP conditions at Great Ormond St Children's Hospital according to established Standard Operating Procedures (see Appendix). Samples of the allodepleted donor T-cells will be tested to ensure the efficacy of allodepletion and sterility and the remainder cryopreserved in aliquots. Sufficient allo-depleted T cells will be prepared and safety tested at one time for a complete course for each patient. The efficacy of allo-depletion will be confirmed pre-infusion using immunophenotyping ($< 1\%$ CD3+71^{+ve} cells), primary MLR ($< 10\%$ residual proliferation to host) and delayed secondary MLR assays (> 10-fold reduction in proliferative responses to host PBMCs compared to unmanipulated donor PBMC) studies. Allodepleted donor-T cells will be tested for bacterial/ fungal sterility (Bactec assay), Mycoplasma (by PCR) and endotoxin (LAL assay).

Patients will then proceed with their transplant with the conditioning/GHVD prophylaxis and a CD34 selected graft. If patients have engrafted with no acute GVHD≥ 2, and allodepleted T-cells meet the release criteria, they will receive intravenous infusions of allodepleted donor T-cells at increasing doses (10^4/kg at day 30 post-SCT, 10^5/kg at day 60 and 10^6/kg at day 90) at monthly intervals post-SCT until either their circulating CD3 count > 1000/L or they develop acute GVHD $\geq$ Grade 2. Patients will be monitored for the outcome measures outlined below by clinical examination and laboratory assays on the for the first year post-SCT to assess for

toxicity and GVHD, incidence of virus-associated/invasive fungal disease, immune reconstitution and survival.

Outcome measures

<u>*Primary*</u>

1. Toxicity attributable to transfer of allodepleted donor T-cells:

(a) Incidence of grade III or grade IV toxicity (graded by the NCI BMT Toxicity Criteria Version 2.0) attributable to transfer of allodepleted donor T-cells

(b) Incidence of Grade II-IV and Grade III-IV acute GVHD before day 100 (graded by Seattle criteria) and limited/extensive chronic GVHD between days 100-365.

2. Time to recovery post-SCT of circulating T-cells to > 1000/µL and CD4 count to > 300/µL

<u>*Secondary*</u>

1. Incidence of virus-associated and invasive fungal disease in the first year post-SCT

2. Time to recovery post-SCT of normal TCR diversity as assessed by Vβ spectratyping

3. *In vitro* anti-viral responses of circulating PBMC after adoptive transfer of allodepleted donor T-cells using interferon-γ ELISPOT and HLA-peptide pentamer assays.

4. Transplant-related mortality and overall survival at 1 and 2 years post-HSCT.

This study will determine if immunotherapy with allodepleted donor T-cells generated using CD25/71 immunomagnetic depletion is safe and improves T-cell reconstitution after HLA-mismatched SCT compared with historical control data. This will lay the foundation for larger, randomised studies to assess the effect of immunotherapy with allodepleted donor T-cells on transplant-related mortality and survival after HLA-mismatched SCT.

APPENDIX

SOP for CD25/71 allodepletion

Purpose and Principle of the Procedure

Viral infections and relapse are the major causes of morbidity and mortality following a haploidentical (genetically mismatched parental) stem cell transplant. Infusion of donor lymphocytes can prevent or treat disease relapse and infections but there is a high incidence of GVHD using unmanipulated donor lymphocytes, due to the presence of alloreactive T-cells. The elimination of alloreactive T-cells may minimize the possibility of GVHD without eliminating T-cells responsible for the desirable anti-viral effects, so that infusion of such T-cells may improve T-cell reconstitution and decrease infections post –haploidentical transplantation. Alloreactive T-cells express CD25 and CD71 and they can be removed by CD25 and CD71 beads in an allergenic MLR. The residual T-cells can then be infused to improve the immune reconstitution of the patient post transfer after bone marrow transplantation

Material

Clinical Grade AIM V	Invitrogen
T75 vented flasks	Nunc (178891)
Ficoll	GE Healthcare (17-1440-03)
EBV–Lymphoblastoid cell lines (LCL)	
Biotinylated anti Human CD71 Antibody	BD Biosciences (555535)
Biotinylated anti Human CD25 Antibody	Roche/Miltenyi (0.1ug/ml)
CliniMACS® ᵖˡᵘˢ Instrument	Miltenyi Biotec (151-01)
CliniMACS® CD25 reagent	Miltenyi Biotec (274-01)
CliniMACS® Flexible Labelling System (FLS)	Miltenyi Biotec (173-01)
CliniMACS® Tubing set	Miltenyi Biotec (161-01)
Presystem –Filter	Miltenyi Biotec (181-01)
CliniMACS® Buffer	Miltenyi Biotec (700-25)
Sample site coupler	Miltenyi Biotec (189-01)
Transfer Set Coupler/Needle	Miltenyi Biotec (185-01)

Transfer Bag 600 mls	Miltenyi Biotec (190-01)
Luer/Spike Interconnector	Miltenyi Biotec (187-01)

Human Serum albumin

Tubing Slide Clamps/ Scissor Clamps

18 G needle

Biological Safety Cabinet

Refrigerator (4-8ºC)

Centrifuge

Incubator (37ºC/5% CO_2)

Haemacytometer and Trypan Blue

Cryovials

FACS tubes

Cell strainer 70µm   BD Biosciences (352350)

FACS Buffer (PBS, 1% HSA)

1. Day-42   Preparation of recipient LCL

2. Day-10   Spin down 1 T-72 flask of recipient LCL and resuspend in 40 mls AIMV with 100µM acyclovir. Transfer 20mls to each of 2 x T-75 flasks

3. **Day -7 Setting up co-culture and primary MLR.** Obtain 300 mls of peripheral blood in preservative free heparin for co-culture and a further 10 mls for ID testing and tissue typing. Donor PBMCs need to be fresh. Prepare donor PBMCs over Ficoll gradient. Freeze 4 aliquots of 5x 10^6 PBMCs for follow up studies.

4. Resuspend donor PBMCs at 2x 10^6/ml in AIMV.

5. Harvest 40 mls of aciclovir treated recipient LCL in mid log phase into a 50 ml centrifuge tube. Centrifuge 400g X 5mins and resuspend in 3 mls AIMV. Count viable cells using haemacytometer and adjust cell concentration to 2x10^6/ml

6. Irradiate LCL to 70 Gy (DCs to 40 Gy)

7. Add 1/40th volume of recipient irradiated LCLs to donor PBMC aliquot

8. Incubate co-culture in 37ºC/5 % CO_2 for 4 days

Day -4 CD25/71 depletion

1. Take 2mls of co-culture using 5 ml Eppendorf Pippettman for FACS analysis

2. Harvest co-culture into 50 ml centrifuge tube. Rinse the flask twice with 5 mls AIMV and pass the contents through a 70μm cell strainer these into a 50 ml centrifuge tube

3. Centrifuge the tube at 400g X 5minutes

4. Remove the supernatant and resuspend in 10 ml CliniMACS® buffer/0.5% HSA ("buffer") to break any clumps. Bring up the volume to 50 mls buffer, count and centrifuge 400g X 5minutes again.

5. Remove the supernatant and resuspend to adjust concentration to 800μl buffer/10^8 PBMCs in buffer in 50 ml centrifuge tube.

6. Clean the bung of one vial biotinylated anti human CD71 antibody with an alcohol wipe and allow to air dry.

7. Draw up using a 18 G needle and syringe. Add 200 μl of biotin CD71 antibody and 2μg of biotin CD25 antibody/10^8 PBMCs. Add the contents to the co-culture and incubate for 15 mins at 4 degrees. (Biotinylated CD71 – clone MA712). Mix well

8. Wash with buffer by filling up the tube (50 mls buffer/10^8 PBMCs) and resuspend cells in 1ml/10^8 PBMCs buffer.

9. Clean the bung with an alcohol wipe of one vial FLS reagent and allow to air dry.

10. Using 18 G needle and syringe add 100 ul FLS reagent each per 10^8 PBMCs. Mix well and incubate at room temperature for 30 minutes on a roller.

11. Wash again with buffer and then resuspend at 1x 10^8 PBMC per ml buffer, in a minimum volume of 40ml

Allodepletion with Chemotherapy Agents

(a)
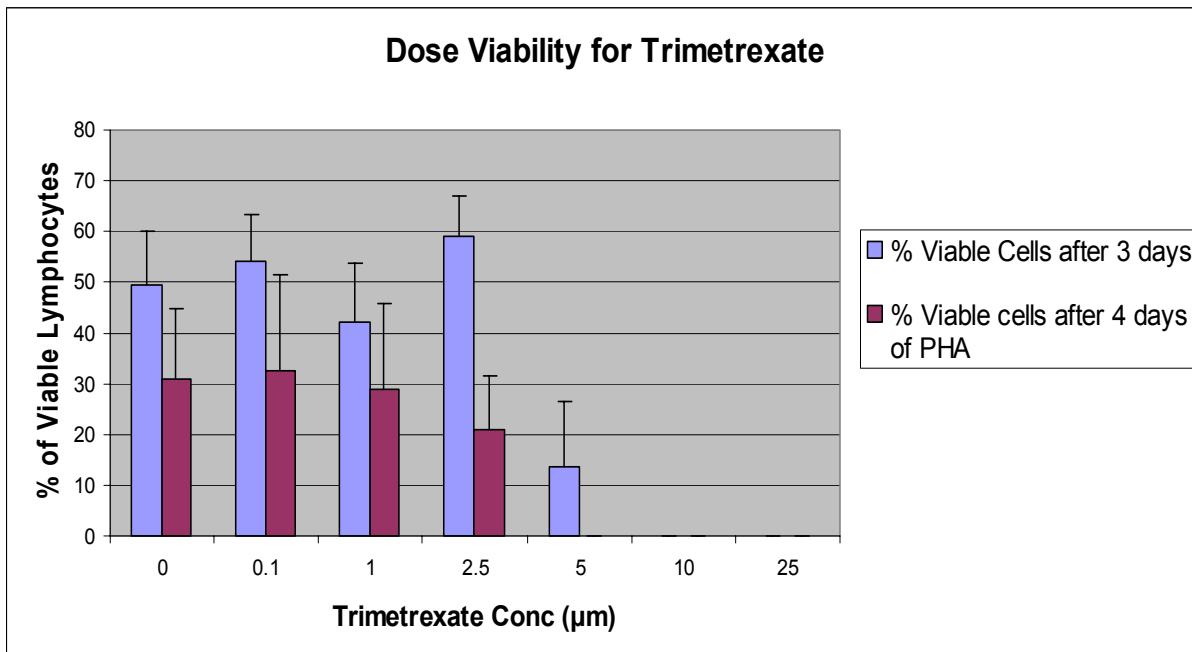
(b)
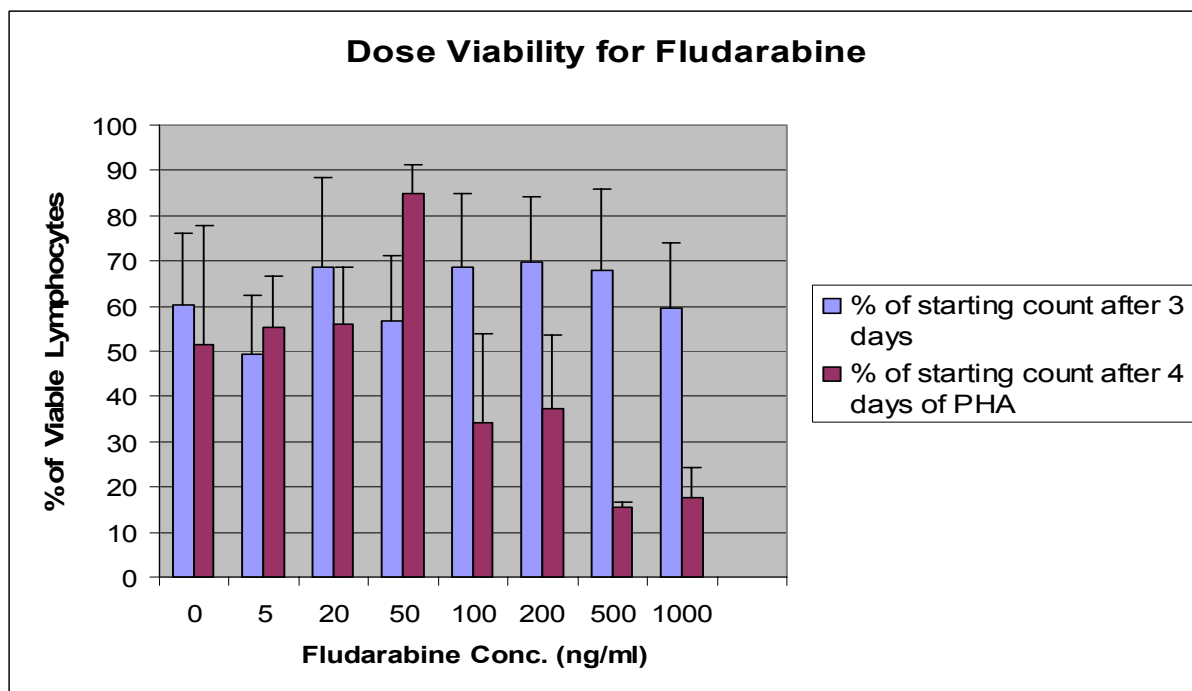
Figure 61:Dose viability Experiments for Trimetrexate (a) and fludarabine (b). Varying concentrations of trimetrexate and fludarabine were added to resting T cells or PHA stimulated T cells. The trimetrexate was added on day 0 and the fludarabine on day 2. The number of viable T cells was then determined by trypan staining and divided by the number of cells on day 0 to determine % viability. The optimum dose for trimetrexate was 1μm and for fludarabine was 50 ng/ml (*n=5*)

213
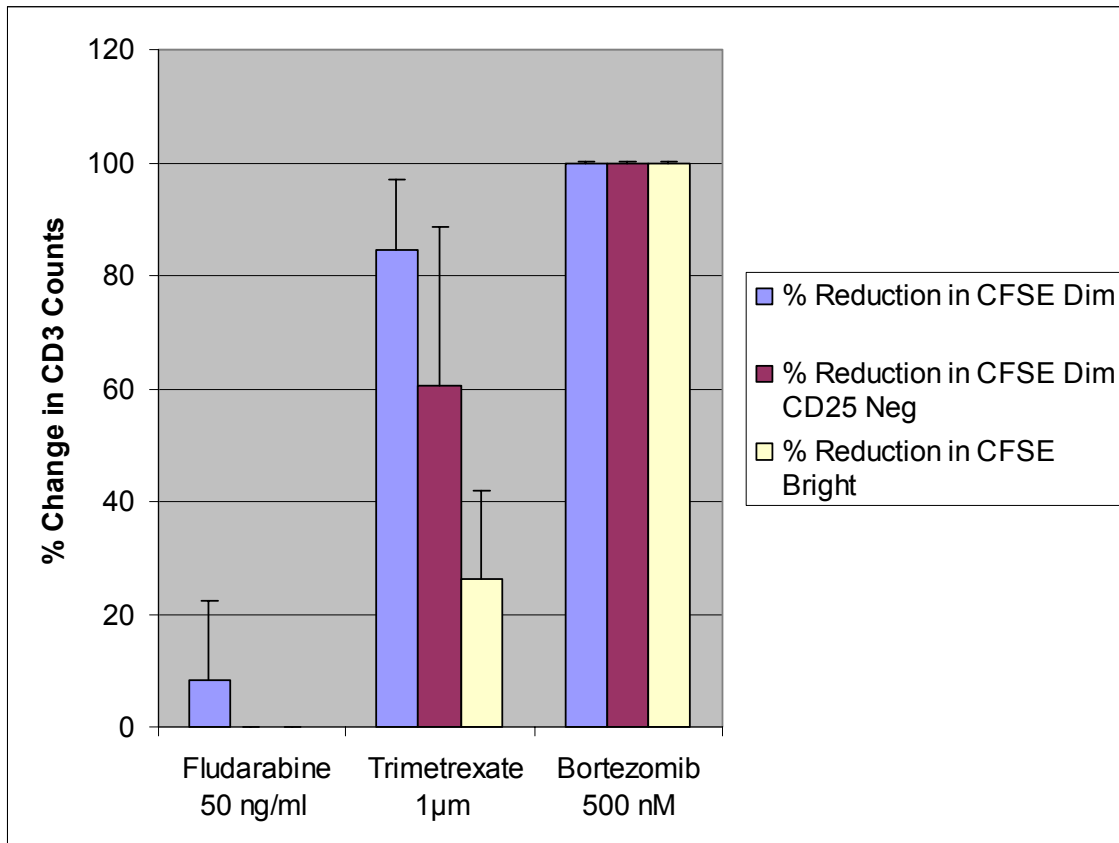
Figure 63:Trimetrexate Selectively deletes Proliferating Alloreactive T cells. *(n=5).*
Fludarabine, Trimetrexate and bortezomib were added to allo MLRs. (consisting of CFSE labelled T cells co
cultured with HLA- mismatched DCs). Controls consisted of CFSE labelled T cells alone or co cultures without
chemotherapy drugs. On day 3, the % of CFSE Dim proliferating alloreactive T cells (including CD25-ve) and
bystander CFSE bright was determined using Trucout beads. Bortezomib led to deletion of all cells, whilst
fludarabine had little effect on proliferating alloreactive T-cells. Trimetrexate led to over 80 % reduction in CFSE
Dim population, a 60 % reduction in CFSE Dim CD25 negative and a 20 % reduction in bystander cells.

Acknowledgements

References

1.   Aversa F. Haploidentical haematopoietic stem cell transplantation for acute leukaemia in adults: experience in Europe and the United States. Bone Marrow Transplant. 2008.

2.   Veys P, Amrolia P, Rao K. The role of haploidentical stem cell transplantation in the management of children with haematological disorders. Br J Haematol. 2003;123:193-206.

3.   Dey BR, Spitzer TR. Current status of haploidentical stem cell transplantation. Br J Haematol. 2006;135:423-437.

4.   Lang P, Greil J, Bader P, et al. Long-term outcome after haploidentical stem cell transplantation in children. Blood Cells Mol Dis. 2004;33:281-287.

5.   Eyrich M, Lang P, Lal S, et al. A prospective analysis of the pattern of immune reconstitution in a paediatric cohort following transplantation of positively selected human leucocyte antigen-disparate haematopoietic stem cells from parental donors. Br J Haematol. 2001;114:422-432.

6.   Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N Engl J Med. 1998;339:1186-1193.

7.   Feuchtinger T, Richard C, Pfeiffer M, et al. Adenoviral infections after transplantation of positive selected stem cells from haploidentical donors in children: an update. Klin Padiatr. 2005;217:339-344.

8.   Aversa F, Martelli MF. Transplantation of haploidentically mismatched stem cells for the treatment of malignant diseases. Springer Semin Immunopathol. 2004;26:155-168.

9.   Bethge WA, Faul C, Bornhauser M, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: An update. Blood Cells Mol Dis. 2008;40:13-19.

10.   Chen X, Hale GA, Barfield R, et al. Rapid immune reconstitution after a reduced-intensity conditioning regimen and a CD3-depleted haploidentical stem cell graft for paediatric refractory haematological malignancies. Br J Haematol. 2006;135:524-532.

11.   Bader P, Soerensen J, Koehl U, et al. 66: Excellent Engraftment and Rapid Immune Recovery in Haploidentical Stem Cell Transplantation Using CD3/CD19 Depleted Peripheral Stem Cell Grafts After Reduced Intensity Conditioning. Biology of Blood and Marrow Transplantation. 2008;14:27.

12.   Ferrara JL, Reddy P. Pathophysiology of graft-versus-host disease. Semin Hematol. 2006;43:3-10.

13.   Kernan NA, Collins NH, Juliano L, Cartagena T, Dupont B, O'Reilly RJ. Clonable T lymphocytes in T cell-depleted bone marrow transplants correlate with development of graft-v-host disease. Blood. 1986;68:770-773.

14.   Barber LD, Madrigal JA. Exploiting beneficial alloreactive T cells. Vox Sang. 2006;91:20-27.

15.   Shlomchik WD. Graft-versus-host disease. Nat Rev Immunol. 2007;7:340-352.

16.   Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med. 2006;354:1813-1826.

17.   Nikolich-Zugich J. High specificity, not degeneracy, allows T cell alloresponses. Nat Immunol. 2007;8:335-337.

18.   Martins SL, St John LS, Champlin RE, et al. Functional assessment and specific depletion of alloreactive human T cells using flow cytometry. Blood. 2004;104:3429-3436.

19.   Jameson BA, McDonnell JM, Marini JC, Korngold R. A rationally designed CD4 analogue inhibits experimental allergic encephalomyelitis. Nature. 1994;368:744-746.

20.   Goulmy E, Schipper R, Pool J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. N Engl J Med. 1996;334:281-285.

21.   Socie G, Loiseau P, Tamouza R, et al. Both genetic and clinical factors predict the development of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Transplantation. 2001;72:699-706.

22.   Chakraverty R, Sykes M. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. Blood. 2007;110:9-17.

23.   Morris ES, Hill GR. Advances in the understanding of acute graft-versus-host disease. Br J Haematol. 2007;137:3-19.

24.   Godfrey WR, Krampf MR, Taylor PA, Blazar BR. Ex vivo depletion of alloreactive cells based on CFSE dye dilution, activation antigen selection, and dendritic cell stimulation. Blood. 2004;103:1158-1165.

25.   Ichiki Y, Bowlus CL, Shimoda S, Ishibashi H, Vierling JM, Gershwin ME. T cell immunity and graft-versus-host disease (GVHD). Autoimmun Rev. 2006;5:1-9.

26.   Debes GF, Arnold CN, Young AJ, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. Nat Immunol. 2005;6:889-894.

27.   Bondanza A, Valtolina V, Magnani Z, et al. Suicide gene therapy of graft-versus-host disease induced by central memory human T lymphocytes. Blood. 2006;107:1828-1836.

28.   Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L-memory T cells without graft-versus-host disease. Blood. 2004;103:1534-1541.

29.   Bleakley M, Mollerup A, Chaney C, Brown M, Riddell SR. Human Minor Histocompatibility Antigen-Specific CD8+ T Cells Are Found Predominantly in the CD45RA+ CD62L+ Naive T Cell Subset. ASH Annual Meeting Abstracts. 2005;106:578-.

30.   Fowler DH. Shared biology of GVHD and GVT effects: potential methods of separation. Crit Rev Oncol Hematol. 2006;57:225-244.

31.   Yakoub-Agha I, Saule P, Depil S, et al. A high proportion of donor CD4+ T cells expressing the lymph node-homing chemokine receptor CCR7 increases incidence and severity of acute graft-versus-host disease in patients undergoing allogeneic stem cell transplantation for hematological malignancy. Leukemia. 2006;20:1557-1565.

32.   Chen BJ, Deoliveira D, Cui X, et al. Inability of memory T cells to induce graft-versus-host disease is a result of an abortive alloresponse. Blood. 2007;109:3115-3123.

33.   Le NT, Chen BJ, Chao NJ. Selective elimination of alloreactivity from immunotherapeutic T cells by photodynamic cell purging and memory T-cell sorting. Cytotherapy. 2005;7:126-133.

34.   Sun Y, Tawara I, Toubai T, Reddy P. Pathophysiology of acute graft-versus-host disease: recent advances. Transl Res. 2007;150:197-214.

35.   Barrett J. Improving outcome of allogeneic stem cell transplantation by immunomodulation of the early post-transplant environment. Curr Opin Immunol. 2006;18:592-598.

36.   Visentainer JE, Lieber SR, Persoli LB, et al. Serum cytokine levels and acute graft-versus-host disease after HLA-identical hematopoietic stem cell transplantation. Exp Hematol. 2003;31:1044-1050.

37.   Przepiorka D, Kernan NA, Ippoliti C, et al. Daclizumab, a humanized anti-interleukin-2 receptor alpha chain antibody, for treatment of acute graft-versus-host disease. Blood. 2000;95:83-89.

38.   Welniak LA, Blazar BR, Anver MR, Wiltrout RH, Murphy WJ. Opposing roles of interferon-gamma on CD4+ T cell-mediated graft-versus-host disease: effects of conditioning. Biol Blood Marrow Transplant. 2000;6:604-612.

39.   Burman AC, Banovic T, Kuns RD, et al. IFNgamma differentially controls the development of idiopathic pneumonia syndrome and GVHD of the gastrointestinal tract. Blood. 2007;110:1064-1072.

40.   Elmaagacli AH, Koldehoff M, Hindahl H, et al. Mutations in innate immune system NOD2/CARD 15 and TLR-4 (Thr399Ile) genes influence the risk for severe acute graft-versus-host disease in patients who underwent an allogeneic transplantation. Transplantation. 2006;81:247-254.

41.   Borsotti C, Franklin AR, Lu SX, et al. Absence of donor T cell derived soluble TNF decreases graft-versus-host-disease without impairing graft-versus-tumor activity. Blood. 2007.

42.   Holler E, Kolb HJ, Moller A, et al. Increased serum levels of tumor necrosis factor alpha precede major complications of bone marrow transplantation. Blood. 1990;75:1011-1016.

43.   Srinivasan R, Chakrabarti S, Walsh T, et al. Improved survival in steroid-refractory acute graft versus host disease after non-myeloablative allogeneic transplantation using a daclizumab-based strategy with comprehensive infection prophylaxis. Br J Haematol. 2004;124:777-786.

44.   Blaser BW, Schwind NR, Karol S, et al. Trans-presentation of donor-derived interleukin 15 is necessary for the rapid onset of acute graft-versus-host disease but not for graft-versus-tumor activity. Blood. 2006;108:2463-2469.

45.   Kappel LW, Goldberg GL, Ivanov II, et al. 27: IL-17 is Required for CD4-Mediated GVHD. Biology of Blood and Marrow Transplantation. 2008;14:12-13.

46.   Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. J Exp Med. 2006;203:2785-2791.

47.   Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. J Exp Med. 1996;183:2645-2656.

48.   Maeda Y, Levy RB, Reddy P, et al. Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. Blood. 2005;105:2023-2027.

49.   Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3:541-547.

50.   Perez VL, Van Parijs L, Biuckians A, Zheng XX, Strom TB, Abbas AK. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. Immunity. 1997;6:411-417.

51.   Peggs KS, Allison JP. Co-stimulatory pathways in lymphocyte regulation: the immunoglobulin superfamily. Br J Haematol. 2005;130:809-824.

52.   Chen BJ, Cui X, Liu C, Chao NJ. Prevention of graft-versus-host disease while preserving graft-versus-leukemia effect after selective depletion of host-reactive T cells by photodynamic cell purging process. Blood. 2002;99:3083-3088.

53.   Ukyo N, Hori T, Yanagita S, Ishikawa T, Uchiyama T. Costimulation through OX40 is crucial for induction of an alloreactive human T-cell response. Immunology. 2003;109:226-231.

54.   Blazar BR, Sharpe AH, Chen AI, et al. Ligation of OX40 (CD134) regulates graft-versus-host disease (GVHD) and graft rejection in allogeneic bone marrow transplant recipients. Blood. 2003;101:3741-3748.

55. Blanco B, Perez-Simon JA, Sanchez-Abarca LI, et al. Bortezomib induces selective depletion of alloreactive T lymphocytes and decreases the production of Th1 cytokines. Blood. 2006;107:3575-3583.

56. Valzasina B, Guiducci C, Dislich H, Killeen N, Weinberg AD, Colombo MP. Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. Blood. 2005;105:2845-2851.

57. Ohata J, Sakurai J, Saito K, Tani K, Asano S, Azuma M. Differential graft-versus-leukaemia effect by CD28 and CD40 co-stimulatory blockade after graft-versus-host disease prophylaxis. Clin Exp Immunol. 2002;129:61-68.

58. Blazar BR, Taylor PA, Noelle RJ, Vallera DA. CD4(+) T cells tolerized ex vivo to host alloantigen by anti-CD40 ligand (CD40L:CD154) antibody lose their graft-versus-host disease lethality capacity but retain nominal antigen responses. J Clin Invest. 1998;102:473-482.

59. Wehler TC, Nonn M, Brandt B, et al. Targeting the activation-induced antigen CD137 can selectively deplete alloreactive T cells from antileukemic and antitumor donor T-cell lines. Blood. 2007;109:365-373.

60. Popma SH, Griswold DE, Li L. Anti-CD3 antibodies OKT3 and hOKT3gamma1(Ala-Ala) induce proliferation of T cells but impair expansion of alloreactive T cells; aspecifc T cell proliferation induced by anti-CD3 antibodies correlates with impaired expansion of alloreactive T cells. Int Immunopharmacol. 2005;5:155-162.

61. Shiow LR, Rosen DB, Brdickova N, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature. 2006;440:540-544.

62. Craston R, Koh M, Mc Dermott A, Ray N, Prentice HG, Lowdell MW. Temporal dynamics of CD69 expression on lymphoid cells. J Immunol Methods. 1997;209:37-45.

63. Holzelova E, Vonarbourg C, Stolzenberg MC, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. N Engl J Med. 2004;351:1409-1418.

64. Shustov A, Nguyen P, Finkelman F, Elkon KB, Via CS. Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-gamma production. J Immunol. 1998;161:2848-2855.

65. Henslee-Downey PJ, Abhyankar SH, Parrish RS, et al. Use of partially mismatched related donors extends access to allogeneic marrow transplant. Blood. 1997;89:3864-3872.

66. O'Flaherty E, Wong WK, Pettit SJ, Seymour K, Ali S, Kirby JA. Regulation of T-cell apoptosis: a mixed lymphocyte reaction model. Immunology. 2000;100:289-299.

67. Zhang HG, Su X, Liu D, et al. Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. J Immunol. 1999;162:1423-1430.

68. Artac H, Coskun M, Karadogan I, Yegin O, Yesilipek A. Transferrin receptor in proliferation of T lymphocytes in infants with iron deficiency. Int J Lab Hematol. 2007;29:310-315.

69. Nguyen XD, Eichler H, Dugrillon A, Piechaczek C, Braun M, Kluter H. Flow cytometric analysis of T cell proliferation in a mixed lymphocyte reaction with dendritic cells. J Immunol Methods. 2003;275:57-68.

70. Macedo MF, de Sousa M, Ned RM, Mascarenhas C, Andrews NC, Correia-Neves M. Transferrin is required for early T-cell differentiation. Immunology. 2004;112:543-549.

71. Li X, Cicalese L, DeMasi M, Benedetti E, Rastellini C. Mechanisms underlying the immunosuppressive activity of antitransferrin receptor monoclonal antibody. Transplant Proc. 2001;33:136.

72. Drobyski WR, Ul-Haq R, Majewski D, Chitambar CR. Modulation of in vitro and in vivo T-cell responses by transferrin-gallium and gallium nitrate. Blood. 1996;88:3056-3064.

73.   Pachnio A, Dietrich S, Klapper W, et al. Proliferation-based T-cell selection for immunotherapy and graft-versus-host-disease prophylaxis in the context of bone marrow transplantation. Bone Marrow Transplant. 2006;38:157-167.

74.   Gale RP, Horowitz MM, Ash RC, et al. Identical-twin bone marrow transplants for leukemia. Ann Intern Med. 1994;120:646-652.

75.   Schetelig J, Kiani A, Schmitz M, Ehninger G, Bornhauser M. T cell-mediated graft-versus-leukemia reactions after allogeneic stem cell transplantation. Cancer Immunol Immunother. 2005;54:1043-1058.

76.   Kennedy-Nasser AA, Bollard CM, Rooney CM. Adoptive immunotherapy for Hodgkin's lymphoma. Int J Hematol. 2006;83:385-390.

77.   Dazzi F, Szydlo RM, Craddock C, et al. Comparison of single-dose and escalating-dose regimens of donor lymphocyte infusion for relapse after allografting for chronic myeloid leukemia. Blood. 2000;95:67-71.

78.   Simula MP, Marktel S, Fozza C, et al. Response to donor lymphocyte infusions for chronic myeloid leukemia is dose-dependent: the importance of escalating the cell dose to maximize therapeutic efficacy. Leukemia. 2007;21:943-948.

79.   Mackinnon S, Papadopoulos EB, Carabasi MH, et al. Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. Blood. 1995;86:1261-1268.

80.   Barrett AJ. Mechanisms of the graft-versus-leukemia reaction. Stem Cells. 1997;15:248-258.

81.   Di Terlizzi S, Zino E, Mazzi B, et al. Therapeutic and diagnostic applications of minor histocompatibility antigen HA-1 and HA-2 disparities in allogeneic hematopoietic stem cell transplantation: a survey of different populations. Biol Blood Marrow Transplant. 2006;12:95-101.

82.   Molldrem JJ, Lee PP, Wang C, Champlin RE, Davis MM. A PR1-human leukocyte antigen-A2 tetramer can be used to isolate low-frequency cytotoxic T lymphocytes from healthy donors that selectively lyse chronic myelogenous leukemia. Cancer Res. 1999;59:2675-2681.

83.   Rezvani K, Grube M, Brenchley JM, et al. Functional leukemia-associated antigen-specific memory CD8+ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. Blood. 2003;102:2892-2900.

84.   Appelbaum FR. Haematopoietic cell transplantation as immunotherapy. Nature. 2001;411:385-389.

85.   Rezvani K, Yong AS, Mielke S, et al. Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood. 2008;111:236-242.

86.   Amrolia PJ, Muccioli-Casadei G, Yvon E, et al. Selective depletion of donor alloreactive T cells without loss of antiviral or antileukemic responses. Blood. 2003;102:2292-2299.

87.   Peggs KS, Sureda A, Qian W, et al. Reduced-intensity conditioning for allogeneic haematopoietic stem cell transplantation in relapsed and refractory Hodgkin lymphoma: impact of alemtuzumab and donor lymphocyte infusions on long-term outcomes. Br J Haematol. 2007;139:70-80.

88.   Rooney CM, Smith CA, Ng CY, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood. 1998;92:1549-1555.

89.   Leen AM, Heslop HE. Cytotoxic T lymphocytes as immune-therapy in haematological practice. Br J Haematol. 2008.

90.   Zakrzewski JL, Kochman AA, Lu SX, et al. Adoptive transfer of T-cell precursors enhances T-cell reconstitution after allogeneic hematopoietic stem cell transplantation. Nat Med. 2006;12:1039-1047.

91.   Guinan EC, Boussiotis VA, Neuberg D, et al. Transplantation of anergic histoincompatible bone marrow allografts. N Engl J Med. 1999;340:1704-1714.

92.   Roskrow MA, Suzuki N, Gan Y, et al. Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. Blood. 1998;91:2925-2934.

93.   Cooper LJ. Adoptive cellular immunotherapy for childhood malignancies. Bone Marrow Transplant. 2007.

94.   Peggs KS, Verfuerth S, Pizzey A, et al. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. Lancet. 2003;362:1375-1377.

95.   Einsele H, Roosnek E, Rufer N, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood. 2002;99:3916-3922.

96.   Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.

97.   Rauser G, Einsele H, Sinzger C, et al. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. Blood. 2004;103:3565-3572.

98.   Cobbold M, Khan N, Pourgheysari B, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med. 2005;202:379-386.

99.   Chatziandreou I, Gilmour KC, McNicol AM, et al. Capture and generation of adenovirus specific T cells for adoptive immunotherapy. Br J Haematol. 2007;136:117-126.

100.   Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol. 2006;134:64-76.

101.   Perruccio K, Tosti A, Burchielli E, et al. Transferring functional immune responses to pathogens after haploidentical hematopoietic transplantation. Blood. 2005;106:4397-4406.

102.   Leen AM, Myers GD, Sili U, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat Med. 2006;12:1160-1166.

103.   Ciceri F, Bonini C, Marktel S, et al. Antitumor effects of HSV-TK-engineered donor lymphocytes after allogeneic stem-cell transplantation. Blood. 2007;109:4698-4707.

104.   Traversari C, Marktel S, Magnani Z, et al. The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. Blood. 2007;109:4708-4715.

105.   Stanghellini MTL, Bonini C, Provasi E, et al. Infusions of HSV-TK Engineered Donor Lymphocytes Effectively Protect Patients Undergoing Haploidentical Stem Cells Trasplantation (HSCT) from Infectious Mortality. ASH Annual Meeting Abstracts. 2007;110:1056-.

106.   Berger C, Flowers ME, Warren EH, Riddell SR. Analysis of transgene-specific immune responses that limit the in vivo persistence of adoptively transferred HSV-TK-modified donor T cells after allogeneic hematopoietic cell transplantation. Blood. 2006;107:2294-2302.

107.   Qasim W, Mackey T, Sinclair J, et al. Lentiviral vectors for T-cell suicide gene therapy: preservation of T-cell effector function after cytokine-mediated transduction. Mol Ther. 2007;15:355-360.

108.   Sauce D, Bodinier M, Garin M, et al. Retrovirus-mediated gene transfer in primary T lymphocytes impairs their anti-Epstein-Barr virus potential through both culture-dependent and selection process-dependent mechanisms. Blood. 2002;99:1165-1173.

109.   Robinet E, Fehse B, Ebeling S, Sauce D, Ferrand C, Tiberghien P. Improving the ex vivo retroviral-mediated suicide-gene transfer process in T lymphocytes to preserve immune function. Cytotherapy. 2005;7:150-157.

110.   Straathof KC, Pule MA, Yotnda P, et al. An inducible caspase 9 safety switch for T-cell therapy. Blood. 2005;105:4247-4254.

111.   Rezvani K, Mielke S, Ahmadzadeh M, et al. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. Blood. 2006;108:1291-1297.

112.   Edinger M, Hoffmann P, Ermann J, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med. 2003;9:1144-1150.

113.   Cohen JL, Salomon BL. Therapeutic potential of CD4+ CD25+ regulatory T cells in allogeneic transplantation. Cytotherapy. 2005;7:166-170.

114.   Cavazzana-Calvo M, Fromont C, Le Deist F, et al. Specific elimination of alloreactive T cells by an anti-interleukin-2 receptor B chain-specific immunotoxin. Transplantation. 1990;50:1-7.

115.   Fehse B, Frerk O, Goldmann M, Bulduk M, Zander AR. Efficient depletion of alloreactive donor T lymphocytes based on expression of two activation-induced antigens (CD25 and CD69). Br J Haematol. 2000;109:644-651.

116.   Hartwig UF, Nonn M, Khan S, Meyer RG, Huber C, Herr W. Depletion of alloreactive T cells via CD69: implications on antiviral, antileukemic and immunoregulatory T lymphocytes. Bone Marrow Transplant. 2006;37:297-305.

117.   Koh MB, Prentice HG, Lowdell MW. Selective removal of alloreactive cells from haematopoietic stem cell grafts: graft engineering for GVHD prophylaxis. Bone Marrow Transplant. 1999;23:1071-1079.

118.   van Dijk AMC, Kessler FL, Stadhouders-Keet SAE, Verdonck LF, de Gast GC, Otten HG. Selective depletion of major and minor histocompatibility antigen reactive T cells: towards prevention of acute graft-versus-host disease. British Journal of Haematology. 1999;107:169-175.

119.   Hartwig UF, Robbers M, Wickenhauser C, Huber C. Murine acute graft-versus-host disease can be prevented by depletion of alloreactive T lymphocytes using activation-induced cell death. Blood. 2002;99:3041-3049.

120.   Giver CR, Montes RO, Mittelstaedt S, et al. Ex vivo fludarabine exposure inhibits graft-versus-host activity of allogeneic T cells while preserving graft-versus-leukemia effects. Biol Blood Marrow Transplant. 2003;9:616-632.

121.   Sun K, Welniak LA, Panoskaltsis-Mortari A, et al. Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. Proc Natl Acad Sci U S A. 2004;101:8120-8125.

122.   Montagna D, Yvon E, Calcaterra V, et al. Depletion of alloreactive T cells by a specific anti-interleukin-2 receptor p55 chain immunotoxin does not impair in vitro antileukemia and antiviral activity. Blood. 1999;93:3550-3557.

123.   Andre-Schmutz I, Dal Cortivo L, Fischer A, Cavazzana-Calvo M. Improving immune reconstitution while preventing GvHD in allogeneic stem cell transplantation. Cytotherapy. 2005;7:102-108.

124.   Vaclavkova P, Cao Y, Wu LK, Michalek J, Vitetta ES. A comparison of an anti-CD25 immunotoxin, Ontak and anti-CD25 microbeads for their ability to deplete alloreactive T cells in vitro. Bone Marrow Transplant. 2006;37:559-567.

125.   Amrolia PJ, Mucioli-Casadei G, Huls H, et al. Add-back of allodepleted donor T cells to improve immune reconstitution after haplo-identical stem cell transplantation. Cytotherapy. 2005;7:116-125.

126.   Davies JK, Koh MB, Lowdell MW. Antiviral immunity and T-regulatory cell function are retained after selective alloreactive T-cell depletion in both the HLA-identical and HLA-mismatched settings. Biol Blood Marrow Transplant. 2004;10:259-268.

127.   Koh MB, Prentice HG, Corbo M, Morgan M, Cotter FE, Lowdell MW. Alloantigen-specific T-cell depletion in a major histocompatibility complex fully mismatched murine model provides effective graft-versus-host disease prophylaxis in the presence of lymphoid engraftment. Br J Haematol. 2002;118:108-116.

128.   Hartwig UF, Nonn M, Khan S, Link I, Huber C, Herr W. Depletion of alloreactive donor T lymphocytes by CD95-mediated activation-induced cell death retains antileukemic, antiviral, and immunoregulatory T cell immunity. Biol Blood Marrow Transplant. 2008;14:99-109.

129.   Jodo S, Kung JT, Xiao S, et al. Anti-CD95-induced lethality requires radioresistant Fcgamma RII+ cells. A novel mechanism for fulminant hepatic failure. J Biol Chem. 2003;278:7553-7557.

130.   Boumedine RS, Roy DC. Elimination of alloreactive T cells using photodynamic therapy. Cytotherapy. 2005;7:134-143.

131.   Mielke S, Nunes R, Rezvani K, et al. A clinical scale selective allodepletion approach for the treatment of HLA-mismatched and matched donor-recipient pairs using expanded T lymphocytes as antigen-presenting cells and a TH9402-based photodepletion technique. Blood. 2007.

132.   Perruccio K, Topini F, Tosti A, et al. Photodynamic purging of alloreactive T cells for adoptive immunotherapy after haploidentical stem cell transplantation. Blood Cells Mol Dis. 2008;40:76-83.

133.   Roy D-C, Cohen S, Busque L, et al. Phase I Clinical Trial of Haplotype Mismatched Myeloablative Stem Cell Transplantation: Higher Doses of Donor Lymphocyte Infusions Depleted of Alloreactive Cells Using ATIR May Improve Outcome without Causing GVHD. ASH Annual Meeting Abstracts. 2007;110:2976-.

134.   Li JM, Giver CR, Waller EK. Graft engineering using ex vivo methods to limit GVHD: fludarabine treatment generates superior GVL effects in allogeneic BMT. Exp Hematol. 2006;34:895-904.

135.   Szabolcs P, Park KD, Marti L, et al. Superior depletion of alloreactive T cells from peripheral blood stem cell and umbilical cord blood grafts by the combined use of trimetrexate and interleukin-2 immunotoxin. Biol Blood Marrow Transplant. 2004;10:772-783.

136.   Vodanovic-Jankovic S, Hari P, Jacobs P, Komorowski R, Drobyski WR. NF-kappaB as a target for the prevention of graft-versus-host disease: comparative efficacy of bortezomib and PS-1145. Blood. 2006;107:827-834.

137.   Sun K, Wilkins DE, Anver MR, et al. Differential effects of proteasome inhibition by bortezomib on murine acute graft-versus-host disease (GVHD): delayed administration of bortezomib results in increased GVHD-dependent gastrointestinal toxicity. Blood. 2005;106:3293-3299.

138.   Andre-Schmutz I, Le Deist F, Hacein-Bey-Abina S, et al. Immune reconstitution without graft-versus-host disease after haemopoietic stem-cell transplantation: a phase 1/2 study. Lancet. 2002;360:130-137.

139.   Solomon SR, Mielke S, Savani BN, et al. Selective depletion of alloreactive donor lymphocytes: a novel method to reduce the severity of graft-versus-host disease in older patients undergoing matched sibling donor stem cell transplantation. Blood. 2005;106:1123-1129.

140.   Mielke S, Rezvani K, Savani BN, et al. Reconstitution of foxp3+ regulatory T cells (Tregs) after CD25-depleted allotransplantion in elderly patients and association with acute graft-versus-host disease (GvHD). Blood. 2007.

141.   Amrolia PJ, Muccioli-Casadei G, Huls H, et al. Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation. Blood. 2006;108:1797-1808.

142.   Mavroudis DA, Dermime S, Molldrem J, et al. Specific depletion of alloreactive T cells in HLA-identical siblings: a method for separating graft-versus-host and graft-versus-leukaemia reactions. Br J Haematol. 1998;101:565-570.

143.   Gao L, Bellantuono I, Elsasser A, et al. Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. Blood. 2000;95:2198-2203.

144.   Amrolia PJ, Reid SD, Gao L, et al. Allorestricted cytotoxic T cells specific for human CD45 show potent antileukemic activity. Blood. 2003;101:1007-1014.

145.   Schuster IG, Busch DH, Eppinger E, et al. Allorestricted T cells with specificity for the FMNL1-derived peptide PP2 have potent antitumor activity against hematological and other malignancies. Blood. 2007.

146.   Heemskerk MH, Hoogeboom M, de Paus RA, et al. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. Blood. 2003;102:3530-3540.

147.   Biagi E, Marin V, Giordano Attianese GM, Dander E, D'Amico G, Biondi A. Chimeric T-cell receptors: new challenges for targeted immunotherapy in hematologic malignancies. Haematologica. 2007;92:381-388.

148.   Pule M, Finney H, Lawson A. Artificial T-cell receptors. Cytotherapy. 2003;5:211-226.

149.   Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther. 2005;12:933-941.

150.   Cooper LJ, Al-Kadhimi Z, DiGiusto D, et al. Development and application of CD19-specific T cells for adoptive immunotherapy of B cell malignancies. Blood Cells Mol Dis. 2004;33:83-89.

151.   Rossig C, Bar A, Pscherer S, et al. Target antigen expression on a professional antigen-presenting cell induces superior proliferative antitumor T-cell responses via chimeric T-cell receptors. J Immunother (1997). 2006;29:21-31.

152.   Brentjens RJ, Latouche JB, Santos E, et al. Eradication of systemic B-cell tumors by genetically targeted human T lymphocytes co-stimulated by CD80 and interleukin-15. Nat Med. 2003;9:279-286.

153.   Rossig C, Brenner MK. Genetic modification of T lymphocytes for adoptive immunotherapy. Mol Ther. 2004;10:5-18.

154.   Howe SJ, Mansour MR, Schwarzwaelder K, et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest. 2008;118:3143-3150.

155.   Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest. 2005;115:1616-1626.

156. Cooper LJ, Al-Kadhimi Z, Serrano LM, et al. Enhanced antilymphoma efficacy of CD19-redirected influenza MP1-specific CTLs by cotransfer of T cells modified to present influenza MP1. Blood. 2005;105:1622-1631.

157. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol. 1997;15:871-875.

158. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998;72:8463-8471.

159. Vigna E, Naldini L. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J Gene Med. 2000;2:308-316.

160. Cullen BR. HIV-1 auxiliary proteins: making connections in a dying cell. Cell. 1998;93:685-692.

161. Cattoglio C, Facchini G, Sartori D, et al. Hot spots of retroviral integration in human CD34+ hematopoietic cells. Blood. 2007;110:1770-1778.

162. Bobisse S, Zanovello P, Rosato A. T-cell receptor gene transfer by lentiviral vectors in adoptive cell therapy. Expert Opin Biol Ther. 2007;7:893-906.

163. Levine BL, Humeau LM, Boyer J, et al. Gene transfer in humans using a conditionally replicating lentiviral vector. Proc Natl Acad Sci U S A. 2006;103:17372-17377.

164. Demaison C, Parsley K, Brouns G, et al. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum Gene Ther. 2002;13:803-813.

165. Karlsson H, Brewin J, Kinnon C, Veys P, Amrolia PJ. Generation of Trispecific Cytotoxic T Cells Recognizing Cytomegalovirus, Adenovirus, and Epstein-Barr Virus: An Approach for Adoptive Immunotherapy of Multiple Pathogens. J Immunother (1997). 2007;30:544-556.

166. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. J Exp Med. 2001;194:1711-1719.

167. Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. J Clin Invest. 2005;115:1953-1962.

168. Lion T, Baumgartinger R, Watzinger F, et al. Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. Blood. 2003;102:1114-1120.

169. Kampmann B, Cubitt D, Walls T, et al. Improved outcome for children with disseminated adenoviral infection following allogeneic stem cell transplantation. Br J Haematol. 2005;130:595-603.

170. Cavalieri S, Cazzaniga S, Geuna M, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. Blood. 2003;102:497-505.

171. Shafer-Weaver K, Sayers T, Strobl S, et al. The Granzyme B ELISPOT assay: an alternative to the 51Cr-release assay for monitoring cell-mediated cytotoxicity. J Transl Med. 2003;1:14.

172. Bader P, Kreyenberg H, Hoelle W, et al. Increasing mixed chimerism is an important prognostic factor for unfavorable outcome in children with acute lymphoblastic leukemia after allogeneic stem-cell transplantation: possible role for pre-emptive immunotherapy? J Clin Oncol. 2004;22:1696-1705.

173. Kolb HJ, Schmid C, Barrett AJ, Schendel DJ. Graft-versus-leukemia reactions in allogeneic chimeras. Blood. 2004;103:767-776.

174. Jurickova I, Waller EK, Yeager AM, Boyer MW. Generation of alloreactive anti-leukemic cytotoxic T lymphocytes with attenuated GVHD properties from haploidentical

parents in childhood acute lymphoblastic leukemia. Bone Marrow Transplant. 2002;30:687-697.

175.   Roucard C, Thomas C, Pasquier MA, et al. In vivo and in vitro modulation of HLA-DM and HLA-DO is induced by B lymphocyte activation. J Immunol. 2001;167:6849-6858.

176.   Brentjens RJ, Santos E, Nikhamin Y, et al. Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. Clin Cancer Res. 2007;13:5426-5435.

177.   Mavroudis DA, Jiang YZ, Hensel N, et al. Specific depletion of alloreactivity against haplotype mismatched related individuals by a recombinant immunotoxin: a new approach to graft-versus-host disease prophylaxis in haploidentical bone marrow transplantation. Bone Marrow Transplant. 1996;17:793-799.

178.   Guimond M, Balassy A, Barrette M, Brochu S, Perreault C, Roy DC. P-glycoprotein targeting: a unique strategy to selectively eliminate immunoreactive T cells. Blood. 2002;100:375-382.

179.   van Meerten T, Claessen MJ, Hagenbeek A, Ebeling SB. The CD20/alphaCD20 'suicide' system: novel vectors with improved safety and expression profiles and efficient elimination of CD20-transgenic T cells. Gene Ther. 2006;13:789-797.

180.   Bonini C, Bondanza A, Perna SK, et al. The Suicide Gene Therapy Challenge: How to Improve a Successful Gene Therapy Approach. Mol Ther. 2007.

181.   Lenaerts L, Verbeken E, De Clercq E, Naesens L. Mouse adenovirus type 1 infection in SCID mice: an experimental model for antiviral therapy of systemic adenovirus infections. Antimicrob Agents Chemother. 2005;49:4689-4699.

182.   Salmeron A, Borroto A, Fresno M, Crumpton MJ, Ley SC, Alarcon B. Transferrin receptor induces tyrosine phosphorylation in T cells and is physically associated with the TCR zeta-chain. J Immunol. 1995;154:1675-1683.

183.   Villa I, Kvale E, Lund-Johansen F, Olweus J. Assay for monitoring in vitro selective depletion strategies in allogeneic stem cell transplantation. Cytotherapy. 2007;9:600-610.

184.   Cavazzana-Calvo M, Stephan JL, Sarnacki S, et al. Attenuation of graft-versus-host disease and graft rejection by ex vivo immunotoxin elimination of alloreactive T cells in an H-2 haplotype disparate mouse combination. Blood. 1994;83:288-298.

185.   Hoffmann P, Eder R, Boeld TJ, et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. Blood. 2006;108:4260-4267.

186.   Papadopoulos EB, Ladanyi M, Emanuel D, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. N Engl J Med. 1994;330:1185-1191.

187.   Small TN, Papadopoulos EB, Boulad F, et al. Comparison of immune reconstitution after unrelated and related T-cell-depleted bone marrow transplantation: effect of patient age and donor leukocyte infusions. Blood. 1999;93:467-480.

188.   Leen AM, Sili U, Vanin EF, et al. Conserved CTL epitopes on the adenovirus hexon protein expand subgroup cross-reactive and subgroup-specific CD8+ T cells. Blood. 2004;104:2432-2440.

189.   Hromas R, Cornetta K, Srour E, Blanke C, Broun ER. Donor leukocyte infusion as therapy of life-threatening adenoviral infections after T-cell-depleted bone marrow transplantation. Blood. 1994;84:1689-1690.

190.   Mackinnon S, Thomson K, Verfuerth S, Peggs K, Lowdell M. Adoptive cellular therapy for cytomegalovirus infection following allogeneic stem cell transplantation using virus-specific T cells. Blood Cells Mol Dis. 2008;40:63-67.

191.   Hebart H, Bollinger C, Fisch P, et al. Analysis of T-cell responses to Aspergillus fumigatus antigens in healthy individuals and patients with hematologic malignancies. Blood. 2002;100:4521-4528.

192.   Peggs KS, Thomson K, Hart DP, et al. Dose-escalated donor lymphocyte infusions following reduced intensity transplantation: toxicity, chimerism, and disease responses. Blood. 2004;103:1548-1556.

193.   Guglielmi C, Arcese W, Dazzi F, et al. Donor lymphocyte infusion for relapsed chronic myelogenous leukemia: prognostic relevance of the initial cell dose. Blood. 2002;100:397-405.

194.   Loskog A, Giandomenico V, Rossig C, Pule M, Dotti G, Brenner MK. Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. Leukemia. 2006;20:1819-1828.

195.   Manuri PR, Olivares S, Dara N, et al. 30: A Fully-Human Chimeric Antigen Receptor for Redirecting Specificity of T Cells to B-Lineage Tumors. Biology of Blood and Marrow Transplantation. 2008;14:13-14.

196.   Waterhouse NJ, Sedelies KA, Clarke CJ. Granzyme B; the chalk-mark of a cytotoxic lymphocyte. J Transl Med. 2004;2:36.

197.   Perez-Diez A, Joncker NT, Choi K, et al. CD4 cells can be more efficient at tumor rejection than CD8 cells. Blood. 2007;109:5346-5354.

198.   Dal Cortivo L, Mahlaoui N, Picard C, et al. Adoptive Immunotherapy with Donor Allodepleted T Cells. ASH Annual Meeting Abstracts. 2005;106:479-.