Regulatory T cells in Haematopoietic Stem Cell Transplantation

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Abstract

Graft-versus-host disease (GvHD) remains the main complication associated with haematopoietic stem cell transplantation (HSCT). GvHD is caused by allo-reactive donor T cells mounting an attack against specific target tissues. $CD4^+CD25^{Hi}Foxp3^+$ regulatory T cells have been shown to modulate GvHD *in vitro* and also *in vivo* animal models. More recently early stage clinical trials have described the successful use of Treg to reduce the incidence of GvHD following HSCT. The aim of this study was to investigate further the suppressive mechanisms by which Treg are able to modulate GvHD and assess the influence of Treg on the beneficial graft-versus-leukaemia (GvL) effect therefore providing further insight into the use of Treg in the therapeutic management of GVHD.

Data presented in this thesis demonstrates the successful isolation and expansion of a highly pure Treg population which maintained suppressive capacity throughout culture. We also confirmed that Treg retain suppressive capacity following cryopreservation resulting in reduced workload and increased consistency when used for *in vitro* functional studies. We also provide the first human *in vitro* evidence that Treg are able to prevent cutaneous GvH reaction by blocking the migration of effector T cells into the target tissues. The presence of Treg during allo-stimulation caused reduced effector cell activation, proliferation, IFNγ secretion and decreased skin homing receptor expression. Further investigation into the Treg modulation of dendritic cells demonstrated, for the first time in experimental *in vitro* human GvHD, that this was due to ineffective effector T cell priming in the presence of Treg caused by impairment of dendritic cell functions. Comprehensive phenotypic and functional analysis of Treg treated moDC showed their decreased antigen processing ability and allo-stimulatory capacity, resulting in a less severe GvH reaction in the skin explant model. Furthermore, this work has revealed that despite Treg impairing *in vitro* GvL mechanisms at a cellular level there was no association observed between increased Treg levels and the incidence of relapse in a small clinical cohort of HSCT patients. In conclusion this study has provided further insight into the mechanisms by which Treg are able to modulate GvHD. This would inform future clinical trials using Treg as a therapeutic alternative to current GvHD treatment and prophylaxis.

Acknowledgements

Contents

List of abbreviations

7AAD	7-amino-actinomycin D
aGvHD	acute GvHD
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APC	antigen presenting cell
APC	allophycocyanin
CBA	cytometric bead assay
CCL(number)	chemokine (C-C motif) ligand (number
CCR(number)	chemokine (C-C motif) receptor (number)
cDNA	copy DNA
CFSE	carboxyfluorescein succinimidyl ester
cGvHD	chronic GvHD
CLA	cutaneous lymphocyte antigen
CML	chronic myelogenous leukaemia
cpm	count per minute
Ct	cycle threshold
cTEC	cortical thymic epithelial cells
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
CXCL(number)	chemokine (C-X-C motif) ligand
CXCR3	chemokine (C-X-C motif) ligand 3
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethly sulphoxide
DTT	dichloro-diphenyl-trichloroethane
ECP	extracorporeal photopheresis
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular-signal-regulated kinase
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
Flt-3L	Fms-related tyrosine kinase 3 ligand
Foxp3	forkhead box P3
FRET	fluorescence resonance energy transfer

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA binding protein 3
GI	gastro-intestinal
GITR	glucocorticoid-induced TNFR family related gene
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practice
GvH	graft versus host
GvHD	graft versus host disease
GvL	graft versus leukaemia
GPCR	G-protein coupled receptor
HLA	human leukocyte antigen
HO-1	heme oxygenase 1
HSCT	haematopoietic stem cell transplantation
IC	intra-cellular
IDO	indoleamine-pyrrole2,3 dioxygenase
IFNγ	interferon γ
IL-(number)	interleukin-(number)
IP-10	interferon gamma-induced protein 10kDa
IPEX	immune-dysregulation-polyendocrinopathy-enteropathy X linked syndrome
iPS	induced pluripotent Stem cells
I-TAC	interferon-inducible T-cell alpha chemoattractant
KIR	killer immunoglobulin-like receptors
LAP	latency associated protein
LBP	LPS binding protein
LFA-1	leukocyte function-associated antigen 1
LPS	lipopolysaccharides
MACS	magnetic activated cell sorting
mDC	myeloid DC
MFI	mean fluorescence intensity
MHC	major histocompatibility
miHA	minor histocompatability antigen
MIG	monokine induced by gamma interferon
MLR	mixed lymphocyte reaction
MMLV-RT	moloney murine leukemia virus reverse transcriptase

moDC	monocyte derived dendritic cells
MS	multiple sclerosis
MCS	mesenchymal stromal cells
mTEC	medullary thymic epithelial cells
MUD	matched unrelated donor
MyD88	myeloid differentiation primary response gene (88)
NK	natural killer cell
NKG2D	natural killer group 2D
Nrp-1	neuropilin-1
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered solution
PBSC	peripheral blood stem cells
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PDL-1	programmed death ligand-1
PE	phycoerythrin
PHA	phytohaemagglutinin
PMA	phorol 12-myristate 13-acetate
RF10	general culture medium
RNA	ribonucleic acid
RORC	RAR-related orphan receptor C
RQ	relative quantification
SHP-1	Src homology region 2 domain-containing phosphatase-1
TBI	total body irradiation
TBX21	T-box transcription factor TBX21
TGFβ	transforming growth factor β
TLR (number)	toll-like receptor (number)
TNFα	tumour necrosis factor α
TolDC	tolerogenic DC
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cells
UCB	umbilical cord blood
VitD3	vitamin D3

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Chapter 1- General Introduction

This chapter will introduce the pathophysiology of graft-versus-host disease – a common complication associated with allogeneic haematopoietic stem cell transplantation – and discuss how and why regulatory T cells may act as a potential treatment or prophylaxis option in the clinic. Introductions for specific research objectives are included in individual results chapters.

1.1　Allogeneic haematopoietic stem cell transplantation

Allogeneic haematopoietic stem cell transplantation (HSCT) is a well-established and effective treatment for haematological malignancies and other inherited or acquired disorders of the haematopoietic system which would otherwise prove fatal (Shlomchik, 2007). Patients are first subjected to a conditioning regimen of chemo/radiotherapy and immuno-suppression which facilitates engraftment, reduces the risk of graft rejection and lowers the number of malignant cells. The patient is then infused with donor haematopoietic stem cells and over time recipient immuno-deficiency improves as their immune system is gradually replaced by donor lymphoid cells (Deeg and Storb, 1985).

Since the first human HSCT pioneered by Thomas *et al.* in 1959 the number of patients receiving HSCTs has continued to rise, with current estimates at around 55,000-60,000 transplants each year (Thomas et al., 1959, Ferrara et al., 2009, Li and Sykes, 2012). However, acute graft-versus-host disease (aGvHD) remains the main complication associated with HSCT; approximately 50% of patients are treated at some stage for aGvHD and it is responsible for up to 26% of transplant related mortality (Appelbaum, 2001, Jagasia et al., 2012, Gooley et al., 2010). aGvHD is an allo-immune disease which occurs when donor T cells recognise a genetic disparity within the host and mount an immune response against various host tissues in particular the skin, GI tract, liver and lungs, typically developing within 100 days of transplantation (Ferrara et al., 2009). The severity of aGvHD and incidence of graft failure can be directly correlated with the level of mismatch between the human leukocyte antigen (HLA) proteins of the donor and the recipient (Atkinson et al., 1990, Loiseau et al., 2007, Petersdorf, 2007). Even in cases where donor and recipient are HLA identical about 50% of recipients require treatment for aGvHD due to differences in the minor histocompatibility (miHA) antigens (Ferrara et al., 2009).

Following HSCT, recipients can also develop chronic GvHD (cGvHD); this typically occurs more than 100 days post-transplant and can occur with or without the patient having aGvHD previously. cGvHD mainly depends on the polarisation of CD4$^+$ T cells towards a Th2 response and it presents as an auto-immune-like syndrome. While cGvHD is a significant issue following transplantation, this introduction will focus on the pathophysiology of aGvHD.

Despite the fact GvHD is a potentially lethal complication associated with HSCT, a certain level of the graft-versus-host reaction is beneficial to maintain the graft-versus-leukaemia (GvL) effect. It has been noted in several studies over the last 30 years that patients who suffered from GvHD had a lower incidence of relapse (Weiden et al., 1981, Weiden et al., 1979). The allo-reactive T cells respond to both the host tissue (causing GvHD) and the leukemic cells (leading to the desirable GvL reaction) (Weiden et al., 1979). Prevention of aGvHD by T cell depletion of the graft is possible however it causes increased relapse and graft failure due to the loss of the beneficial GvL effect of the donor T cells (Shlomchik, 2007, Martin et al., 1985). Also the use of donor lymphocyte infusions to treat recurring or residual malignancy post-transplantation illustrates the GvL effect (Kolb, 2008). Therefore treatment of patients in the clinic is a balancing act between the beneficial and detrimental graft-versus-host reactions. The GvL effect will be discussed in detail in Chapter 6.

Over the last 20 years the procedures used to treat patients with HSCT have advanced tremendously. The source of donor stem cells has expanded from bone marrow harvested under general anaesthesia to also now include peripheral blood stem cells (PBSS) and umbilical cord blood (UCB). The source of the stem cells used for transplantation has historically been implicated in transplant outcome and GvHD status. The use of peripheral blood stem cells (PBSC) requires administration of growth factors such as G-CSF to the donor, stimulating haematopoietic stem cell mobilisation into the peripheral blood of the donor, which can then be harvested by apheresis. Despite the highest relative increase in the use of cord blood as a stem cell source between 2006-2008 (Figure 1.1), PBSC remains the most common source of donor cells (Gratwohl et al., 2013). The use of UCB as a stem cell source is associated with a decrease in the incidence of GvHD which is thought to be due to the immaturity

of the donor T cells and the higher regulatory T cell content within UCB (Li and Sykes, 2012). The disadvantage of UCB is the low number of stem cells available from each unit – however transplantation of UCB from two donors can actually lead to improved engraftment. The 'sacrifice' of 1 cord unit enables successful engraftment of the other (Barker et al., 2009). The use of PBSC had previously been associated with increased incidence of aGvHD. However a recent study reported the stem cell source is associated with incidence of chronic GvHD rather than aGvHD (Flowers et al., 2011).
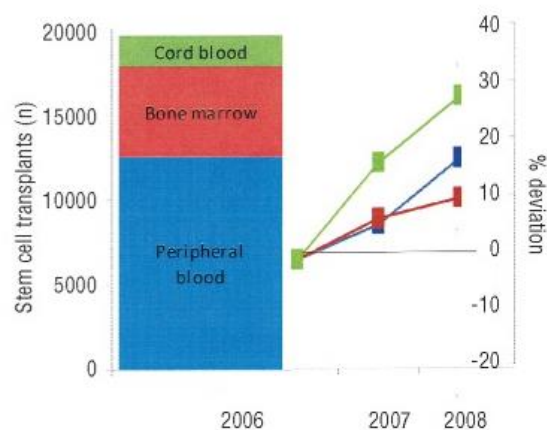
Figure 1.1 Stem cell sources for transplants performed in 2006-2008 (Gratwohl et al., 2013).

Additionally there have been considerable advances in the conditioning regimens used to prepare patients for transplantation; the objectives of conditioning being immunosuppression and the eradication of underlying disease (Figure 1.2). Originally, myeloablative conditioning regimens were used to eradicate leukemic cells, and then HSCT was used as a rescue therapy. Myeloablative regimens involve high doses of chemotherapy which is often combined with total body irradiation (TBI). However the intensive cytotoxic therapy itself can cause morbidity and mortality and is considered to be too toxic for elderly patients.

The development of non-myeloablative conditioning regimens has allowed for more patients, particularly older patients, to be considered for HSCT due to reduced toxicity. The reduced intensity conditioning regimens provide sufficient immune-suppression to allow for engraftment of the donor cells and rely on the GvL activity of the graft followed by donor lymphocyte infusion post-transplant to eradicate the tumour cells (Martino et al., 2002).

a Myeloablative allogeneic haematopoietic-stem-cell transplantation
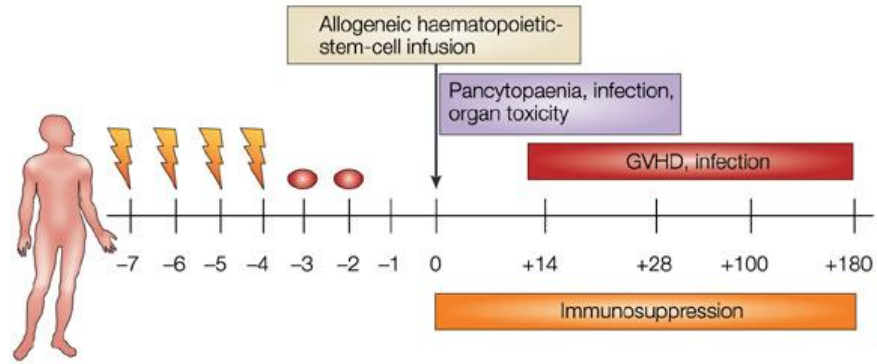
b Non-myeloablative allogenic haematopoietic-stem-cell transplantation
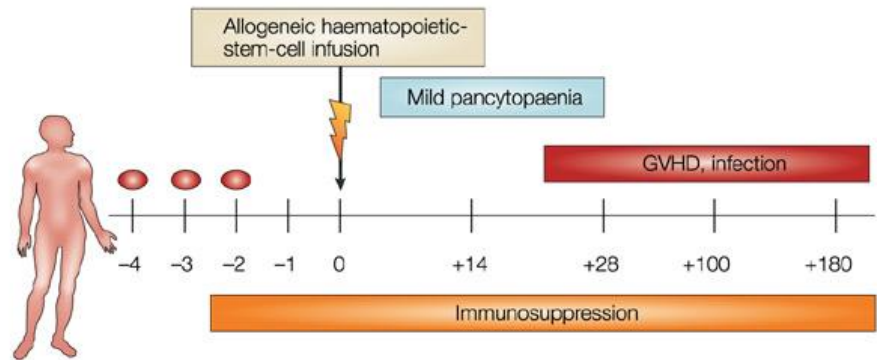
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Figure 1.2 Myeloablative and non-myeloablative conditioning regimens (Bleakley and Riddell, 2004). Ovals represent chemotherapy and lightning bolts represent TBI.

When deciding upon the transplant protocol there are many factors that influence the transplant and the conditioning regimen including age, disease, overall health, gender mismatch between donor and recipient and stem cell source. Donors who are considered first for transplantation are HLA matched siblings; if this is not possible then matched unrelated donors (MUD) or haplo-identical related donors are used. Of the allogeneic transplants that occurred worldwide between 2006-2008, 51% were performed from family members and the remaining 49% from MUD donors (Gratwohl et al., 2013).

1.2 The Human Leukocyte Antigen complex

The level of HLA matching between the donor and the recipient is crucial to the outcome of HSCT. The HLA system (also known as the human major histocompatibility (MHC) region) is located on the short arm of chromosome 6. The HLA region contains over 220 genes, 40 of which are believed to function in the immune response (Horton et al., 2004, Klein and Sato, 2000). Three distinct regions have been identified (shown in Figure 1.3) that encode proteins responsible for allo-antigen recognition and greatly influence the outcome of allogeneic transplantation as well as being responsible for normal immune defence against pathogens.
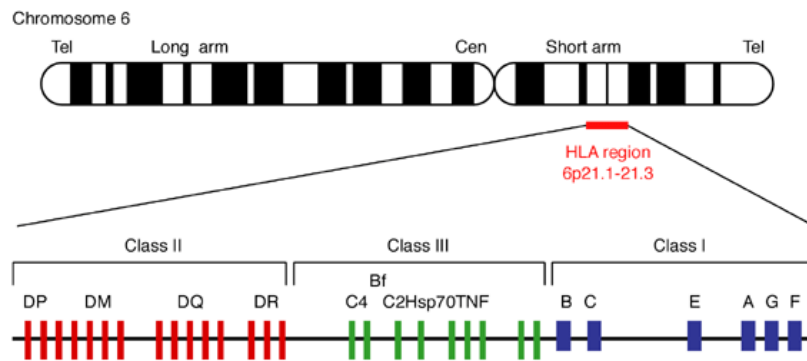
Figure 1.3 Gene map of the HLA region (Mehra and Kaur, 2003).

Class I molecules (HLA-A, HLA-B and HLA-C) are expressed on the surface of all nucleated cells and are responsible for the presentation of peptides derived from intracellular proteins to $CD8^+$ T cells. Class I molecules consist of two polypeptide chains, the β chain is encoded by a gene on chromosome 15 and the α chain encoded by genes from the class I HLA region (Figure 1.4). Class II molecules are structurally different to the class I molecules, however they both function to present peptides to T cells as part of the adaptive immune response. Both the Class II α and β chains are coded for by genes from within the class II region (Klein and Sato, 2000). Class II molecules are expressed on cells involved in the immune response, in particular antigen presenting cells (APC) – presenting peptides from extra-cellular proteins to $CD4^+$ T cells. The class III region, located between Class I and Class II regions, contains non-HLA genes which are involved in the immune system such as TNFα (Robinson et al., 2013).
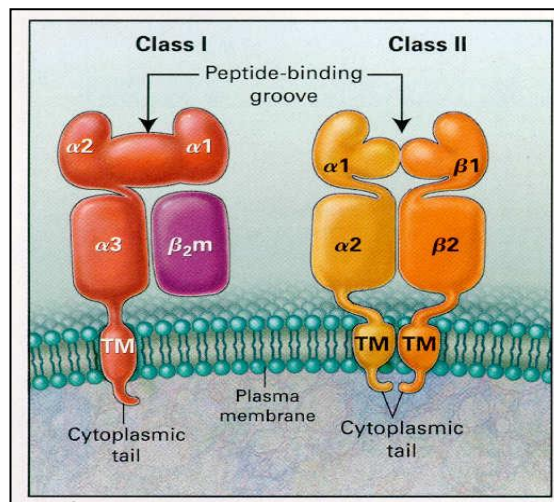
Figure 1.4 The structure of HLA class I and II molecules (Klein and Sato, 2000).

The main function of HLA molecules is to present pathogen derived peptides to T cells, initiating the adaptive immune response. In HSCT settings recipient HLA Class I and Class II molecules function as allo-antigens which can be recognised by engrafted donor T cells initiating a powerful GvH immune response, which contributes to both beneficial GvL and detrimental GvHD. Following HSCT antigen presentation can occur through the direct pathway where donor T cells recognise allogeneic host HLA molecules either with or without peptide present (Chakraverty and Sykes, 2007). Antigen presentation can also occur through the indirect pathway where host allo-antigens are presented by donor APC following the uptake and processing of host material. There are many cell types which are able to act as APC including dendritic cells (DC), monocytes, macrophages and occasionally B cells. These APC are able to take up antigens through a variety of methods including phagocytosis, endocytosis and scavenger receptors (Merad et al., 2007). Once DCs have been exposed to antigen in the periphery they mature and migrate to the lymph nodes. When in the lymph nodes the mature APC are able to present these HLA-peptide complexes to T cells and initiate T cell activation (Merad et al., 2007). Typically donor CD4$^+$ T cells are activated via class II HLA-peptide presentation and CD8$^+$ T cells via class I HLA-peptide presentation (Goker et al., 2001). However, in the HSCT setting donor dendritic cells are also able to cross-present allo-antigens on Class I MHC to CD8$^+$ T cells (Shlomchik, 2007).

Additional signals provided by co-stimulatory molecules engaging complete T cell activation as TCR-HLA/peptide binding in the absence of co-stimulatory signals drives T cells to an anergic state. Co-stimulatory signals also govern the

outcome of T cell activation as either stimulatory or regulatory depending on the molecules and ligands involved. This will be discussed in the following sections.

HLA matching between donor and recipient is crucial in the outcome of HSCT. The overall survival of patients is significantly improved when patient and donor are HLA identical (Figure 1.5) and as the degree of mismatch increases patient survival decreases (Loiseau et al., 2007). Therefore the number of mismatches between donor and patient is a major risk factor post HSCT.
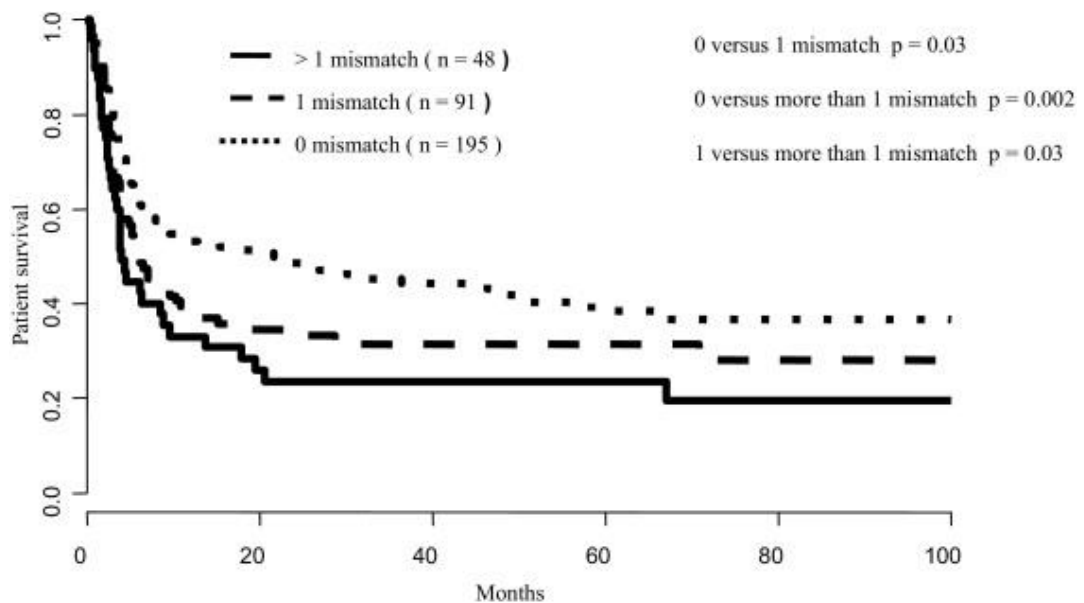
Figure 1.5 Effect of the number of HLA mismatches on overall survival post HSCT (Loiseau et al., 2007).

Recent advances have resulted in an increase in both the number of known HLA alleles and an understanding of their importance in HSCT. The HLA system is one of the most complex regions of the human genome with, to date, over 9000 known polymorphisms (Figure 1.6) (Robinson et al., 2013). Although registries of unrelated donors now include 20 million HLA-typed volunteers worldwide, the complexity of the HLA system remains a barrier to the success of HSCT with many patients unable to find a suitable match. Only 30% of patients have a suitable HLA matched or single mismatch related donor (Li and Sykes, 2012, Lee et al., 2007, Gratwohl et al., 2013). An ideal donor has matches at 5 HLA loci; HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1. Some mismatches are worse than others in terms of transplant outcome and GVHD (Petersdorf and Hansen, 2008).
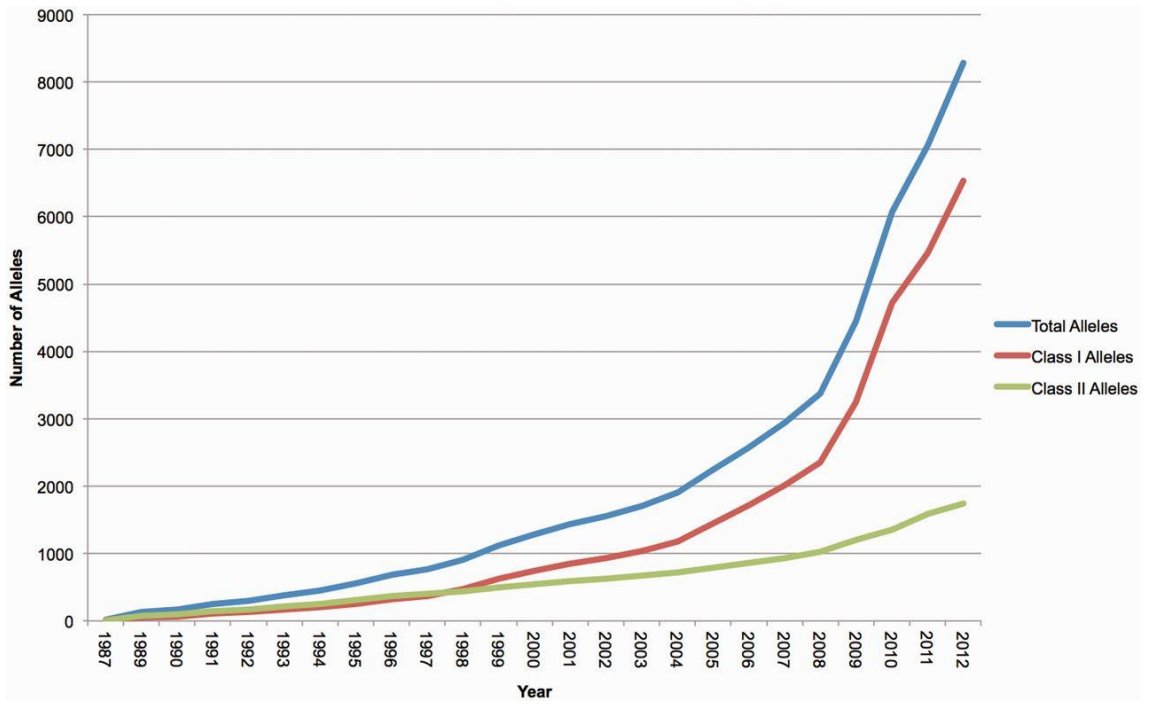
Figure 1.6 The number of named HLA alleles (Robinson et al., 2013).

1.3 Graft-versus-host disease

Graft-versus-host disease (GvHD) is generally classified into acute and chronic forms, commonly distinguished by the time of onset after HSCT. Early work suggested that aGvHD occurred within 100 days of transplantation and cGvHD was >100 days, however late onset acute GvHD and overlap syndrome (with features of both chronic and acute) show that this classification is not strictly accurate (Ferrara et al., 2009). Although considered to be different conditions, there is evidence to suggest a close relationship between risk factors for chronic and acute GvHD (Atkinson et al., 1990).

The mechanisms behind chronic GvHD are complex and are not entirely understood with a more diverse range of symptoms; it often resembles autoimmune disorders (Shlomchik, 2007). It can be progressive (acute to chronic), quiescent (acute then chronic) or *de novo* (chronic only) (Ferrara et al., 2009). This review will focus on the mechanisms of aGvHD rather than cGvHD.

1.3.1 The pathophysiology of acute graft-versus-host-disease

In the 1960s Billingham proposed a set of three criteria for the development of aGvHD (Billingham, 1966). By the mid-1990s a revised set of 4 criteria had been proposed: they state that (1) the host immune system must be incapable of mounting an immune response, (2) the graft must contain immune-competent cells, (3) host cells must express tissue antigens not present in the donor and therefore appear foreign to the donor cells, and (4) the effector cells must be able to migrate to the target tissues (Sackstein, 2006). When all these criteria are fulfilled the patient is then at risk of developing aGvHD following HSCT.

The pathophysiology of aGvHD is commonly described as a three phase process: the effects of conditioning, donor T cell activation and finally cellular and inflammatory effector phase (Figure 1.7). Each phase will be discussed in more detail.
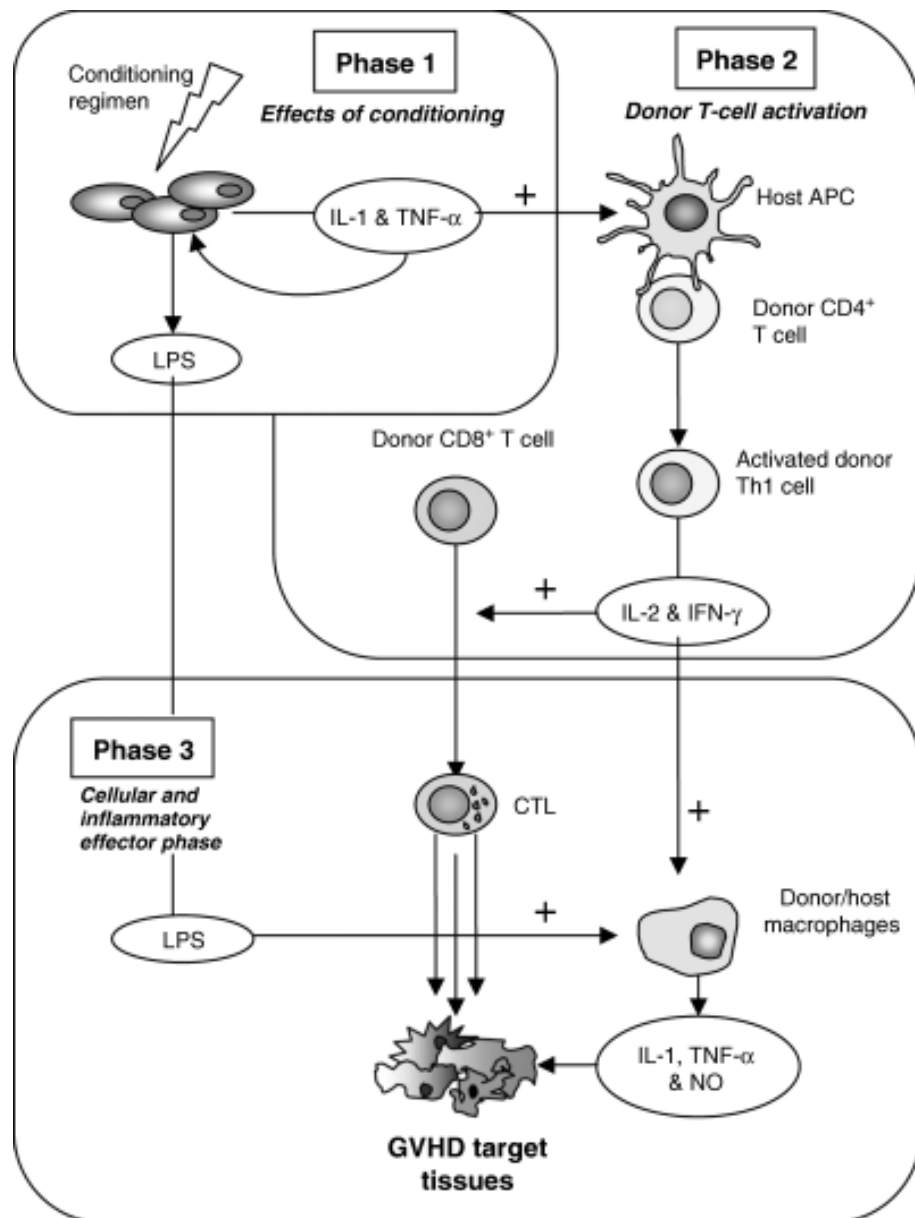
Figure 1.7 The three phase model of aGvHD (Jaksch and Mattsson, 2005).

Phase I: Conditioning regimen induces up-regulation of HLA molecule expression on host APC and inflammatory cytokine production by host target tissues.

A side effect of conditioning regimens is injury to GvHD target tissues, in particular the intestinal mucosa and liver. These damaged tissues produce several pro-inflammatory cytokines, chemokines and adhesion molecules (Ferrara et al., 2009). The cytokines most commonly secreted during phase I by activated host cells are TNFα, IL-1 and IL-6 (Cooke et al., 1998, Matzinger, 2002). These pro-inflammatory cytokines in turn increase expression of further adhesion molecules, HLA and co-stimulatory molecules activating residual host dendritic cells (DC) cells (Matzinger, 2002).

Conditioning also causes increased serum levels of lipopolysaccharides (LPS) which have been able to leak through the damaged intestinal mucosa; there is a direct correlation between the serum levels of LPS and the degree of intestinal damage after HSCT (Cooke et al., 1998). Following conditioning additional damage to the intestinal mucosa can occur when LPS leaks through triggering further production of TNFα and IL-1 by macrophages (Nestel et al., 1992). Increased levels of TNFα receptor 1 have been detected early post HSCT which were associated with the development of GvHD (Choi et al., 2008).

Several studies have been carried out in murine models to demonstrate the importance of LPS in the pathophysiology of GvHD. Toll-like-receptor-4 (TLR-4) detects LPS and experiments in TLR-4 mutant mice, compared to wild type, have shown a reduction in GvHD risk (Imado et al., 2010). In addition LPS antagonists have been shown to reduce GvHD while maintaining GvL in murine models (Cooke et al., 2001). In murine modes recombinant human keratinocyte growth factor has been shown to reduce GvHD mortality, GvH pathology in the GI tract and lower levels of serum LPS while still maintaining the beneficial GvL and could therefore provide a potential therapeutic option (Krijanovski et al., 1999).

Gut decontamination using antimicrobial chemotherapy prior to transplantation has been reported to reduce the severity of GvHD, supporting the principle that gut micro flora are important in the pathogenesis of GvHD (Beelen et al., 1999). Damage to the GI tract is particularly important as it allows

the translocation of inflammatory stimuli such as LPS which then lead to further stimulation of APCs in phase 2 of aGvHD (Ferrara et al., 2009)

The conditioning regimen prior to HSCT in essence primes the host for induction of GvHD. Production of danger signals and maturation of host DC mean that upon infusion of the donor cells all the signals are already there to activate the donor T cells and allow for the induction of phase II of aGvHD.

Phase II: Allo-antigen recognition induces donor T cell activation, proliferation and differentiation.

Phase II of GvHD occurs once the transplant has taken place. The maturation of immature host DCs under the influence of inflammatory cytokines and LPS continues during and immediately after HSCT (Janeway and Medzhitov, 2002). Following HCST both host and donor APC are able to process and present allo-peptides to donor T cells, resulting in T cell activation. The donor T cells then proliferate and secrete cytokines including IL-2 and IFNγ, causing a cytokine storm further promoting antigen presentation and T cell recruitment; this sequence of events is crucial to the pathophysiology of GvHD (Jaksch and Mattsson, 2005).

Many different APC are involved in phase II of aGvHD; both those from the host and the donor, including non-haematopoietic APC. DCs which are situated in various barrier tissues, such as the skin and GI tract are particularly important. Interestingly the location of DCs correlates with the specific group of target tissues associated with aGvHD. Using animal models it has been shown that the removal of DCs from an organ may prevent aGvHD in that particular organ but have no effect on aGvHD in other target organs (Zhang et al., 2002). The presence of donor DCs in peripheral blood can be detected as soon as 1 day post-transplant implicating both host and donor DC in the pathophysiology of aGvHD (Merad, Collin et al. 2007). It has been suggested that depletion of DCs from the graft may reduce GvHD as donor APC are not required for GvL effect (Matte et al., 2004) and donor derived APC may increase severity of GvHD following transplant (Shlomchik, 2007).

The key process in phase II is activation of T cells by APC (Figure 1.8), and as previously mentioned this requires both the presentation of antigen to the T

cell and a co-stimulatory signal, the absence of a co-stimulatory signal results in T cell anergy rather than T cell activation (Banchereau et al., 2000).
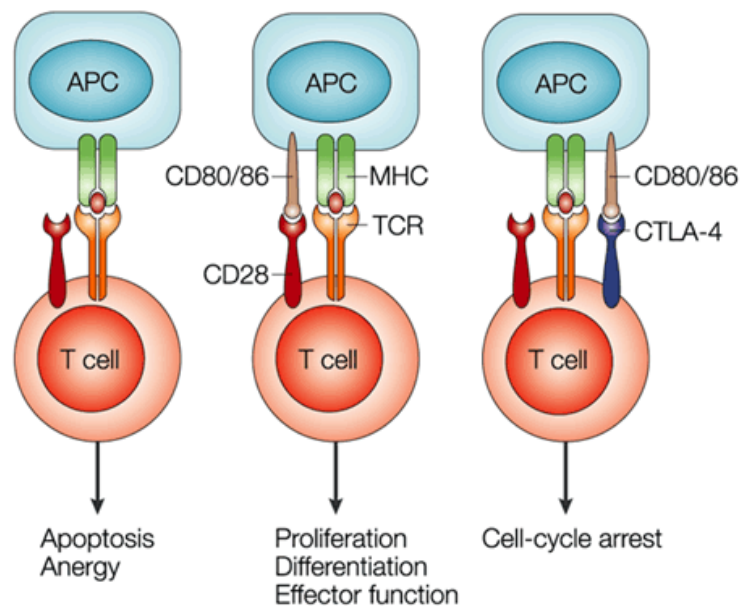
Figure 1.8 T cell activation (Alegre et al., 2001).

The consequence of T cell receptor signalling by cognate antigenic peptides is determined by the co-signalling receptor - which can be co-stimulatory or co-inhibitory. The CD80/86-CD28 co-stimulatory receptor-ligand interaction (Figure 1.8) was the first to demonstrate the two-signal process of T cell activation (Bretscher and Cohn, 1970, Lafferty and Cunningham, 1975). The discovery of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) as a co-inhibitory CD80/86 ligand highlighted the complexity of the T cell activation system (Linsley et al., 1991). CD28 is constitutively expressed on T cells and provides a co-stimulatory signal following ligation with CD80/86, conversely interactions between CTLA-4 and CD80/86 result in cell cycle arrest. CTLA-4-CD80/86 interactions can also induce the expression of indoleamine-pyrrole 2,3 dioxygenase (IDO) in the APC which can act in trans to supress T cell responses and induce Regulatory T cells (Treg). Furthermore when CLTA-4 expression is increased CD28 is down regulated by endocytosis providing another level of regulation of the T cell responses (Rudd et al., 2009). In addition to the CD80/86-CD28/CTLA-4 receptor ligand interactions there are dozens of other interactions between co-signalling receptors, forming a highly complex regulatory system (Chen and Flies, 2013).

T cell activation is a highly regulated process with multiple levels of control. It requires the correct spatial and temporal expression of receptors and their ligands on the different cell types, and co-signalling receptors/ligands are able to interact with more than 1 ligand/receptor resulting in multiple functions and bi-directional co-signalling provides additional control. T cell activation is a balance between various stimulatory and inhibitory factors; these pathways and interactions between T cells and APC provide a potential method for treatment and prevention of GHVD and therefore has been the subject of various investigations.

The importance of the co-stimulatory B7 antigens expressed on APC, B7-1 (CD80) and B7-2 (CD86) was shown by blockage of B7-CD28 interaction inhibiting aGvHD in murine models (Ogawa et al., 2001). Additionally when cells were transplanted from CD28(-/-) mice there was a reduction in T cell activation, proliferation and ultimately better survival following transplantation (Yu et al., 1998). Furthermore blockage of interactions between CD40 and CD154 co-stimulatory pathway has been shown to prevent CD28 independent aGvHD (Saito et al., 1998). Over the course of APC-T cell interaction various cytokines and chemokines are released. The secretion of IL-1 by APC stimulates TNFα production and also the production of IL-2 by T cells. IL-2 is commonly considered the most important cytokine in the pathophysiology of aGvHD because of its central role as a T-cell growth factor; IL-2 causes allo-reactive T cells to expand and differentiate into CD8$^+$ cytotoxic T lymphocytes (Jaksch and Mattsson, 2005).

In addition IFN-γ is produced during the cytokine storm and it has many roles in aGvHD. Mice with aGvHD have higher levels of IFN-γ than those without aGvHD (Hill et al., 1997). Along with IL-2, IFN-γ is able to promote T-cell expansion and induce cytotoxic T lymphocyte and NK cell responses (Jaksch and Mattsson, 2005). IFN-γ also induces increased expression of chemokines, adhesion molecules and HLA molecules and it appears to mediate nitric oxide production and FAS expression (Jaksch and Mattsson, 2005). Although it is commonly seen as detrimental, IFN-γ can have opposing functions in the pathophysiology of aGvHD. IFN-γ is also able to suppress the immune system by inducing FAS expression on donor cells and causing activation-induced cell death (Goker et al., 2001). This provides an example of why targeting cytokines

as a method of GvHD prophylaxis or treatment would prove challenging. It is a highly complex system requiring a fine balancing act, reducing levels and increasing levels may both have beneficial and detrimental outcomes.

Chemokines have an important role in the pathophysiology of aGvHD. The process of recruitment of effector T cells to target tissues involves all three families of migration molecules including chemokines, integrins and lectins. Many of the inflammatory cytokines (for example IL-1, TNFα and IFNγ) produced during phase I and II induce expression of chemokines and chemokine receptors in GvHD target tissue and on effector T cells respectively (Jaksch and Mattsson, 2005). Increased gene expression of CCR5, CXCR3, CCR1 and CCR2 has been shown to correlate with aGvHD following HSCT (Jaksch et al., 2005). Chapter 4 contains a more detailed review of the role of chemokines in the pathophysiology of GvHD.

Phase 2 of aGvHD is one of T cell activation and proliferation and also production of pro-inflammatory cytokines and chemokines which recruit effector T cells to aGvHD target tissues.

Phase III: Effector cells and inflammatory cytokines induce target tissue damage.

The third and final phase of aGvHD is a complex cascade of effector processes. Effector cells which were activated during phase II induce target cell damage during phase III. Through the action of chemokines and adhesion molecules allo-reactive T cells (CD4$^+$ and CD8$^+$) are able to migrate into the target tissue and cause damage characteristic of aGvHD. The ability of allo-reactive cytotoxic T cells to migrate to target tissues will be discussed in more detail in Chapter 4.

There are three main pathways responsible for tissue damage in aGvHD target tissues; The FAS/FASL pathway, the perforin/granzyme pathway and direct injury mediated by cytokines. The importance of each of these processes has been shown in knockout mouse models (Jaksch and Mattsson, 2005). Figure 1.9 from Shlomchik, 2007, summarises various methods of tissue injury caused by infiltrating T cells. Perforin and FAS mechanisms account for most CD8$^+$ T cell effector function in aGvHD (Via et al., 1996). CD8$^+$ T cells tend to favour FAS/FASL pathway (shown in figure 1.11 as CD95/CD95L), but also

cause target cell death via the perforin/granzyme cytolytic granules pathway (Lowin et al., 1994). The perforin/granzyme pathway requires binding of perforin – following direct cell contact – then penetration of the cell granule (granzyme A and B) which then leads to activation of the caspase cascade and cell cytolysis takes place (Goker et al., 2001). The FAS receptor (CD95) is expressed on many tissues and expression levels increase during inflammation and FAS receptor ligand (FASL/CD95L) is predominantly expressed on activated T cells, macrophages and neutrophils. Interaction between FAS and FASL results in FAS mediated apoptosis (Nagata and Golstein, 1995). During aGvHD FASL expression is increased on CD8^{+} T cells and CD4^{+} T cells and serum levels of both FAS and FASL correlate with GvHD severity grade (Jaksch and Mattsson, 2005). The FAS/FASL mechanism appears to be particularly important in hepatic GvHD as FAS deficient recipients are protected from hepatic GvHD (van Den Brink et al., 2000).
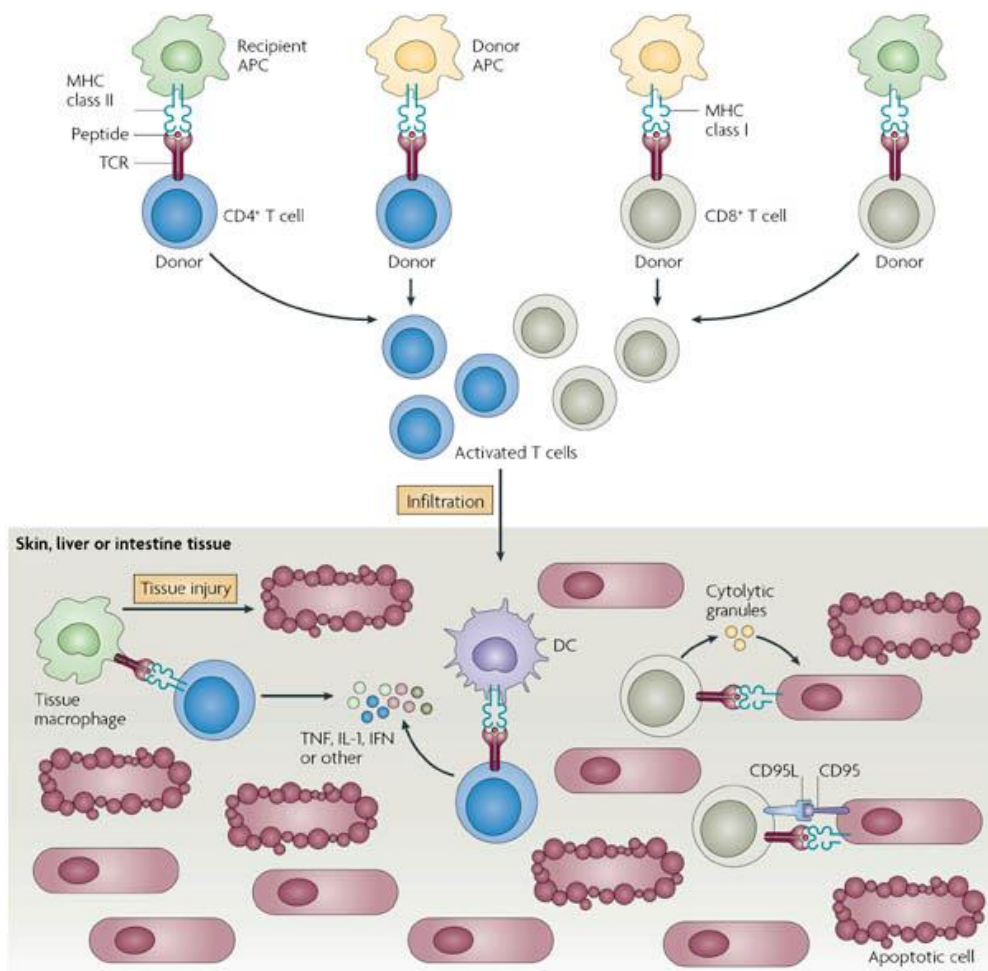
Figure 1.9 Mechanisms of tissue injury by T cells in GVHD (Shlomchik, 2007).

Further TNFα and IL-1 are produced by APC once they have been stimulated. TNFα activates further DCs thereby increasing allo-antigen presentation, production of cytokines and recruitment of more effector cells exacerbating the situation (Jaksch and Mattsson, 2005). It is also able to induce tissue destruction through apoptosis (Wall and Sheehan, 1994).

The pathophysiology of GvHD is highly complex with several important mechanisms acting together resulting in damage to the specific set of target organs. GvHD still presents a very real challenge in the clinic and therefore remains the focus of much research both into the cellular mechanisms of the disease and new therapeutic options.

1.3.2 *Current clinical strategies for management of GVHD*

The aim of clinical management of GvHD is to limit the risk of patients developing GvHD without reducing engraftment or the beneficial GvL effect. Unfortunately despite all the advances made over the past decades the overall outcome of GvHD treatment has not improved significantly therefore prevention of severe GvHD is imperative. A combination of prophylactic tactics can be used in the clinic such as high resolution HLA typing, manipulation of donor T cells, immuno-suppressants including methotrexate, cyclosporine and corticosteroids (Goker et al., 2001). Another approach is to T cell deplete the graft either before infusion or post-transplant using mono-clonal antibodies; this however often results in a higher incidence of graft failure and relapse (Shlomchik, 2007, Martin et al., 1985). The administration of monoclonal antibodies against leukocytes (for example alemtuzumab-anti CD52) has also been used as a method of GvHD prophylaxis (Ferrara et al., 2009).

Current first line treatment for GvHD is still steroids, however being a pan immunosuppressive there is an association with prolonged infection, graft failure and relapse (Ferrara et al., 2009). Furthermore in some cases patients do not respond to steroids therefore alternative treatments must be explored. An alternative treatment option for GvHD sometimes used in the clinic is extracorporeal photopheresis (ECP). In ECP white blood cells are removed by apheresis, incubated with DNA-intercalating agent and exposed to UV light before being returned to the patient. The first report of ECP treatment of patients with GvHD came in 1996 (Rossetti et al., 1996) Murine models suggest that ECP works by increasing the number of donor regulatory T cells (Treg) (Gatza et al., 2008).

More recently the use of cellular therapy for clinical management has become an interesting prospect. Clinical trials have been carried out using different populations of suppressive or regulatory cells to prevent GvHD. Treg have been identified as a particular population that are able to suppress allo-immune responses in a HSCT setting and therefore interest for use in the prevention of GvHD. Various studies both in murine models and human studies progressing as far as clinical trials have shown that potentially Treg are able to reduce incidence of aGVHD possibly by prevention of T cell activation in phase 2 of aGVHD.

1.4   Immune tolerance and regulatory T cells

Immune tolerance is a state of un-responsiveness to particular antigens. Immune tolerance can be established during T cell development in the thymus (central tolerance through depletion of T cells with a high affinity TCR to self-antigens) or in the periphery through anergy induction, Treg suppression and inhibitory molecules. Central tolerance is an important gatekeeper stopping large numbers of auto-reactive T cells being released into the periphery, while the small numbers of auto-reactive T cells which escaped thymic negative selection are eliminated or kept under control by peripheral tolerance mechanisms.

Progenitor double negative T cells enter the thymus at the cortico-medullary junction (Lind et al., 2001) and migrate through the cortex. As cells migrate through the thymus they pause as their TCR interacts with peptide-HLA complexes; low avidity interactions induce survival signals and enrich for useful T cells able to recognise HLA molecules (Takahama, 2006). High avidity interactions between the TCR and peptide-HLA complexes lead to the deletion of the thymocytes, through the induction of apoptosis. This negative selection results in the deletion of self-reactive T cells. The positively selected double positive thymocytes then differentiate into single positive thymocytes, either CD4^{+} or CD8^{+} (Egerton et al., 1990). Following negative selection in the thymus naïve T cells are released back into circulation, and only around 3-5% of cells survive this process (Takahama, 2006).

The thymus also functions in the generation of naturally occurring Foxp3^{+} Treg. Most Foxp3^{+} cells are found in the medulla region of the thymus, suggesting this is where Treg development occurs (Takahama, 2006). The current model states that when the TCR avidity lies between the levels which drive positive and negative selection Treg development occurs (Maloy and Powrie, 2001). This model is supported by more recent data showing self-antigens for Treg are relatively uncommon (Hsieh et al., 2012). The APC present in the thymus are also believed to be influential in the development of Treg as co-stimulation promotes maturation of Treg precursors, preventing their deletion (Hinterberger et al., 2011). The control of central tolerance is a highly regulated process, controlling the number of auto-reactive T cells and generating Treg. Any auto-reactive T cells which do escape central tolerance

are then controlled by Treg mediated tolerance induction in the periphery. The mechanisms by which Treg do this are discussed in section 1.6.

In the context of normal physiology, both central and peripheral tolerance function to abolish auto-reactive T cells, preventing autoimmune responses and therefore protecting individuals from developing autoimmune diseases. In allogeneic transplant settings, peripheral tolerance plays a key role to maintain certain levels of immune tolerance against allo-antigens. Therefore most transplant tolerance induction utilises peripheral tolerance mechanisms, and of particular interest are Treg.

1.4.1 Regulatory T cells and their role in immune regulation

Treg are a heterogeneous sub population of T cells; the best defined population of Treg is the naturally occurring Treg, commonly characterised as being CD4$^+$CD25^{Hi}Foxp3$^+$. They have a vital role in maintaining self-tolerance and preventing proliferation of auto-reactive T cells (Sakaguchi et al., 1995). Tregs have been shown to be able to influence most immune responses: autoimmunity, transplantation tolerance, anti-tumour immunity and anti-infectious responses (Tang and Bluestone, 2008). Undoubtedly Treg are an exciting component of the immune system and are worthy of significant further study. In order to study their mechanisms of action in more detail further *in vitro* studies are required.

After controversy in the late 1980s and early 1990s as to the existence of Treg, Sakaguchi and colleagues identified a population of murine T cells which were CD4$^+$ and expressed high levels of the IL-2Rα subunit CD25. This subpopulation, between 5-10% of CD4$^+$ cells, was shown to have suppressive properties (Sakaguchi et al., 1995). Further characterisation and isolation of this population of murine Treg and confirming their existence in humans attracted a huge amount of attention in the following 5 years. By 2001 several groups had confirmed the existence of a CD4$^+$CD25^{Hi} sub-population of Treg in human peripheral blood (Baecher-Allan et al., 2001, Dieckmann et al., 2001, Jonuleit et al., 2001, Ng et al., 2001, Taams et al., 2001, Levings et al., 2001). Although Treg had been successfully characterised as CD4$^+$CD25^{Hi} the identification of more markers was required in order to distinguish regulatory T cells from activated T cells. CD62L adhesion molecule L selectin was first

identified in NOD mice as a Treg marker, although it is not exclusively expressed on Treg and is therefore of little use as an isolation aid (Lepault and Gagnerault, 2000). The co-signalling molecule CTLA-4 was also shown to be expressed on Treg, however again this marker is also present on other T cell subsets (Takahashi et al., 2000).

The discovery of Foxp3 as a marker for Treg came about in 2001 through studies into severe auto-immune disorders both in humans and mice. Immune-dysregulation-polyendocrinopathy-enteropathy X linked syndrome (IPEX) is a rare autoimmune disease in humans with the murine equivalent being scurfy (Powell et al., 1982, Godfrey et al., 1991). These diseases were found to be caused by mutations in the gene for the transcription factor Foxp3 which in turn resulted in a lack of functional Treg (Wildin et al., 2002). In 2003 two groups reported the importance of Foxp3 in the development and regulation of Treg illustrating its functional importance and confirming its reliability as an identification marker for Treg (Fontenot et al., 2003, Hori et al., 2003). Fontenot *et al* showed adaptive transfer of wild type Treg could rescue disease development when transferred to neo-natal Foxp3 deficient mice. Additionally Hori *et al* used retro-viral transfer to convert naïve T cells into Treg which were phenotypically similar to naturally occurring Treg. Recent work has shown that Foxp3 gene transfer into CD4$^+$ T cells from IPEX patients allows for the conversion into functionally stable Treg emulating the function of Treg from healthy donor cells thereby confirming Foxp3 as the master transcription factor for functional Treg (Passerini et al., 2013). Therefore Foxp3 is now considered to be the gold standard identifier of Treg.

CD4$^+$CD25^{Hi}Foxp3$^+$ Treg can be further classified into naturally occurring Treg (nTreg) which develop in the thymus and induced Treg (iTreg) which are induced in the periphery. Naïve CD4$^+$CD25$^-$ cells can become regulatory T cells in the periphery or *in vitro* through a variety of signals including IL-10 and TGFβ (Sojka et al., 2008). Expression of the transcription factor Helios was thought to differentiate between nTreg and iTreg; Thornton *et al.* showed that 100% of CD4$^+$Foxp3$^+$ thymocytes expressed Helios but only 70% CD4$^+$Foxp3$^+$ peripheral cells expressed Helios (Thornton et al., 2010). This notion has recently been challenged by an observation showing Helios expression in Treg induced in the periphery (Gottschalk et al., 2012) However the current

consensus is that Helios expression is a reasonable, although not definitive, marker for distinguishing nTreg from iTreg.

1.4.2 Other regulatory cell populations

In addition to CD4$^+$CD25^{Hi}Foxp3$^+$ Treg there are other populations of T cells able to promote tolerance and regulate immune functions. One such population of regulatory T cells which have been suggested to have a beneficial role in aGvHD are CD8$^+$Foxp3$^+$ Treg. These cells were shown, in murine models, to be induced during early GvHD and have the ability to potently suppress allo-reactive T cell responses *in vitro* (Beres et al., 2012). CD8$^+$ Treg cells, also identified in humans express many of the same surface markers as their CD4$^+$ equivalents; such as glucocorticoid-induced TNFR family related gene (GITR), CD103 and CTLA-4. Although Foxp3 is often seen as the master regulator of Treg other populations of regulatory CD4$^+$ T cells which lack Foxp3 expression have also been described, including CD4$^+$ Tr1 cells which secrete IL1-0 and TGFβ in response to antigen stimulation (Liu et al., 2014, Banchereau et al., 2012).

Other subpopulations include "adaptive" or "induced" regulatory CD4$^+$ T cells which are created *ex vivo*. Haque *et al* have used a mouse model to demonstrate a potential method of generating large numbers of Treg – they were able to programme Treg from induced pluripotent stem (iPS) cells which functioned to reduce arthritis development (Haque et al., 2012). This provides an interesting and exciting alternative to the isolation and expansion of naturally occurring Treg. Zhang *et al* reported the conversion of human conventional CD4 and CD8 donor T cells into large numbers of iTreg in just 1 week (Zhang et al., 2013). However unlike nTreg these cells lost expression of Foxp3. Administration of rapamycin and IL-2 to the patients did significantly improve Foxp3 stability. This study demonstrated the feasibility of generating large numbers of iTreg which function to induce tolerance post transplantation in the presence of systemic rapamycin (Zhang et al., 2013).

Various populations of regulatory T cells exist, some naturally occurring *in vivo* and others generated *ex vivo*, although all important and of potential use in the prevention and/or treatment of aGvHD only the function and role of nTreg will be discussed and investigated further within this thesis.

1.4.3 Treg in autoimmunity

Autoimmune disorders are caused by a breakdown in the mechanisms which balance tolerance and protection against invading pathogens. Treg are able to subdue peripheral activation of auto-reactive T cells which escaped negative selection in the thymus thereby preventing auto-immune diseases. The importance of Treg in the control of autoimmunity is seen in conditions such as IPEX where a lack of Treg results in severe autoimmune disorders (Wildin et al., 2002). Peripheral Treg are polyclonal and undergo a similar selection process in the thymus with the exception of positive selection for recognition of auto peptides (Andre et al., 2009). Once Treg are exposed to antigen in the lymph nodes they become activated and exert their suppressive properties. Treg are activated at a much lower concentration of antigen than conventional T cells preventing autoimmunity (Sakaguchi et al., 2008). The important role of Treg has been shown in several auto-immune disorders, and in some cases administration of Treg is being investigated as a possible treatment option.

Multiple Sclerosis (MS) is a progressive auto-immune disorder characterised by demyelination of axonal tracks (Sospedra and Martin, 2005). In MS the suppressive function and number of Treg was seen to be reduced, along with lower Treg proliferative capacity (Viglietta et al., 2004, Carbone et al., 2014). Murine models of MS showed that depletion of Treg accelerated disease progression while adaptive transfer of Treg was able to attenuate disease progression (McGeachy et al., 2005, Kohm et al., 2002).

Type 1 Diabetes is caused by a breakdown in regulation leading to the activation of β-cell specific auto-reactive CD4/CD8 cells. Several studies observed that supplementation of Treg prevented disease whereas administration of anti-CD25 exacerbates progression (Anderson and Bluestone, 2005, Tarbell et al., 2007). Various clinical trials are ongoing to evaluate the infusion of Treg for the prevention/treatment of autoimmune disorders and Treg have been successfully used to delay the onset of type 1 diabetes in children (Marek-Trzonkowska et al., 2012).

1.4.4 Treg in tumour immunity

Treg are abundant in solid tumours which raises the question do Treg prevent immune surveillance against tumours? Treg depletion in rodents has been shown to enhance tumour immunity and therefore provides a potential therapeutic option for the treatment of solid tumours (Shimizu et al., 1999, Sakaguchi et al., 2008). The ability of Treg to prevent tumour immunity becomes a concern in the use of Treg as a method of managing GvHD following HSCT as Treg may be detrimental to the GvL effect of the graft. This will be discussed further in Chapter 6.

1.4.5 Treg in solid organ transplant

Over 4,200 solid organ transplants were carried out in the UK last year (www.uktransplant.org.uk) and these transplants are able to take place due to the development of immunosuppressive drugs which are able to prevent organ rejection. However, the long term use of immuno-suppressants is not ideal due to an increased risk of infection and side effects which can reduce the both the life of the graft and of the patient. In rare cases some patients have a functioning graft in the absence of any immunosuppression and Treg are one reason implicated in the tolerance of these grafts (Tokita et al., 2008, Wood et al., 2012, Mazariegos, 2011).

In animal models of transplantation, including humanized mouse models, the infusion of Treg has been shown to prevent both acute and chronic graft rejection. Treg are able to protect against the adverse effects of transplantation including transplant arteriosclerosis, the thickening of arteries in transplanted grafts, and allo-immune damage to skin grafts can be controlled by Treg, with a more potent suppression by antigen specific Treg (Nadig et al., 2010, Sagoo et al., 2011). In addition depletion of Treg from hosts in experimental models of transplantation appears to accelerate rejection of the donor organ (Tang et al., 2012). These studies in animal models provide evidence for the 'proof of concept' supporting the translation of Treg therapy into a clinical transplantation setting. A large multi-centre safety and feasibility clinical trial has been initiated assessing the use of Treg in kidney transplant patients (Geissler, 2012). Additionally a recent report has described the safe use of induced Treg in living donor liver transplant patients, permitting weaning of immunosuppression (Juvet

et al., 2014) however these results are only preliminary and further follow up of these patients is required.

1.4.6 Treg in allo HSCT

The role of Treg in HSCT has been of great interest for many years. Co-transfer of Treg in murine models was shown to prevent lethal GvHD both *in vitro* and *in vivo* (Hoffmann et al., 2002, Taylor et al., 2002, Cohen et al., 2002). More importantly, in addition to prevention of GvHD, infusion of Treg maintained the GvL effect mediated by the conventional T cells (Edinger et al., 2003). Not only is the effect of Treg important on the incidence of GvHD following transplantation but also on the levels of immune reconstitution and engraftment. In murine models the immune reconstitution following transplantation is unaffected by the co-administration of Treg (Gaidot et al., 2011). Several murine studies had therefore indicated that infusion of Treg following transplantation had the potential to be an effective treatment for GvHD.

In addition to experimental aGvHD there were several observations from clinical cohorts of patients suggesting the beneficial role of Treg following transplantation. Wolf *et al* reported that in a cohort of 58 patients, split into either high or low Treg content within the graft, the patients with a lower content had a much higher incidence of aGvHD that those with a higher Treg content without any difference in incidence of relapse (Wolf et al., 2007). Further observations have supported that high levels of Treg result in a reduced risk of aGvHD (Rezvani et al., 2006, Li et al., 2010, Ukena et al., 2011). The role of Treg in cGvHD remains controversial. Clark *et al* suggested that increased peripheral blood levels of Treg are associated with cGvHD (Clark et al., 2004) whereas Zorn *et al* report an association between low levels of Treg and chronic GvHD (Zorn et al., 2005).

In vitro studies have shown that Tregs are able to suppress GvHD, and still allow for beneficial GvL, this opened the door for a small number of clinical trials aimed at safety testing and prevention of GvHD. A small number of clinical trials have shown Treg administration to have no safety issues (Di Ianni et al., 2011, Brunstein et al., 2011, Trzonkowski et al., 2009).

1.4.7 Clinical application of Treg in HSCT

Just 8 years after the first reports in murine models clinical trials began investigating *ex vivo* isolated and expanded Treg for the treatment/prevention of GvHD. The first-in-man case report from Trzonkowski *et al* included just two patients, one successfully treated for *de novo* chronic GvHD by expanded donor Treg cells. The other patient unfortunately was not successfully treated for their severe acute GvHD, however the administration of Treg was considered to be rather late at 75 days post transplantation and the authors did note a slight improvement in the patient following infusion of Treg (Trzonkowski et al., 2009). The *ex vivo* expanded Treg used in this trial were isolated by FACS cell sorting, although not under GMP conditions.

The results of two early stage clinical trials were recently published. One trial enrolled 28 patients with high risk malignancies where freshly isolated Treg using cliniMACS were infused after conditioning and then again 4 days prior to transplantation (Di Ianni et al., 2011). This trial provided evidence to suggest that Treg did not prevent engraftment, 26 patients achieved full engraftment and in a high risk cohort of patients there were only 2 cases of aGvHD (Di Ianni et al., 2011). Patients treated for high risk malignancies are still associated with high transplant-related mortality; in this report after 12 months, 12 of the 26 evaluable patients were alive and disease free and only 1 report of relapse which was improved when compared to a cohort of historical controls. The other trial included 23 patients - also with high risk malignancies - for a dose-escalation trial. These patients received non-myeloablative conditioning regimens and were treated with a double UCB transplant (Brunstein et al., 2011). Treg were isolated from partially HLA-matched third party UCB by cliniMACS and *ex vivo* expanded prior to infusion on day 1 and day 15 post transplantation. There were no dose-limiting toxicities or changes in the frequency of adverse events when compared to a historical cohort or patients. Importantly a significant decrease in the incidence of severe acute GvHD was seen in patients who received Treg therapy.

1.4.8 Future directions of the clinical use of Treg for transplantation

Over recent years significant advances in the understanding of Treg in transplantation have resulted in the completion of early stage clinical trials in HSCT and the initiation of clinical trials in sold organ transplantation. Although these early results look promising it is important to continue the research and to follow up the patients involved in these early stage trials. The two HSCT clinical trials have demonstrated the safety and feasibility of early Treg infusion with the added benefit of a reduction in the incidence of GvHD. The Treg infusion did not lead to impaired immunity against infections or lead to global immunosuppression. Questions still remain as to the effect of Treg infusion on the GvL effect, this issue will be explored in Chapter 6.

The use of Treg to promote tolerance in solid organ transplantation is an appealing prospect and unlike HSCT there are no potential problems with Treg impairment of the beneficial GvL effect. Currently global immunosuppression is used to prevent solid organ graft rejection however this impairs immune responses to infections and tumour surveillance as well as leading to diabetes, nephrotoxicity and cardiovascular disease (Tang and Bluestone, 2013). Ideally post-transplant immunosuppression would be able to prevent immune responses against the graft whilst maintaining normal protective immune functions, and Treg may provide the answer to this problem. In mouse models, Treg therapy after transplantation prevents rejection and encourages further induction of Treg which promotes long term survival of the graft (Waldmann, 2008). It has been reported that allo-antigen specific Treg have a more potent effect than polyclonal Treg (Tang et al., 2012). However this could be problematic, especially in the case of deceased donor transplants, if the Treg were to be administered early for maximum potency.

An additional consideration is the cost of Treg therapy in comparison to currently used therapies. Estimates for the cost of Treg administration to patients on the ONE study are around £30 000, which when compared to the cost of typical kidney transplant immunosuppression of £6000 for a year appears rather high (Juvet et al., 2014). Although the initial cost is off-putting if Treg treatment enables immunosuppression weaning and reduces adverse side effects of transplantation it may become economically viable.

1.5 Mechanism of action of Foxp3⁺ Treg

Over the last 20 years there has been significant research into the action of Treg which has revealed a broad range of mechanisms. It is this wide range of mechanisms available to Treg and their versatility which makes them so effective at coping with auto-reactive cells that escape central tolerance control (Tang and Bluestone, 2008). Similar mechanisms may also apply to Treg mediated tolerance induction to allo-reactive T cell responses in transplantation. Treg have been shown to act through both contact independent and dependant mechanisms some of which are summarised in Figure 1.10.

Treg are able to act through several mechanisms simultaneously, some mechanisms may still remain un-discovered. The mechanisms by which Treg exert their suppressive function depends upon the particular micro-environment of the Treg. Once Treg have become activated they are able to supress in a non-specific manner, termed "bystander suppression". This bystander suppression has been shown to remain even after the original Treg population has been either lost or removed (Qin et al., 1993, Tarbell et al., 2007). Understanding the mechanisms behind the suppressive action of Treg is important as it provides opportunities for the development of the use of Treg in a clinical setting, in particular a understanding of the role of Treg in the prevention of GvHD following HSCT.
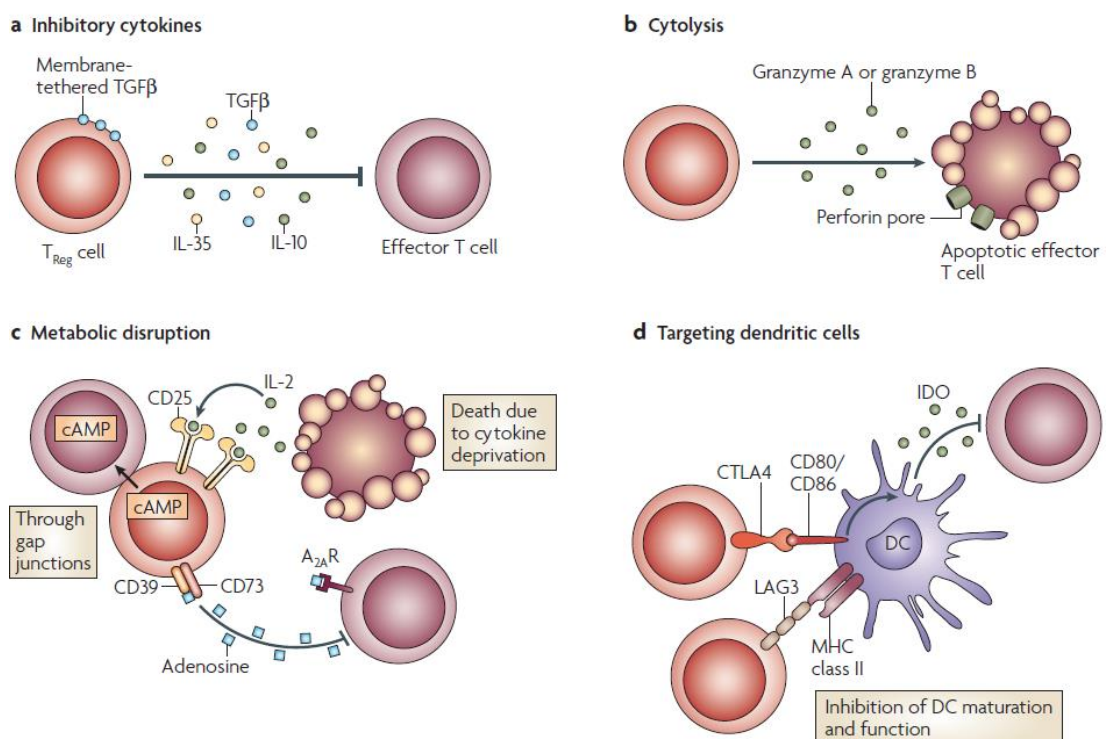
Figure 1.10 Suppressive mechanisms of Treg (Vignali et al., 2008).

1.5.1 Treg suppression by inhibitory cytokines

The suppressive cytokines IL-10, TGFβ and IL-35 are all produced by Treg and therefore have received significant attention in investigations into Treg function. The secretion of suppressive cytokines provides a mechanism for a non-cell contact dependent Treg mediated suppression (Vignali et al., 2008). The specific role of IL-10 has proved controversial with opposing reports as to the importance of IL-10 in Treg suppressive function. Mouse models have shown the function of IL-10 to be important in transplantation tolerance (Hara et al., 2001) but not in diabetes (Balasa et al., 2000). Treg cell-specific deletion of IL-10 does not result in systemic auto-immunity, however it did result in enhanced pathology of the lungs following induced hypersensitivity (Rubtsov et al., 2008). Therefore it appears that the importance of IL-10 in Treg function depends on the disease or experimental system being investigated (Vignali et al., 2008).

TGFβ produced by Treg can either be membrane bound - and therefore act in a cell-cell contact dependent manner - or the TGFβ can be secreted acting in a contact independent mechanism. Similar to IL-10 there were opposing views as to the importance of TFGβ in Treg suppressive action. Early work suggested that TGFβ was not essential for Treg function as TGFβ knock-out mice and TGFβ blocking antibodies did not result in Treg dysfunction (Nakamura et al., 2001, Vignali et al., 2008, Piccirillo et al., 2002). Alternative studies demonstrated a crucial role for membrane bound TGFβ; membrane bound TGFβ on Treg has been shown to control the infiltration of CD8$^+$ cells into islets resulting in a delay in the progression of diabetes (Green et al., 2003). More recent reports have reported the ability of TGFβ to skew T cells away from effector functions towards a regulatory phenotype (Clayton et al., 2007), and TGFβ is commonly used for the generation of induced regulatory T cells both *in vivo* and *in vitro* (Vignali et al., 2008).

The inhibitory cytokine IL-35 contributes to Treg suppressive function; it is a hetero-dimeric cytokine member of the IL-12 family. IL-35 is up regulated in Treg cells which are actively supressing (Collison et al., 2007). Recombinant IL-35 is able to supress T cell proliferation in vitro and ectopic expression of IL-35 has been shown to be sufficient for suppressive capacity in naïve T cells (Collison et al., 2007).

1.5.2 Suppression by cytolysis

In addition to the production of suppressive cytokines Treg are able to supress through cytolysis of target cells through mechanisms more commonly associated with cytotoxic T lymphocytes (CTL) and NK cells (Lieberman, 2003). Treg are able to kill target cells through granzyme A and perforin (Grossman et al., 2004). Treg have been shown to prevent tumour clearance by CTL and NK cells by killing them in a granzyme/perforin dependent mechanism (Cao et al., 2007). In addition to target cell cytolysis by granzyme/perforin Treg are able to induce effector cell apoptosis through the TNF-related apoptosis-inducing ligand (TRAIL) DR5 pathway (Ren et al., 2007). The D5 ligand of TRAIL is the only ligand capable of transducing the apoptotic signal in humans and is thought to induce apoptosis on transformed cells, but spare normal healthy cells (Smyth et al., 2003).

1.5.3 Treg secretion/deprivation of cytokines-metabolic disruption

Imaging studies have shown that suppression can occur with no direct cell-cell contact (Tang and Krummel, 2006). Deprivation of cytokines provides a cell-cell contact independent mechanism for their action. A significant point of debate in the Treg field is the ability of Treg to starve their surroundings of IL-2 due to their high expression of the IL-2 receptor (CD25). Some studies have suggested that IL-2 deprivation is not a bona fide mechanism of action (Vignali et al., 2008) however other evidence suggests that it is possible for Treg to induce apoptosis in effector T cells due to cytokine deprival, in particular IL-2 (Pandiyan et al., 2007). Additional disruption of effector cell metabolism by Treg can occur through CD39/CD73 ectoenzyme interactions and the transmission of cAMP directly into effector cells through gap junctions (Vignali et al., 2008).

1.5.4 Treg suppression by modulation of dendritic cells

It is well established that Treg's have a central role in tolerance by acting directly upon effector T cells by a combination of the previously discussed mechanisms (Thornton and Shevach, 1998, Takahashi et al., 2000). However Treg have a close relationship with dendritic cells, and are also able to modulate immune responses through interactions with APC (Vignali et al., 2008). Treg are able to influence dendritic cell maturation and function through a variety of mechanisms including preventing the maturation of dendritic cells by

LAG-3-MHC class II interactions (Liang et al., 2008) and inducing the secretion of indoleamine 2,3-dioxygenase (IDO) which depletes local tryptophan, limiting T cell proliferation and survival (Grohmann et al., 2003). More detail of interactions between Treg and dendritic cells will be discussed in Chapter 5.

1.6 Treg stability and heterogeneity

Treg are able to influence the immune system through a variety of mechanisms and are crucial in the prevention of auto-immune disorders. Proof-of-concept studies and early stage clinical trials are showing promising results however concerns still remain as to the stability of Treg during cellular therapy. It has been suggested that under some inflammatory circumstances Treg become unstable and adopt an effector cell function and phenotype (Duarte et al., 2009). However, there are reports demonstrating notable stability even in highly inflammatory conditions (Rubtsov et al., 2010). General consensus is that under normal circumstances naturally occurring thymus derived Treg are stable and long lived due to many mechanisms ensuring maintenance of Foxp3 expression (Sakaguchi et al., 2013).

Within the $CD4^+CD25^+Foxp3^+$ population of Treg there is both functional and phenotypic heterogeneity (Shevach, 2006, Campbell and Koch, 2011). The Treg population can be separated into suppressive subsets which mirror the T-helper subsets based on chemokine receptor expression suggesting they are able to co-locate, which is important for the maintenance of tolerance (Duhen et al., 2012). Treg expressing the master transcription factor for Th1 cells T-bet and the chemokine receptor CXCR3 accumulate at the sites of Th1 inflammation (Koch et al., 2009). The Th2 specific differentiation factor IRF4 facilitates Treg control of Th2 mediated autoimmune responses (Zheng et al., 2009). Likewise expression of the Th17 transcription factor STAT3 is required for the control of Th17 responses (Chaudhry et al., 2009). In addition a subset of Treg expressing the lectin-like receptor $CD161^+$ has been shown to produce the pro-inflammatory cytokines IL-17 and IFNγ whilst remaining suppressive (Pesenacker et al., 2013). This heterogeneity of Treg occurs at a single-cell level, determined by single cell profiling, rather than lineage reprogramming (Dong et al., 2013). This adaptability and variation in Treg subsets has raised questions as to the stability of the Treg when faced with changing

microenvironments, particularly with the use of Treg in a clinical setting (Sawant and Vignali, 2014).

The stability of Treg requires the maintenance of Foxp3 expression. Foxp3 gene is seen to be the master regulator of self-tolerance, not only is it of importance in the development of Treg, but also in the function of Treg (Hori et al., 2003, Fontenot et al., 2003). Foxp3 is able to bind to the promoter of hundreds of genes and influence their expression, including promotion of its own expression through interactions with other transcription factors (Zheng et al., 2007). Foxp3 works with other transcription factors to establish the Treg phenotype and it is becoming clear that epigenetic changes are important in Treg lineage (Ohkura et al., 2012). Treg lineage commitment relies on de-methylation of a CpG island (CNS2 element) in the promoter region of the Foxp3 locus. Foxp3$^+$ Treg which are generated *in vitro* in the presence of TGFβ initially express high levels of Foxp3, however due to failure to de-methylate the CNS2 element these cells loose Foxp3 expression (Miyao et al., 2012). Therefore differences in the methylation status of this region between nTreg and iTreg are of importance, suggesting that it is the iTreg population which is unstable rather than the nTreg (Rudensky, 2011, Sakaguchi et al., 2013). Consequently the isolation of nTreg for use in a clinical setting is important to ensure they maintain their phenotype and function. Issues regarding identification and isolation of nTreg will be discussed in Chapter 3.

1.7 General aims and hypothesis

It was hypothesised that Treg are able to prevent GvHD without adversely affecting GvL. Hence the general aim of this study was to investigate the suppressive mechanisms by which Treg are able to modulate GvHD whilst assessing the influence of Treg on the GvL effect. This was to be done by:-

1. Optimising and validating methods for isolation, expansion and storage of suppressive Treg.

2. Assessment of whether Treg prevention of GvH tissue damage is associated with blocking of effector T cell migration.

3. Investigating whether Treg induced impairment of DC functions had an impact on cutaneous GvH reactions.

4. Investigation if Treg are able to impair GvL reactions and therefore impact on relapse using *in vitro* cellular assays and phenotypic analysis of patient samples post transplantation.

Chapter 2 - General Materials and Methods

2.1   Sample source

All human peripheral blood and skin samples were collected under approval of the local ethics committee with informed consent.

2.2   General cell culture

All cultured cells were incubated at 37ºC with 5% CO_2 in a humidified incubator (Flow Laboratories IR 1500 incubator). All cell culture was carried out in class II containment cabinets to minimise the risk of infection.

2.2.1   Isolation of PBMC

PBMC were isolated by density centrifugation from heparinised peripheral blood or leukocyte reduction system (LRS) cones using Lymphoprep™ (Axis-Sheilds). Whole blood was diluted 1:1 with phosphate buffered saline (PBS) and carefully layered over the Lymphoprep™ solution. Samples were then centrifuged at 800g for 20 minutes. Most of the top phase was discarded and PBMC removed from the interface with a sterile Pasteur pipette. PBMC were then washed in sterile PBS.

2.2.2   Cell counting

Cell viability was assessed by trypan blue (Gibco) dead cell exclusion dye. Cells were washed in appropriate media/PBS and re-suspended in a known volume; 20µl of cell suspension was then mixed 1:1 with trypan blue dye. Counting was performed using Improved Neubauer cell counting chamber (Weber Scientific International). Cells within five squares were counted and multiplied by 10^5 to determine the cell concentration per ml.

2.2.3   Cell culture media and buffers

General culture medium (RF10) contained Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamate and 10% Heat Inactivated Foetal Calf Serum (HI FCS) (Invitrogen). Fluorescence activated cell sorting (FACS) buffer consisted of PBS, 2% heat inactivated FCS and 1mM endotoxin free Ethylenediaminetetraacetic acid (EDTA) (Invitrogen). Magnetic activated cell sorting (MACS) buffer contained PBS supplemented with 1mM EDTA and 0.5%

HI FCS. Skin explant media consisted of RPMI 1640 supplemented with 100IU-ml penicillin, 100µg/ml streptomycin, 2mM L-glutamate and 20% human AB serum (Sera Lab).

2.2.4 Cryopreservation and thawing of isolated cell populations and PBMC

Isolated cell populations were cryo-preserved in freezing solution which contained 90% HI FCS (Sera Lab) and 10% dimethly sulphoxide (DMSO, Kock-light Ltd). Whole PBMC fractions were cryo-preserved in 70% RPMI, 20% HI FCS and 10% DMSO. All cells were dispensed into cryovials (Nunclon), wrapped in bubble plastic and stored at -80°C for 24 hours. Cells were then transferred to -140°C for long term storage. Thawing of the cells was carried out by gently thawing them in a 37°C water bath and washing twice with pre-warmed RF10. Cells were then counted as previously described and re-suspended in appropriate media at the correct concentration, depending on the requirements of the experiment.

2.3 Cell isolation/depletion

RosetteSep® (StemCell Technologies) and immuno-magnetic separation were used to isolate cell populations from PBMC. Methods used to isolate each cell population are summarised in Table 2.1

Table 2.1 Summary of methods used to isolate cell populations. PBMC was isolated by density centrifugation either from peripheral blood or leukocyte reduction system (LRS) cones.

Cell population	Samples source	Isolation method
CD8	LRS cone	RosetteSep®
CD4	LRS cone	RosetteSep®
Treg	LRS cone	RosetteSep® then RoboSep®
CD14	PBMC	Miltenyi MACS
CD4 naive	PBMC	RoboSep®
NK	PBMC	Miltenyi MACS

Tregs were isolated from LRSC in a two-step process; CD4$^+$ negative enrichment (StemCell Technologies RosetteSep® CD4$^+$ Human T cell enrichment cocktail) then RoboSep® automated selection of CD25^{Hi} (CD25 positive selection kits StemCell Technologies).

2.3.1 RosetteSep enrichment

RosetteSep® negative enrichment cocktail contains tetrameric antibody complexes which cross-link unwanted cells to red blood cells forming immuno-rosettes. The unwanted cells then pellet with the red blood cells when layered over Ficol and centrifuged at 1200g for 20 minutes at room temperature. This allows desired cells to be removed from the interface. This method is advantageous as the desired cells themselves are not labelled and it results in a high purity. A diagrammatic representation of the Rosettes formed by the tetramaric antibody complex between unwanted cells and red blood cells is shown in Figure 2.1.

2.3.2 RoboSep positive selection

RoboSep® cell isolation is an automated immuno-magnetic separation method where cells are labelled with a tetrameric antibody complex. RoboSep®

can be used for either positive selection or negative enrichment. The magnetically labelled cells are then separated from unlabelled ones using RoboSep® automated separation. A diagram of the tetrameric antibody complex used for the magnetic labelling of required cells is shown in Figure 2.1B.
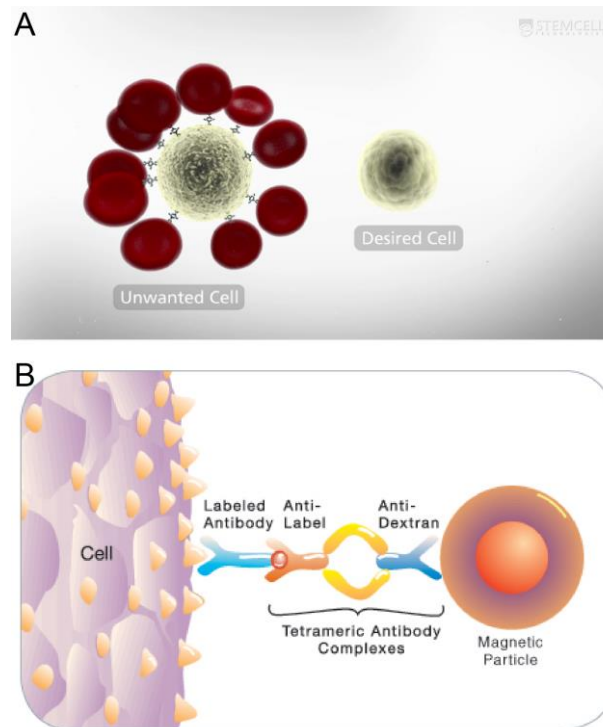
Figure 2.1 StemCell Technologies cell isolation.
A) RosetteSep® Negative enrichment cocktail B) CD25 positive selection cocktail.
Images from www.stemcell.com.

2.3.3 Miltenyi MACS separation

Miltenyi MACS separation is a column based immuno-magnetic separation technique. Cells are labelled with MACS MicroBeads then applied to a MACS column. Un-labelled cells pass through the column (negative fraction) while labelled cells remain in the column (positive fraction). After washing the columns can be removed from the magnet and the positive fraction eluted. This technique can be used for positive cell isolation as well as depletion of unwanted cells.

2.4 In vitro cell culture

2.4.1 Expansion of Treg

Isolated Treg were expanded for up to 3 weeks, unless otherwise stated, in RF10 supplemented with additional 500IU/ml IL-2 (Roche), 100nM Rapamycin (Sigma-Aldrich) and CD3/CD28 Dynabeads® T cell expander (Invitrogen). Initially the ratio of Beads:Cells was 3:1, this ratio was reduced to 1:1 after Treg numbers had begun to increase, usually days three-seven after isolation. Prior to use, Treg were detached from the Dynabeads using EasySep® magnet (Stemcell Technologies).
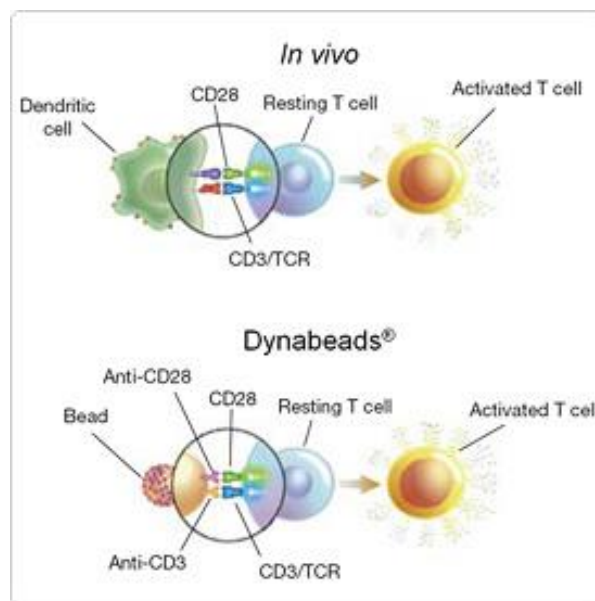
Figure 2.2 T cell activation by Dynabeads®. Picture from Invitrogen.com.

2.4.2 Generation of moDC

Monocyte-derived dendritic cells were generated using CD14$^+$ MACS isolated cells. CD14$^+$ cells were cultured in 24 well plates at 5×10^5 cells/ml for 7 days in RF10 supplemented with IL-4 (50ng/ml, ImmunoTools) and GM-CSF (50ng/ml, ImmunoTools). After three days half the media was replaced with fresh media. Mature moDCs were generated by the addition of 0.1µg/ml LPS for the final 24 hours of culture.

2.5 Flow Cytometry

Flow cytometry is a technique which is used for multi-parametric analysis of cells, including information about cell size, cell type, complexity and viability. Flow cytometers are made up of a fluidics system to present the cells and remove the waste; lasers to generate fluorescence; optics and detectors to gather and detect the light, and a computer system to collect and store the data.

Cells are passed through the flow cytometer by means of hydrodynamic focusing ensuring that only one cell is analysed at once. Laser beams are aimed at this stream of cells which then scatter. Side scatter and forward scatter are used to assess the granularity and size of cells respectively. Larger, more complex cells generate a higher side scatter and forward scatter than smaller less granular cells.

The addition of antibodies labelled with flourophores can identify specific markers either on the cell surface or within the cell. Each flourophore has a characteristic excitation and emission spectra allowing several to be used at once for multi-parameter analysis. When several flourophores are being used together compensation is required, as the flourophores may have slightly overlapping excitation and emission spectra resulting in a false elevation of the signal. Compensation is easily achieved through the use of single colour stained cells or compensation beads.

Unless otherwise stated for staining of cell surface markers 1×10^{5}-1×10^{6} cells were re-suspended in FACS buffer and incubated with appropriate concentrations of antibodies at 4°C for 20 minutes. Table 2.2 contains a complete list of antibodies used. Cells were then washed with FACS buffer and 300µl FACS buffer was added prior to flow cytometry analysis. All flow cytometry data was collected on a BD Canto II flow cytometer and data analysed with FlowJo software, version 7.4 (TreeStar).

Table 2.2 Antibodies used with fluorochrome, isotype, clone and supplier. Summary table of antibodies used showing the marker, fluorochrome, isotype, clone and supplier.

Marker	Flourochrome	Species	Isotype	Manufacturer	Clone
7AAD				BD	
CCR10	PE	Rat	IgG2a	R&D	314305
CCR7 (CD197)	PEcy7	Rat	IgG2a	BD	3D12
CD107a	PE	Mouse	IgG1	BD	H4A3
CD127	Brilliant Violet	Mouse	IgG1	Biolegend	AD19D5
CD14	FITC	Mouse	IgG2b	BD	MφP9
CD16	FITC	Mouse	IgG1	BD	NKP15
CD1a	APC	Mouse	IgG1	BD	HI149
CD25	APC	Mouse	IgG1	BD	M-A251
CD40	APC	Mouse	IgG1	BD	5C3
CD25	FITC	Mouse	IgG1	BD	2A3
CD25	PE	Mouse	IgG1	BD	2A3
CD25	PEcy7	Mouse	IgG1	BD	2A3
CD3	PerCPcy5.5	Mouse	IgG1	BD	SK7
CD4	FITC	Mouse	IgG1	BD	SK4
CD4	V500	Mouse	IgG1	BD	RPA-T4
CD4	Pacific Blue	Mouse	IgG1	BD	RPA-T4
CD45RA	V450	Mouse	IgG2b	BD	HI100
CD56	APC	Mouse	IgG1	BD	B159
CD69	PEcy7	Mouse	IgG1	BD	FN50
CD8	APC	Mouse	IgG1	BD	SK1
CD8	APCcy7	Mouse	IgG1	BD	SK1
CD8	PerCPcy5.5	Mouse	IgG1	BD	SK1
CD80	PE	Mouse	IgG1	BD	L307.4
CD83	FITC	Mouse	IgG1	BD	HB15e
CD86	PEcy7	Mouse	IgG1	BD	FUN-1
CLA	FITC	Rat	IgM	BD	HECA-452
CTLA-4	PE	Mouse	IgG2a	eBioscience	14D3
CXCR3	APC	Mouse	IgG1	BD	1C6/CXCR3
Foxp3	APC	Rat	IgG2a	eBioscience	PCH101
GranzymeB	FITC	Mouse	IgG1	BD	GB11
Helios	PE	Hamster	IgG	BioLegend	22F6
HLA-ABC	FITC	Mouse	IgG1	BD	G46-2.6
HLA-DR	PerCP	Mouse	IgG2a	BD	L243
IFNγ	PE	Mouse	IgG2b	BD	25723.11
IL-10	PE	Rat	IgG2a	BD	JES5-16E
IL-17a	PE	Mouse	IgG1	BD	N49-653
IL-2	APC	Mouse	IgG1	BD	5344.111
IL-4	APC	Mouse	IgG1	BD	8D4-8
LAP(TGFβ)	PE	Mouse	IgG1	R&D	27232
NKG2D	PerCPcy5.5	Mouse	IgG1	BD	1D11
Perforin	PE	Mouse	IgG2b	BD	δG9

2.5.1 Phenotypic assessment of Treg

Unless otherwise stated Treg purity was assessed by flow cytometry using antibodies CD3 PerCPcy5.5, CD4 FITC, CD25 PE, CD127 Brilliant Violet and Foxp3 APC (Antibody supplier and clone shown in Table 2.2). Treg were defined as $CD4^+CD25^{Hi}CD127^{Low}Foxp3^+$. Surface staining was carried out as previously described. Cells were then washed with FACS buffer and then intracellular Foxp3 staining was carried out according to manufacturer's instructions (eBiosciences Fix/Perm staining kit).

2.5.2 Assessment of intra-cellular cytokine production

Intra-cellular (IC) staining for cytokine production was carried out by stimulating cells with phorhol 12-myristate 13 acetate (PMA) (20ng/ml) and Ionomycin (500ng/ml) (Sigma) in RF10 and incubating at 37°C for one hour. Brefeldin A (10µg/ml) (BD Biosciences) was added and incubated for a further three hours. Cells were then washed in FACS buffer, and then fixed and permebalised using eBioscience Fix/Perm staining kit. Cells were blocked with normal mouse serum (Dako) and stained with appropriate antibodies then washed in perm buffer for flow analysis.

2.5.3 Flow cytometry to measure activation markers

Markers CD69 and CD25 were used to assess the level of activation of $CD8^+$ T cells by flow cytometry. Early activation marker CD69 was assessed 24 hours after stimulation by allogeneic moDC. The late activation marker CD25 was measured five days following allo stimulation.

2.5.4 CBA analysis of culture supernatants

As well as detailed phenotypic analysis of cells, flow cytometry is a technique able to assess soluble factors present in serum or culture supernatants. Cytometric bead assay (CBA) is a particle based immunoassay which is able to measure levels of multiple soluble proteins in supernatants or serums. The assay involves the 'capture' of the soluble protein of interest by a capture bead (Figure 2.3A). A PE-conjugated antibody forms a complex with the protein and capture bead; it is this complex which is detected. Each detection bead population has a discrete fluorescence intensity which is assigned an alpha-numeric position (Figure 2.3B). Comparison to a standard curve allows

the ability to use several of these kits in a multiplex situation and allow for accurate measurement of the concentration of several soluble factors simultaneously. Culture supernatants were collected and analysed using CBA Flex sets, included in Figure 2.3C, according to manufacturer's instructions (BD Biosciences). Data was analysed using BD FCAP array software to calculate the protein concentration in culture supernatants.
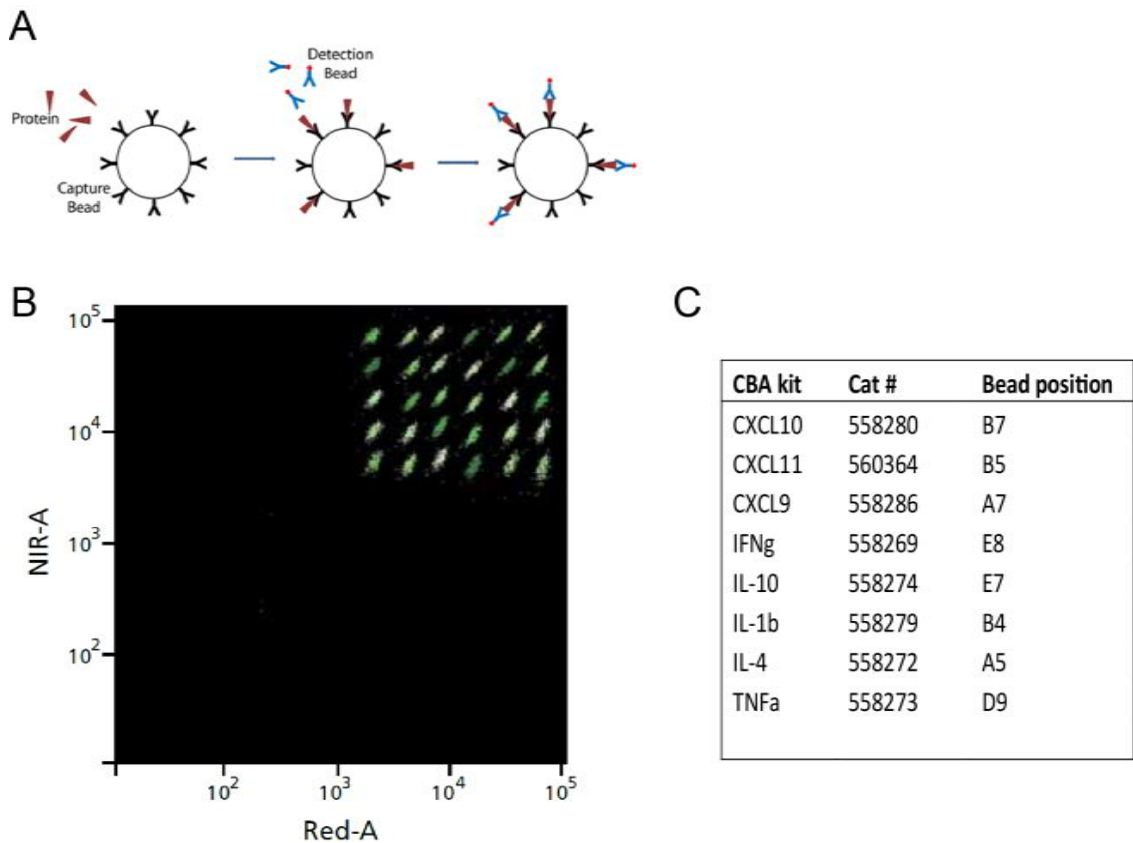
Figure 2.3 Diagram of the process of a CBA assay.
A) The protein of interest is captured on a bead. Detection beads are then added which bind to the protein forming a complex. This complex has a distinct fluorescence intensity which is assigned an alpha-numeric position.
B) Alpha-numeric positions of all the beads as they appear after flow cytometry. Mean fluorescence intensity is calculated and compared with the standard curve in order to calculate protein concentration. Image from www.bdbiosciences.com.
C) Summary table of CBA kits used, including bead position.

2.6   Assessment of Treg suppressive properties

Mixed lymphocyte reactions (MLRs) were used to assess the suppressive properties of Treg. CD8$^+$ T cells were stimulated with allogeneic moDC, either in the presence or absence of autologous Treg. A minimum of three conditions were set up for each MLR: CD8$^+$ T cells alone (1x10^6 cells per ml), CD8$^+$ T cells plus moDC (1x10^6 CD8 cells per ml and 1x10^5 moDC cells per ml) and thirdly CD8$^+$ T cells with moDC and Treg (1x10^6 CD8 cells per ml, 1x10^5 moDC cells per ml and between 6.25x10^4-5x10^5 Treg per ml). The ability of the Treg to suppress the activation and proliferation of CD8$^+$ T cells was measured using flow cytometry for activation markers CD69 and CD25 as well as 3H-thymidine incorporation and carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution.

Table 2.3 Cell concentrations used for MLR. MLR reactions were cultured in RF10 with 25IU/ml IL2 added.

	CD8$^+$	moDC	Treg
CD8	1x10^{6}cells/ml	-	-
+moDC	1x10^{6}cells/ml	1x10^{5}cells/ml	-
+2:1 Treg	1x10^{6}cells/ml	1x10^{5}cells/ml	5x10^5 cells/ml
+4:1 Treg	1x10^{6}cells/ml	1x10^{5}cells/ml	2.5x10^5 cells/ml
+8:1 Treg	1x10^{6}cells/ml	1x10^{5}cells/ml	1.25x10^5 cells/ml
+16:1 Treg	1x10^{6}cells/ml	1x10^{5}cells/ml	6.25x10^4 cells/ml

2.6.1   3H-thymidine incorporation

Radioactive 3H-thymidine incorporation is a method used to measure the proliferation of cells. Each time a cell replicates it incorporates radioactive thymidine and this can then be analysed to give a measure of proliferation. MLR reactions were set up in 96 well U-bottomed plates with a range of Treg concentrations (Table 2.3). After five days the cells were harvested using micromate 196 cell harvester (Packard) onto a filter. Cells were pulsed with 3H-thymidine 16 hours prior to harvesting. The filter was left to dry at room temperature for 24 hours and assessed for their level of proliferation by sealing in a plastic pocket with scintillation fluid (PerkinElmer) and counting radioactivity using a Betaplate TriLux counter (PerkinElmer). The whole process was carried out following all local rules on the safe use of radioactive material.

2.6.2 CFSE dilution

CFSE dilution was used to measure the proliferation of allo-stimulated cells. Briefly CD8$^+$ T cells were labelled with CFSE on day 0. Cells were labelled by incubating with 1µM CFSE in PBS for 5 minutes in the dark and then washed 3 times in RF10. Labelled cells were then used to set up MLR reactions as previously described. After 5 days proliferation was assessed using flow cytometry.

2.7 Skin explant assay

The skin explant assay was originally developed to aid the prediction of developing GvHD following allo HSCT. It was first described by Vogelsang *et al* in 1985 and has been widely used since as an effective tool to study the immuno-biology of GvHD and predict the outcome of allo-HSCT (Vogelsang et al., 1985, Wang et al., 2009, Dickinson et al., 1998, Wang et al., 2006, Kim et al., 2011). Briefly, "donor" PBMC are sensitised to host cells by means of a MLR. The cells are then co-cultured with small sections of recipient skin and then analysed for histologic changes associated with GVHD.

This method has been modified to detect the capacity of Treg to prevent allo-reactive CD8$^+$ T cell induced tissue damage in the skin and is summarised in Figure 2.4. MLR were set up with CD8$^+$ T cells, allo-stimulated (CD8+moDC) and allo-stimulated in the presence of Treg (CD8+moDC+4:1 Treg). After seven days the cells were harvested and were subjected to MACS CD4 depletion (Miltenyi Biotech) to remove any remaining moDC (which are CD4^{low}) and any Treg, leaving only the allo stimulated CD8$^+$ T cells, allo stimulated CD8$^+$ T cells in the presence of Treg and the control CD8$^+$ un-stimulated T cells. These cells were then re-suspended in skin explant media (RPMI 1640 supplemented with 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamate and 20% human AB serum). One million CD8$^+$ T cells from each condition were placed into a 96 well U-bottomed plate with a small piece of skin. The assay was then incubated at 37°C for three days when the skin was either stored in RNA later (Ambion) or fixed in formalin then embedded in paraffin wax. Sections were stained with haematoxylin and eosin (H&E) by the department of Cellualr Pathology Royal Victoria Infirmary, Newcastle UK. Remaining MLR cells and supernatants were

stored for future use. The damage to the skin was graded according to the criteria described by Lerner *et al* 1974.

A
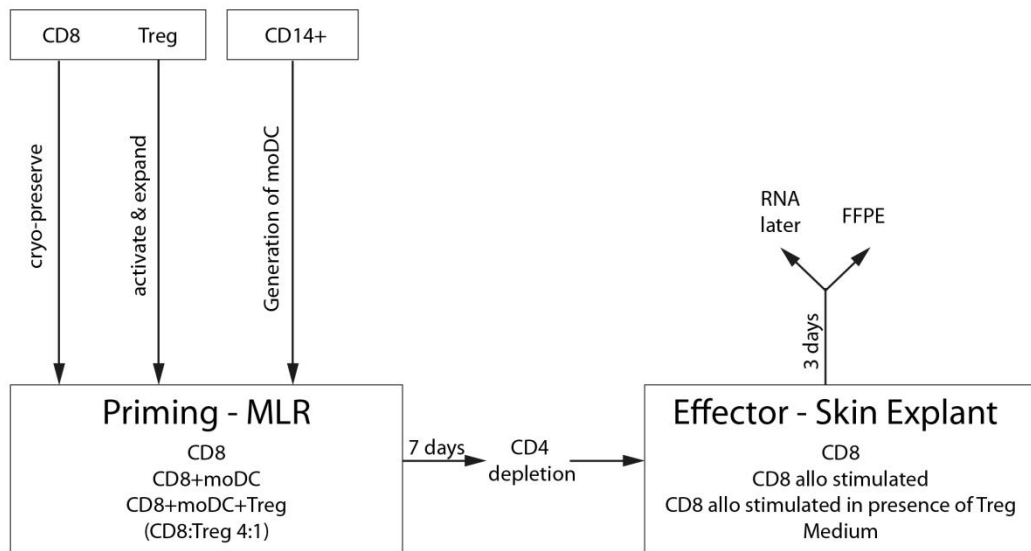
B
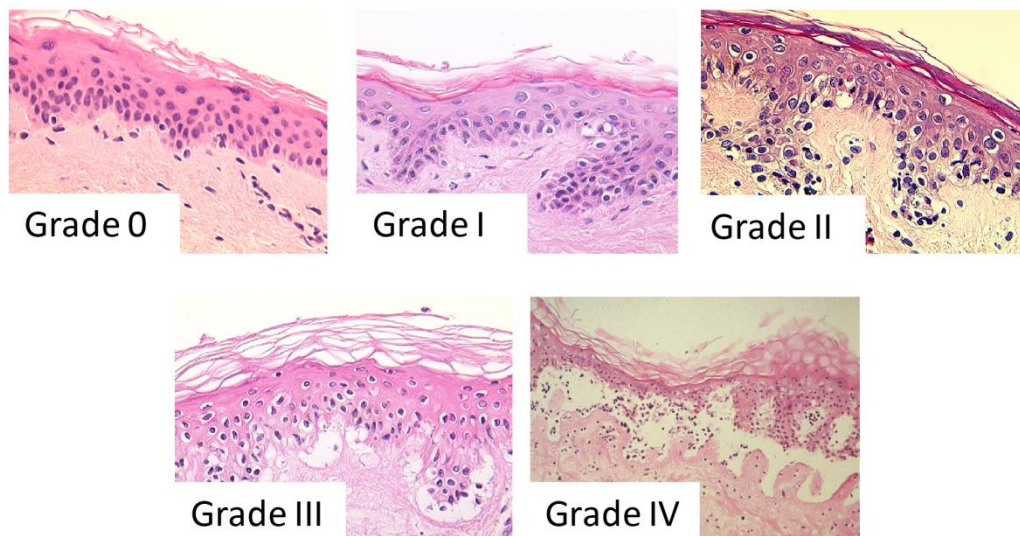
Figure 2.4 Skin Explant assay methods.
A) Diagram to summarise the methods used in the modified skin explant assay. B) Images of skin sections following skin explant; Grade 0 is normal healthy skin; Grade I shows very mild vacuolization of basal cells; Grade II has diffuse vacuolization of basal cells with dyskeratotic bodies; Grade III has cleft formation between dermis and epidermis; Grade IV demonstrates complete separation of the dermis & and the epidermis (Lerner et al., 1974).

2.8 Gene expression

Total RNA was extracted from cryo-preserved cell pellets using Qiagen RNeasy mini kit, following manufacturer's instructions. RNA purity and quantity was assessed using NanoDrop (NanoDrop®, ND-1000). Complementary DNA (cDNA) was generated by reverse transcription of the RNA using an equal volume of cDNA master mix containing random hexamers (Thermo Scientific), dichloro-diphenyl-trichloroethane   (DTT) (Invitrogen), first strand buffer (Invitrogen), dNTP (Roche Diagnostics) RNAsin (Promega) and reverse transcriptase, MMLV-RT (Invitrogen). The reaction mix was incubated at 37°C for 80 minutes then 65°C for a further 10 minutes. The final cDNA concentration was estimated to be half the concentration of the RNA, assuming 100% efficiency of cDNA generation. Once generated the cDNA was stored at -20°C until used for quantitative real-time PCR using Taqman® gene expression assays.

Taqman® gene expression assays use a fluorogenic probe to detect PCR products as they are generated. The probe sets, which have a fluorescent dye on the 5' end and a quencher dye on the 3' end, anneal downstream from one of the primer sites. Prior to DNA polymerase acting the quencher dye reduces the fluorescence from the reporter dye by the use of fluorescence resonance energy transfer (FRET). As Taq DNA polymerase extends the primer it displaces and cleaves the 5' reporter dye, increasing the fluorescence signal. Taq polymerase then continues to the end of the template strand completely displacing the probe. During each cycle additional reporter molecules are cleaved from the quencher molecules meaning the fluorescence intensity is proportional to the amount of amplification. Taqman® gene expression assays were used to measure expression of several genes of interest, summarised in Table 2.4. Reactions were carried out in triplicate where possible using 7900HT Fast Real Time PCR System and standard thermal cycling conditions (Applied Biosystems). C_t values were normalised to the reference gene GAPDH using the comparative C_T threshold method.

Table 2.4 Primer/probe sets used for qPCR.

Gene of interest	Primer/Probe set
GAPDH	Hs99999905_m1
CXCR3	Hs00171041_m1
IFNγ	Hs00989291_m1
SELPG/CLA	Hs00380945_m1
CD80	Hs00175478_m1
CD86	Hs01567026_m1
MARCH1	Hs00215631_m1
TBX21	Hs00203436_m1
GATA3	Hs00231122_m1
Foxp3	Hs01085834_m1
RORC	Hs01076122_m1

2.8.1 Calculation of RQ values

Relative quantification (RQ) is used to describe the change in gene expression in relation to a reference group. When analysing MLR gene expression the CD8 cultured alone were used as the reference group. C_t is the cycle number where the number of amplified targets crosses the threshold. The C_t was normalised (ΔCt) by subtracting the C_t of the reference gene from the test gene C_t.

$$RQ = 2^{-(\Delta Ct\ of\ test\ group-\ \Delta Ct\ of\ reference\ group)}$$

Where test group is either CD8+DC or CD8+DC+Treg and reference group is CD8 only (Livak and Schmittgen, 2001).

2.9 Statistical analysis

All statistical analysis was performed using Graphpad Prism™ software. Either Mann-Whitney U tests or paired t tests were used with a p value of less than 0.05 being considered significant.

2.10 Phenotypic analysis of patient PBMC

Serial samples of patient peripheral blood were taken 7 days prior to HSCT then at regular intervals post transplantation under approval of the local ethics committee. PBMC were isolated by density centrifugation and stored as previously described. Samples were stored where possible 7 days prior to transplant, then 28 days, 3 months, 6 months, 9 months and 12 months post transplantation. Samples were then thawed and stained with either panel A and B or panel C alone (Table 2.5). Sample data was collected with Canto II and analysed using FlowJo (TreeStar). Patient characteristics are shown in Table 2.6

Table 2.5 Patient PBMC staining panels.

	Panel A	Panel B	Panel C
FITC	CLA	CD4	CLA
PE	CD4	CCR10	CCR10
PerCPcy5.5	CD3	CD3	CD3
PEcy7	CD25	CD25	CD25
APC	CXCR3	Foxp3	CXCR3
APCcy7	CD8	CD8	CD8
Brilliant violet 450/V450	CD45RA	CD45RA	CD127
V500	-	-	CD4

Table 2.6 Patient characteristics.

Number	30
Median age	48 (range 20-67)
Sex	
Male	12
Female	18
Diagnosis	
Acute lymphoblastic leukaemia	2
Acute myeloid leukaemia	14
Non-Hodgkins lymphoma	6
Hodgkins lymphoma	4
Myelodysplastic syndrome	2
Chronic myelogenous leukaemia	1
Chronic Granulomatous disease	1
Reduced Intensity Conditioning	
Yes	23
No	7
Stem Cell Source	
PBSC	24
BM	6
aGvHD Grade	
0	13
I	2
II	12
III	3
cGvHD	
Yes	15
No	15
Relapse	
Yes	8
No	22
Donor Type	
MUD	25
SIB	5

Chapter 3 - Isolation, Expansion and Cryo-preservation of suppressive Regulatory T cells

3.1 Introduction

One significant barrier to the use of Tregs in a clinical setting is their low number in peripheral blood. The *ex vivo* expansion of regulatory T cells has therefore been important for both development of their use in a clinical setting and functional *in vitro* studies. This chapter aims to show the successful isolation and expansion of suppressive Treg for their use in *in vitro* studies and discuss issues relevant to the clinical translation of Treg therapy. This chapter provides the basis for the entire thesis, validating the methods used to isolate and expand Treg *ex vivo* and characterising the Treg both phenotypically and functionally.

3.1.1 In vitro identification and isolation of Treg

The gold standard identifier for Treg is the transcription factor Foxp3 although its intracellular location disqualifies it's use as an isolation marker in human studies. In murine studies it is possible to sort Treg based on Foxp3 expression using GFP-Foxp3 mice (Simonetta et al., 2012). Tregs do all express CD4 and CD25 so consequently this combination of markers has formed the basis of many commercially available isolation kits for Treg (Sakaguchi et al., 1995). However Treg isolation based on these two markers does not exclude the selection of other highly activated T cells along with Treg especially in an inflammatory situation. Liu *et al* (Figure 3.1) reported that the brightest 2% of CD25 cells were more than 90% Foxp3$^+$ but looking at the brightest 5%, 10% and 15% there was variation between individuals (Liu et al., 2006). It is therefore not possible to rely fully on CD4$^+$CD25$^+$ selection to achieve high Foxp3$^+$ purity. However, selecting only the cells which expressed the highest levels of CD25 could result in a population of cells with a high percentage of Foxp3 expression. There are other surface markers which are constitutivelty expressed on Treg such as, HLA-DR, GITR, CTLA-4 and CD45RA however, they are also present on other cell lineages and may not be expressed by 100% of Treg, so unless used with a combination of other markers they are of little use in the identification and isolation of Treg.
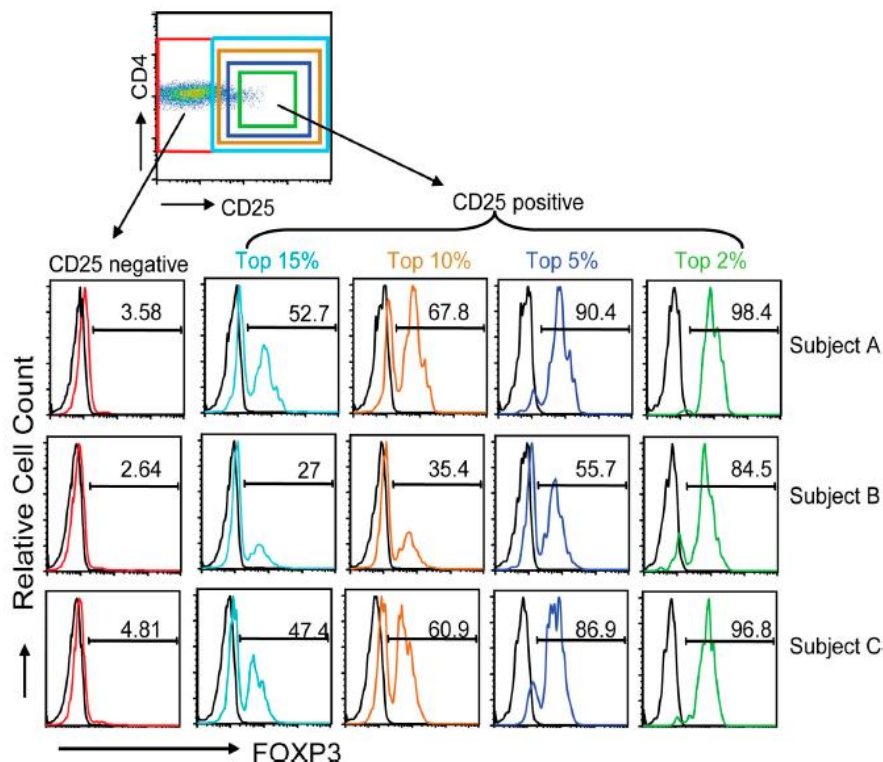
Figure 3.1 Foxp3 expression correlates with CD4 cells expressing high levels of CD25 (Liu et al., 2006).

Also of relevance in the isolation of Treg is the absence, or low expression, of cell surface markers. The IL-7 receptor α chain, CD127 can be used as a negative marker of Treg and its expression inversely correlates with Foxp3 expression and Treg suppressor activity (Liu et al., 2006). Therefore CD127^{low} could be used as an additional marker to aid in the identification and separation of Treg cells (Seddiki et al., 2006). More recently CD26^{low} has been suggested as a marker for Treg. CD26 is a serine protease which is expressed at high levels of activated T cells and low levels on Treg which may aid in the isolation of Treg in inflammatory situations (Salgado et al., 2012). As an alternative to CD4 positive selection, CD8 and CD19 depletion, followed by CD25 positive selection has been reported, resulting in a GMP population of Treg suitable for use in clinical trials (Di Ianni et al., 2012).

Undoubtedly there are several potential combinations of markers which are capable of isolating Treg from whole blood, leukapheresis products or other cell/tissue sources and the required use, yield and purity of isolated Treg must be taken into consideration when deciding which markers and methods to use for Treg isolation.

3.1.2 In vitro activation and expansion of Treg

Due to the low number of Treg in peripheral blood it is often necessary to *ex vivo* expand them prior to use. Several methods for the expansion of Treg have been reported over the years. In 2001 Levings *et al* showed that it was possible to expand human Tregs *in vitro* using exogenous IL-2 (40U/ml) and feeder cells (Levings et al., 2001). This study stimulated Treg with anti-CD3 and used a cocktail of irradiated allogeneic cell types as feeder cells similar to one described in the 1980s by van de Griend to expand allo-specific cytotoxic T lymphocytes (van de Griend and Bolhuis, 1984). In a move away from feeder cells and towards a more clinical grade Treg product in 2004 Hoffman *et al* described the successful use of CD3/CD28 T cell activator beads to activate and expand Treg. Initially cells were cultured at 4 beads per cell, but then reduced to 1 bead per cell, in the presence of high dose (300IU/ml) IL-2 up to 40 000 fold expansion could be achieved without the need for feeder cells. (Hoffmann et al., 2004).

Earle *et al* also demonstrated that *in vitro* expansion using anti CD3/CD28 beads was possible without the loss of suppressor function (Earle et al., 2005). This study cultured Treg in media supplemented with much higher concentration of recombinant human IL-2 (2000 IU/ml) and anti CD3/CD28 beads at a 1:1 ratio. They reported a lag in expansion for the first week, sometimes even seeing cell death, but after this initial lag phase cells expanded well. Reporting cell death in the early stages of expansion is not seen in any other studies which use an increased bead number and then reduce the number of beads per cell after the cells have begun to expand.

A 2006 study by Hoffmann *et al* suggested that only naive $CD4^+CD25^{Hi}$ Treg ($CD45RA^+$) were able to maintain high purity during expansion, they used 300U/ml recombinant IL-2 with either CD3 and CD28 antibodies or T cell expander beads coated with antibody, and suggested RA- Treg were more likely to lose Foxp3 expression and have less suppressive properties (Hoffmann et al., 2006). It was shown by the same group that upon repetitive *in vitro* stimulation $CD4^+CD25^+$ Tregs lose Foxp3 expression, in particular memory-type Treg (Hoffmann et al., 2009). Prevention of this loss of Foxp3 expression was to be the next advance in methods to expand Treg *ex vivo*.

In 2007 Strauss *et al* described the use of rapamycin (sirolimus) in Treg expansion culture to preferentially select for Tregs and prevent the loss of Foxp3 expression (Strauss et al., 2007). Rapamycin is a powerful immunosuppressive reagent, blocking signalling in response to the T cell growth factor IL-2 (Abraham and Wiederrecht, 1996). The exact mechanism by which Treg preferentially expand is still unknown however it is believed that rapamycin induces apoptosis in TCR-activated T cells but Treg appear to be resistant. In conventional T cells the PI3K/mTOR survival signalling pathway is blocked by rapamycin leading to activation induced apoptosis (Zeiser et al., 2008). In addition the presence of rapamycin leads to differential expression of anti-apoptotic Bcl-2 family of proteins – levels are increased in Treg and down regulated in conventional T cells leading to an increase in Bax and subsequent apoptosis (Strauss et al., 2009). In this study the optimal rapamycin concentration was decided to be 1nM. They stated any more than 10nM decreased expansion rates, and less than 1nM had no desirable effect, however this data was not reported. This study also used high concentration exogenous recombinant human IL-2 (1000IU/ml). They also confirmed that phenotypic characteristics were unaffected by rapamycin, measuring expression of CD25, Foxp3, CD62L, GITR, CTLA-4, CCR7, FAS and HLA-DR. Battaglia *et al* showed that, after proving rapamycin expanded murine Treg, it also promoted the expansion of human Treg (Battaglia et al., 2006).

There are undoubtedly several factors which need to be taken into consideration for the *in vitro* culture of Treg. Within our lab a Treg expansion method has been developed which takes into account several aspects of the studies previously mentioned (Wang et al., 2009). This method uses high concentration IL-2 (500IU/ml), 100nM Rapamycin and CD3/CD28 T cell expander beads, initially 3 beads per cell but reduced to 1 bead per cell once cell numbers began to increase (Wang et al., 2009).

3.1.3 The effect of cryo-preservation on Treg

The effects of cryo-preservation on the suppressive properties of Treg is becoming of more importance with the increasing use of cryo-preserved Treg in clinical trials (Brunstein et al., 2011). Despite the interest in the use of cryo-preserved Treg for clinical use, little is known about the effects of cryo-preservation on their potency. The ability to cryo-preserve Treg for use in *in vitro* studies would also be highly beneficial as it would allow for more experimental flexibility and efficient use of resources. To date no comprehensive study has tested the effects of cryo-preservation on the suppressive capacity of Treg.

Cryo-preservation of PBMC is commonly used for subsequent analysis, however many studies have reported cryo-preservation to be detrimental to some subpopulations of cells (Golab et al., 2013). A study by Constantini *et al* reported a comparison of PBMC from HIV patients and healthy controls prior to and following cryo-preservation. They suggested that cryo-preservation could affect surface marker expression and also the proportion of cells within the PBMC. In the healthy controls they reported a decline in central memory (CD45RO$^+$CD62L$^-$) and naïve (CD45RA$^+$CD62L$^+$) cells and an increase in effector CD8$^+$ cells (Costantini et al., 2003). This study made no mention of the proportion of Treg but raised questions as to the effects of cryo-preservation on cell populations within PBMC.

In 2009 Elkord reported a decrease in the proportion of Treg following cryo-preservation (Elkord, 2009). This study only investigated the percentage of cells, no assessment of their suppressive capacity was carried out and only 6 healthy volunteer samples were used. A letter in direct response to this paper was then published stating that they had observed no such decrease in Treg percentage following cryopreservation and that this difference could be caused by differences in methodology (Van Hemelen et al., 2009). Despite differing results in the effect of cryo-preservation on Treg it was generally agreed that it was necessary to keep protocols consistent, so if a particular study used cryo-preserved samples then it was important to always use previously cryo-preserved samples.

There is little in the literature as to the effects of cryo-preservation on the suppressive properties of isolated Treg. Peters *et al*, in 2008, reported successful isolation and expansion of clinical grade Treg; they also tested the suppressive capacity of Treg following cryo-preservation. It was observed that freshly isolated Treg required both a recovery period and expansion following thawing in order to retain their suppressive capacity; Treg which were expanded prior to cryo-preservation did not require a recovery period and were highly suppressive upon thawing (Peters et al., 2008). However in this study they only looked at the Treg capacity to prevent 3H thymidine incorporation of autologous naive $CD4^+CD25^-$ cells. It is therefore necessary to test the effects of cryo-preservation on the suppressive effects of Treg prior to routinely using them for further *in vitro* experiments.

The specific aims of this chapter were to:-

- Optimise and validate a method for *in vitro* isolation and expansion of Treg.
- Monitor the suppressive properties of Treg throughout *ex vivo* expansion.
- Investigate the effects of cryo-preservation on Treg ability to mediate suppression of T cell allo-responses.
- Compare the suppressive properties of donor or third party derived Treg in the context of HSCT.

3.2   Methods

3.2.1   Cell isolation and culture

The isolation of CD8$^+$ cells, isolation and expansion of Treg and the generation of moDC was carried out as described previously (Chapter 2). Treg were cryo-preserved as previously described in 90% FCS and 10% DMSO.

3.2.2   Expansion rate and culture conditions for Treg

Treg were expanded as previously described. Calculation of cell yield and assessment of Treg stability were assessed by maintaining Treg in expansion culture for up to 5 weeks with regular cell counts and Foxp3 staining to assess purity. Culture medium was changed every 2-7 days when required. Treg were also expanded either in the presence or absence of rapamycin.

3.2.3   The effect of cryo-preservation on Treg

A summary of the methods used to test the effects of cryo-preservation is shown in Figure 3.2. Briefly detailed phenotypic analysis of Treg was carried out by flow cytometry prior to and following cryo-preservation. In order to ensure that the cryo-preservation of Treg did not affect their ability to suppress allo-reactive CD8$^+$ T cells Treg were tested fresh, following expansion, and then again after being cryo preserved for a minimum of 7 days. Suppressive capacity was assessed by measuring allo-stimulated CD8$^+$ proliferation, activation, cytokine production and the extent of GvH reaction using the modified skin explant assay.

3.2.4   Use of third party Treg to suppress GvH reactions

A summary of the methods used to test the potency of third party Treg in ameliorating GvH reactions is shown in Figure 3.3 CD8$^+$ T cells were allo-stimulated as previously described, either in the presence of Treg from the same donor as the CD8$^+$ T cells, or from a third party donor. The CD8$^+$ T cell response to allo-stimulation was measured using CD8$^+$ T cell activation and proliferation. In addition third party Treg were tested in the skin explant model, briefly responder PBMC were incubated with irradiated stimulator PBMC either in the presence or absence of third party Treg for 7 days. The responder PBMC were then incubated with small sections of skin (from the stimulator donor) which was then graded for cutaneous GvH damage as previously described.
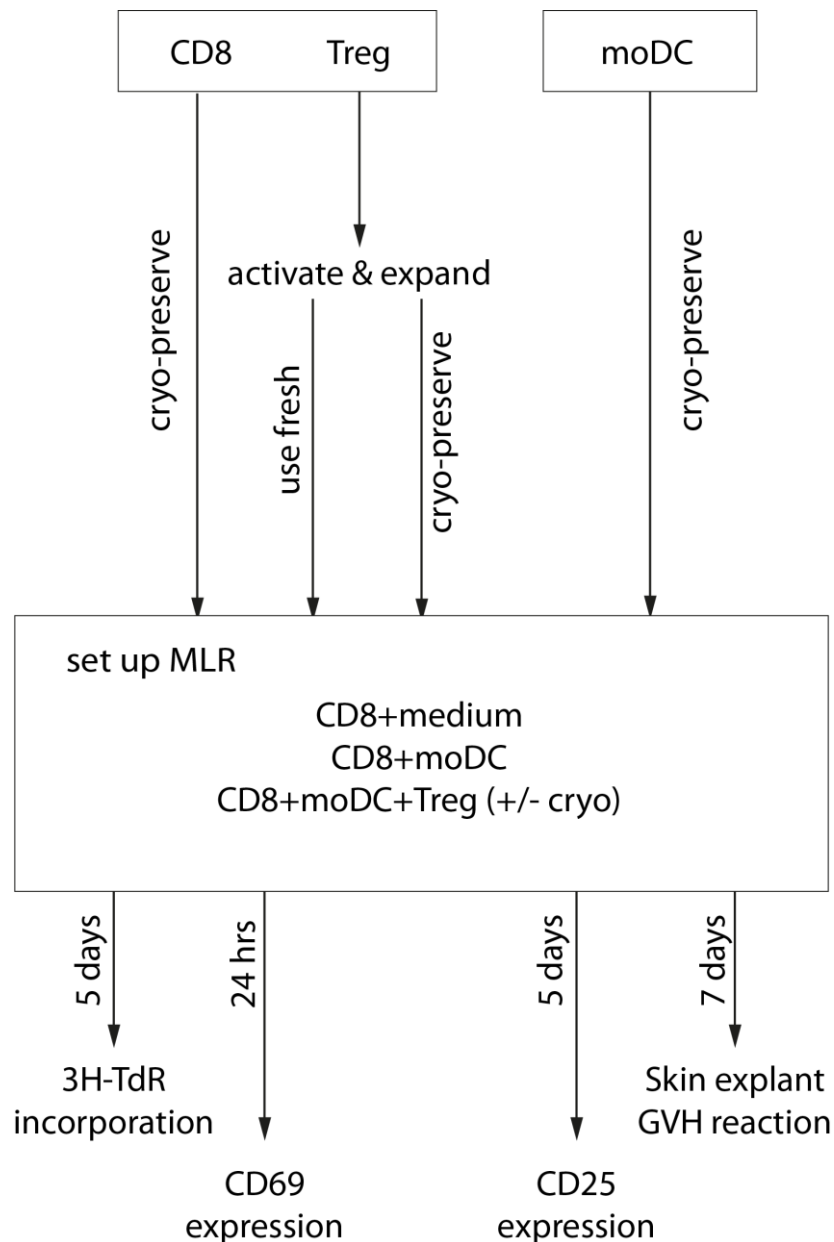
Figure 3.2 The effects of cryo-preservation on Treg function.
Cells were isolated from LRS cones. Some Treg were used fresh following expansion, other aliquots were cryo-preserved in 90% FCS 10% DMSO for a minimum of 7 days prior to use. Treg were added into MLR with ratios of 2:1, 4:1, 8:1 and 16:1 for proliferation assays and 2:1 and 4:1 for flow cytometry to measure activation markers. CD8^{+} T cell proliferation was measured by 3H-thymidine (3H-TdR) incorporation on D5. Activation markers were measured by flow cytometry (CD69 after 24 hours and CD25 after 5 days). Skin explant GVH reaction was carried out as previously described; briefly CD8+ cells were isolated from MLR on D7 by CD4 depletion of Treg and any remaining moDC. CD8 cells were then incubated with small sections of skin (from the stimulator) for 3 days, which were then routinely fixed and H/E stained then graded according to the Lerner scale (Lerner et al., 1974).
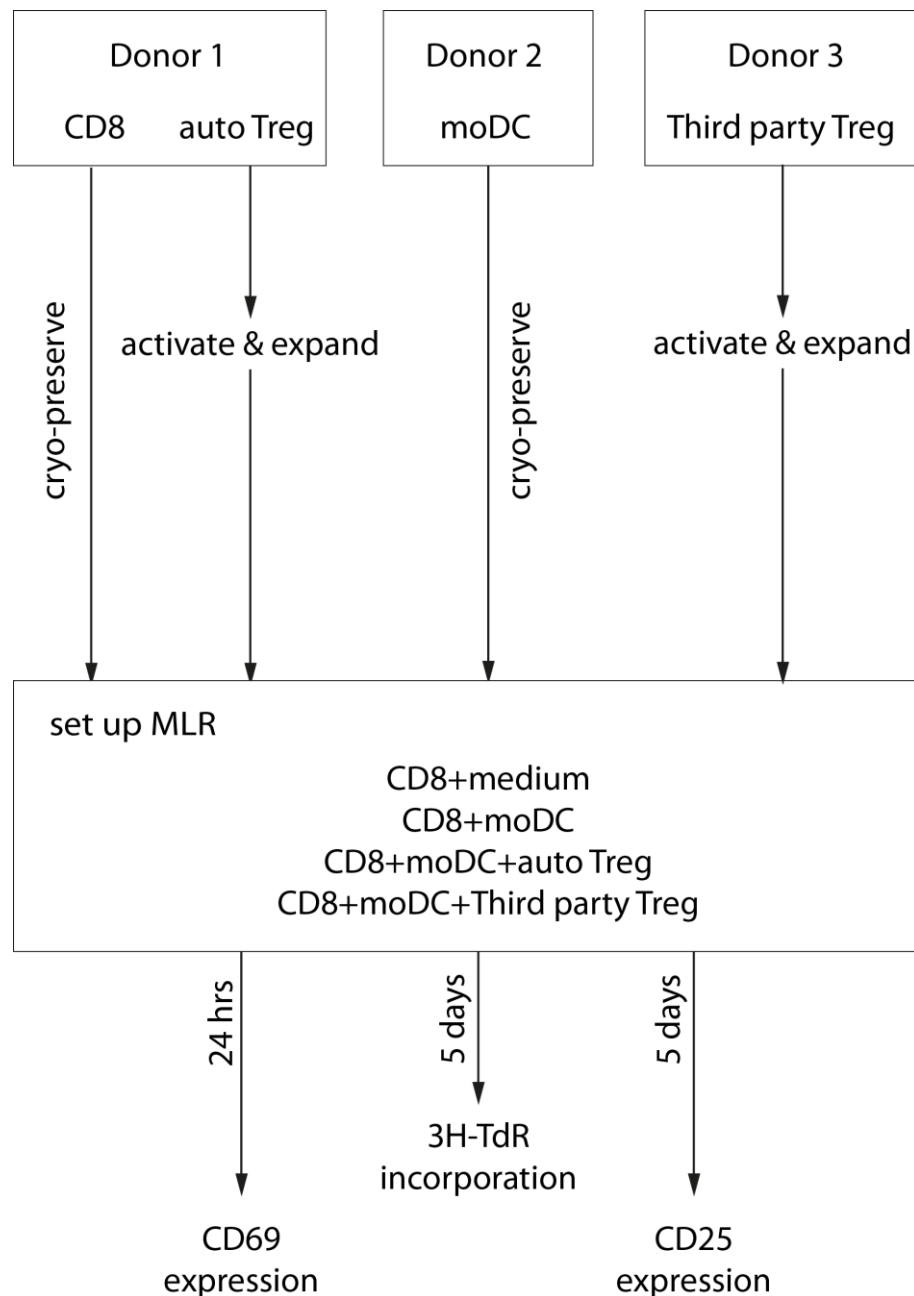
Figure 3.3 The use of third party Treg to supress GvH reaction.
Cells were isolated from LRS cones. CD8+ T cells and Treg were isolated from one donor, moDC were generated from a second donor and Treg isolated from the third donor. Treg were activated and expanded prior to use. Either autologous or third party Treg were added into mixed lymphocyte reactions at ratios of 4:1, 8:1 and 16:1. CD8 T cell activation was measured after 24 hours by expression of CD69, and after 5 days by expression of CD25. 3H-TdR incorporation on D5 was used to assess the capacity of Treg to supress CD8+ T cell proliferation.

Acknowledgements

3.3 Results

Data included in this chapter has been published as a letter to the journal Transplantation 95;e68-70 (2013) Do cryopreserved regulatory T cells retain their suppressive potency? (Mavin et al., 2013).

3.3.1 *Isolation and Expansion of Regulatory T cells*

Treg were successfully isolated from LRS cones following CD4 negative enrichment and CD25^{Hi} positive selection. The average Treg purity upon isolation, identified by Foxp3 expression, was 81.9%±2.17 SEM, rising to 90.7%±1.52 SEM following expansion (Figure 3.4 & Figure 3.5).
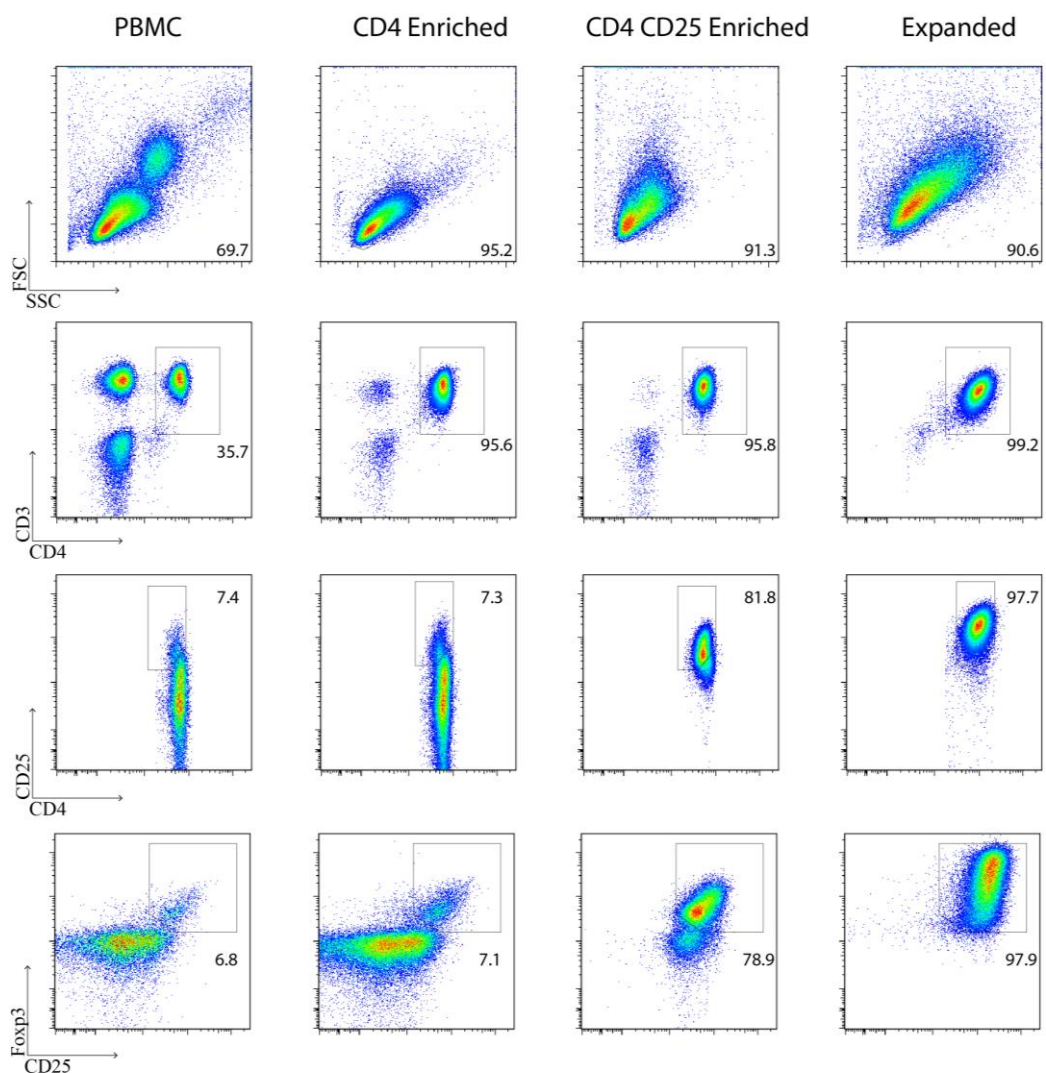
Figure 3.4 Successful isolation of Treg from LRS cones.
Representative plots from multi-parameter flow cytometry analysis (n=35). Data shown includes analysis of PBMC before isolation, CD4 enriched lymphocytes, CD4 CD25^{Hi} enriched cells and Treg after activation and expansion. Treg purity was calculated by gating on lymphocyte populations based on FSC and SSC then the CD3$^+$CD4$^+$ population. Within the CD4$^+$ population both the CD4$^+$ CD25^{Hi} and the the CD25^{Hi}Foxp3$^+$ populations are shown. Appropriate isotype controls were used to determine gate position.

3.3.2 Rapamycin is required to maintain Foxp3 expression

To ensure that Treg were successfully expanded Foxp3 purity was routinely checked throughout expansion. Treg were expanded in RF10 with exogenous IL-2 and T cell expander beads. In the presence of rapamycin Treg increased in purity (p=0.0015) and maintained Foxp3 expression of over 90% for up to 3 weeks in expansion culture and over 70% for up to 4 weeks (Figure 3.5). In the absence of rapamycin during expansion culture Treg purity decreased rapidly. This is consistent with the findings by Strauss *et al* (Strauss et al., 2007). This data confirms that the Treg isolation and expansion protocol used results in a highly pure population of Treg which were stable in expansion culture and therefore suitable for further use in *in vitro* studies.
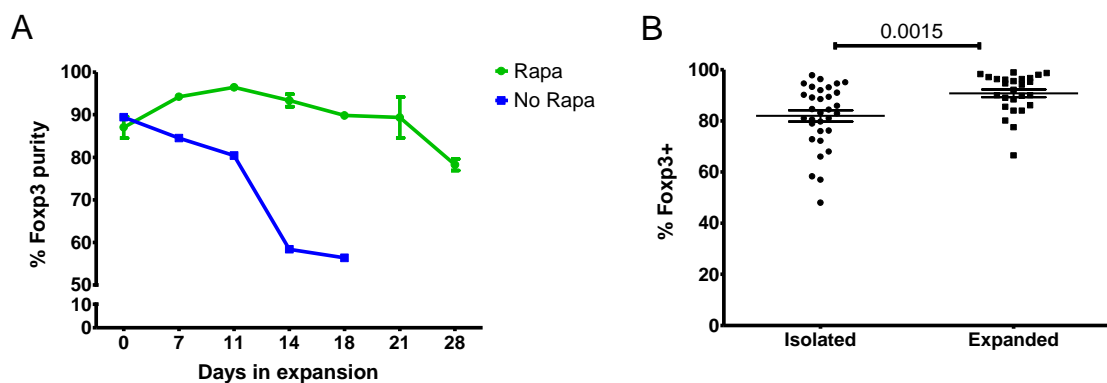
Figure 3.5 Rapamycin maintains Treg phenotype during expansion.
A. Rapamycin is required to maintain Treg purity; upon isolation purity was 80.6%. Purity increased to >95% during week 1 of expansion in the presence of rapamycin (n=1-26).
B. Purity of Treg upon isolation then following expansion in the presence of rapamycin (n=26) purity significantly increased from 81%±2.17 to 90%±1.52, p=0.0015.

3.3.3 Expression of Helios in freshly isolated/expanded Treg

Commonly the transcription factor Helios is used to differentiate between natural and induced Treg. It has been reported that naturally occurring thymus derived Treg express Helios and Treg induced in the periphery do not express Helios (Thornton et al., 2010). However, this appears to be controversial since there have been reports of induced Treg expressing Helios *in vitro* (Gottschalk et al., 2012). In the absence of any other known marker to differentiate between them Helios was used in this study to assess the proportion of induced and naturally occurring Treg during expansion. The percentage of Helios[+] Treg seen

was in line with what is previously reported in the literature (Thornton et al., 2010) and it remained consistent throughout Treg expansion (Figure 3.6).
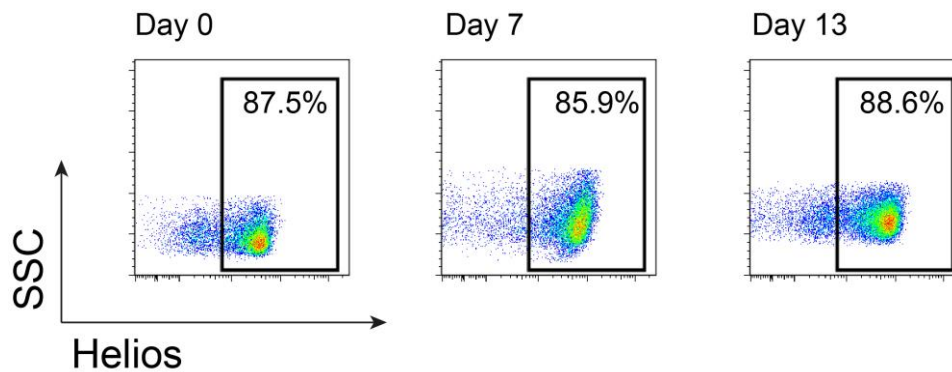
Figure 3.6 Helios expression in Treg cells throughout expansion culture.
Representative plots (n=2). Prior to plots shown, gates were applied on live cells and Foxp3$^+$ Treg. Gate position was determined using the appropriate isotype control.

3.3.4 *Expansion rate of isolated Treg*

The cell number and phenotype of Treg were monitored throughout expansion by cell counting and IC Foxp3 staining. Over 3 weeks of expansion culture, in the presence of IL-2, rapamycin and CD3/CD28 beads, cell numbers increased by an average of 57 fold ±18.6 SEM. Trypan blue dead cell exclusion dye was used to monitor cell viability (Figure 3.7).
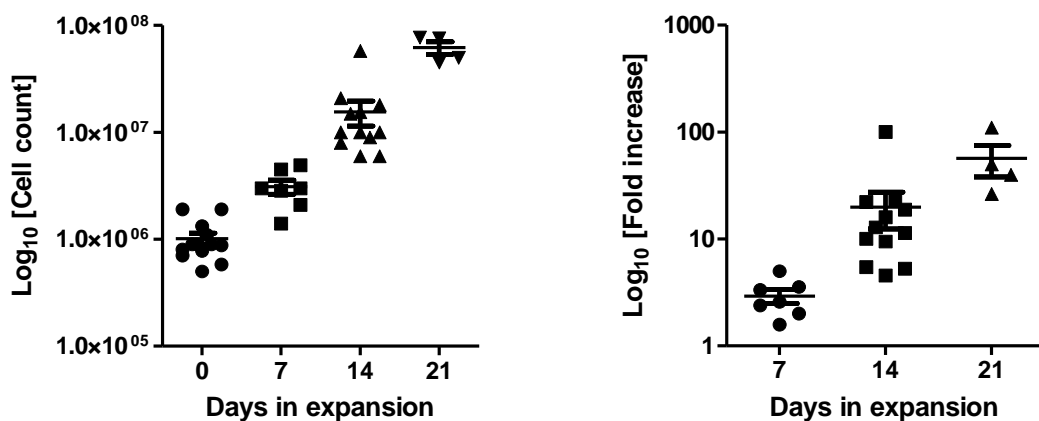
Figure 3.7 Expansion of cultured Treg.
Growth curve is shown for Treg expanded for up to 3 weeks (n=4-13).

3.3.5 Treg prevent allo-moDC stimulated CD8⁺ T cell proliferation in a dose dependent manner

Mixed lymphocyte reactions (MLR) were used to assess the suppressive ability of Treg. As this study was to focus on the potential beneficial properties of Treg in ameliorating GVHD following HSCT allo stimulated CD8$^+$ T cells were chosen to test Treg suppressive properties due to the prominent role of CD8$^+$ cells in the pathophysiology of GVHD.

Treg suppression of CD8$^+$ T cell proliferation was measured using 3H-TdR incorporation (Figure 3.8), after 4 days the MLR were pulsed with 3H-TdR for a further 16 hours. The count per minute (cpm) recorded by CD8$^+$ T cells alone was considered to be the background level. It was shown that Treg were able to suppress proliferation by allo-stimulated CD8$^+$ T cells to a level not significantly different from background levels when used at a ratio of 2:1 CD8:Treg ($p=0.100$). As the number of Treg in the MLR decreased, to a ratio of 16 CD8 to 1 Treg, the proliferation of CD8$^+$ T cells increased but always remained significantly lower than allo-stimulated CD8$^+$ T cells in the absence of Treg ($p=<0.0001$).
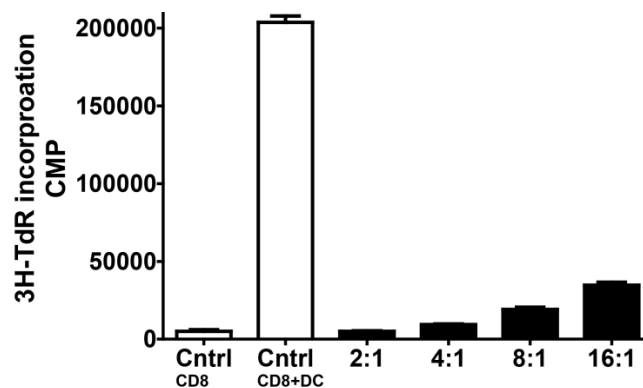
Figure 3.8 Treg prevent allo-stimulated CD8$^+$ T cell proliferation in a dose dependent manner.
3H-TdR incorporation was used to measure CD8$^+$ T cell proliferation; un-stimulated CD8$^+$ T cells and allo-moDC stimulated CD8$^+$ T cells acted as negative and positive controls respectively (□). Autologous Treg were present in a range of CD8:Treg ratios (■). Suppression was seen to be greater with higher concentrations of Treg. Representative data is shown (n=5), triplicate MLR wells were analysed for each experiment (as indicated by error bars).

3.3.6 Treg inhibit allo-moDC stimulated CD8$^+$ T cell activation

Activation of CD8$^+$ T cells following allo-moDC stimulation results in increased expression of activation markers CD69 and CD25, measured by flow cytometry (Figure 3.9). The early activation marker CD69 increased in expression 24 hours after allo-stimulation from 2.40%±0.59SEM to 13.1%±1.34SEM, but in the presence of Treg (at a CD8:Treg ratio of 4:1) this early activation was reduced (only 6.36%±1.09SEM expression). After 5 days expression of CD25 increases from 0.34%±0.04SEM to 31.6%±2.77SEM, but was again suppressed in the presence of Treg (4.97%±0.94SEM). This data, along with Treg prevention of allo-moDC stimulated CD8$^+$ T cell proliferation, confirms that the expanded Treg are highly suppressive and suitable for use in further studies.
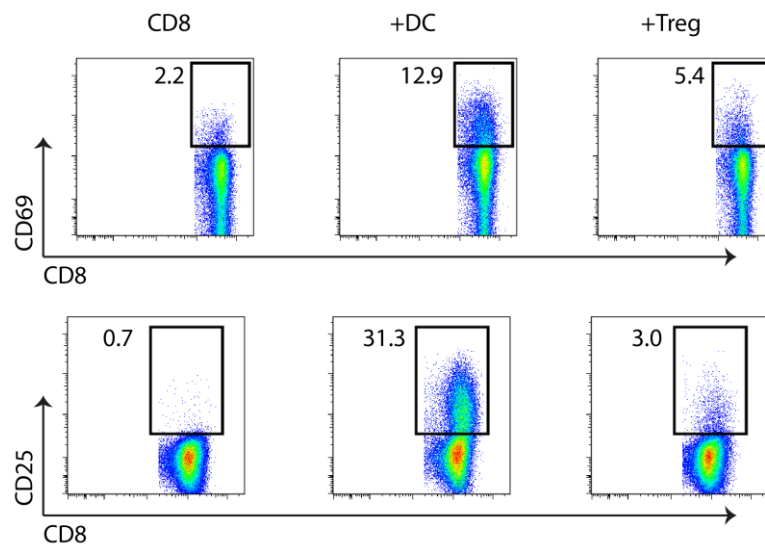
Figure 3.9 CD69 and CD25 suppression by Treg on allo-stimulated CD8$^+$ T cells.
Cells were gated on lymphocytes based on FSC SSC and then CD8$^+$ T cells prior to representative plots shown. MLR were used to assess CD8$^+$ T cell activation, CD8$^+$ T cells alone were considered to be background expression and allo-moDC stimulated CD8$^+$ T cells either in the presence or absence of autologous Treg at a ratio of 4:1 (n=5).

3.3.7 Treg retain their suppressive properties in expansion culture

The suppressive capacity of Treg was measured at regular time points following isolation using 3H-TdR incorporation to ensure it was maintained throughout expansion. Suppressive capacity of the Treg increased following 1 week of expansion culture, falling slightly in weeks 2 and 3 with a more marked decreased when Treg were expanded for more than 4 weeks (Figure 3.10). The decrease in suppressive capacity was more apparent when less Treg were present (8:1 and 16:1 CD8:Treg ratios).

The decrease in suppressive capacity of the Treg corresponded with a decrease in Foxp3 purity of the Treg (Figure 3.10 shown in red). This suggests that while the Treg express high levels of Foxp3 they will remain suppressive for several weeks in expansion culture and are therefore suitable for use in further *in vitro* studies. Because of the decrease in suppressive capacity associated with the decline in Foxp3 expression further functional studies were carried out using Treg expanded for only 2-3 weeks, with close monitoring of Foxp3 expression levels.
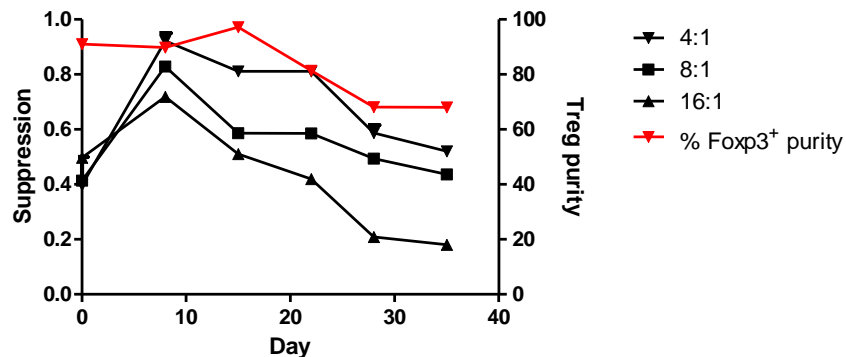
Figure 3.10 Association of Treg Foxp3$^+$ purity and their suppressive potency.
CD8$^+$ T cells were allo-moDC stimulated in the presence of 4:1 Treg (▼) 8:1 Treg (■) or 16:1 Treg (▲). Treg were freshly isolated and expanded and suppressive potency as assessed at 5 time points. Proliferation was measured by 3H-TdR incorporation. Levels of Treg suppression were calculated by subtracting background cpm (CD8$^+$ T cells alone) and dividing by the positive control (CD8$^+$T cells allo-stimulated with moDC). Values were then subtracted from 1 to give a suppression value. Each data point represents mean of 3 replicates in one experiment. Treg Foxp3$^+$ purity is shown in red (▼).

3.3.8 Isolation based upon CD4$^+$CD25^{Hi} is sufficient for suppressive Treg

As previously described the use of CD4$^+$CD25$^+$ to isolate Treg may not be sufficient to achieve high purity, particularly in an inflammatory situation. Two commercially available Treg kits were compared, based upon purity following isolation, purity of expanded cells, expansion capacity and ability to suppress allo-stimulation in a MLR. Both kits, obtained from Stemcell Technologies, relied on CD25^{Hi} immuno-magnetic positive selection following a negative RossetteSep enrichment. One kit uses CD4$^+$ enrichment and the other CD4$^+$CD127$^-$ enrichment.

The purity of freshly isolated Treg was not significantly different between the two kits (Figure 3.11) and following activation and expansion of this isolated Treg the purity remained comparable between cells isolated by both kits; 96.47%±0.56SEM for CD4$^+$CD25^{Hi} and 97.3%±0.91SEM for CD4$^+$CD127$^-$ CD25^{Hi}. In addition to the purity of the cells the number isolated was investigated. From the same starting number the CD4$^+$CD25^{Hi} selection resulted in a higher yield than the CD4$^+$CD127$^-$CD25^{Hi} selection (Figure 3.11). Throughout expansion the growth rate of Treg isolated by both methods was comparable, however the final cell number was much greater for the CD4$^+$CD25^{Hi} selected cells due to the higher starting number (p=0.049).
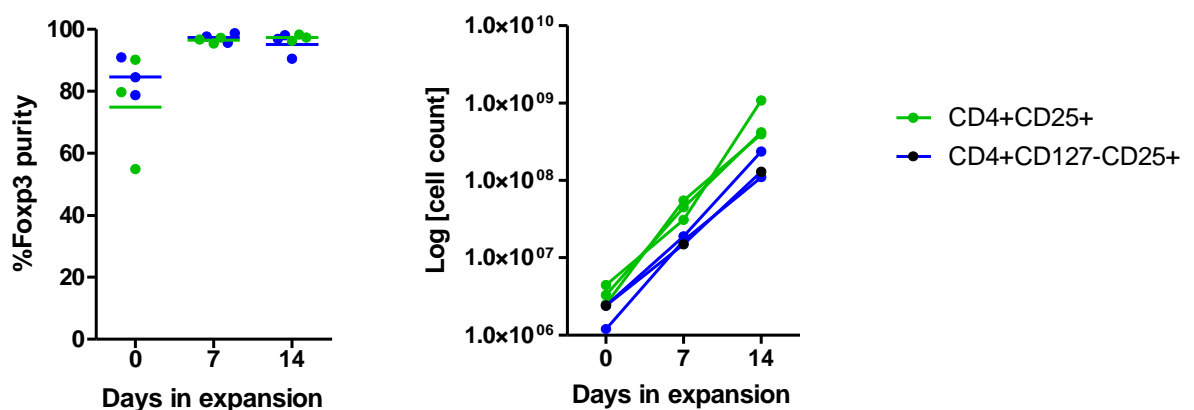
Figure 3.11 Selecting Treg by CD4$^+$CD25^{Hi} results in greater yield without a reduction in purity following expansion.
Foxp3 purity was assessed by flow cytometry and cell number was measured by cell counting using Trypan blue dead cell exclusion dye at regular points throughout Treg expansion.

To ensure both populations of Treg had equally potent suppressive capacity the ability of the cells to supress allo-stimulation of CD8$^+$ T cells was assessed. The ability of the Treg to prevent allo-stimulated CD8$^+$ T cell proliferation, measured by CFSE dilution, and activation by expression of CD25 and CLA was comparable between CD4$^+$CD25^{Hi} Treg and CD4$^+$CD127$^-$CD25^{Hi} Treg (Figure 3.12). This supports current literature therefore was only carried out once.
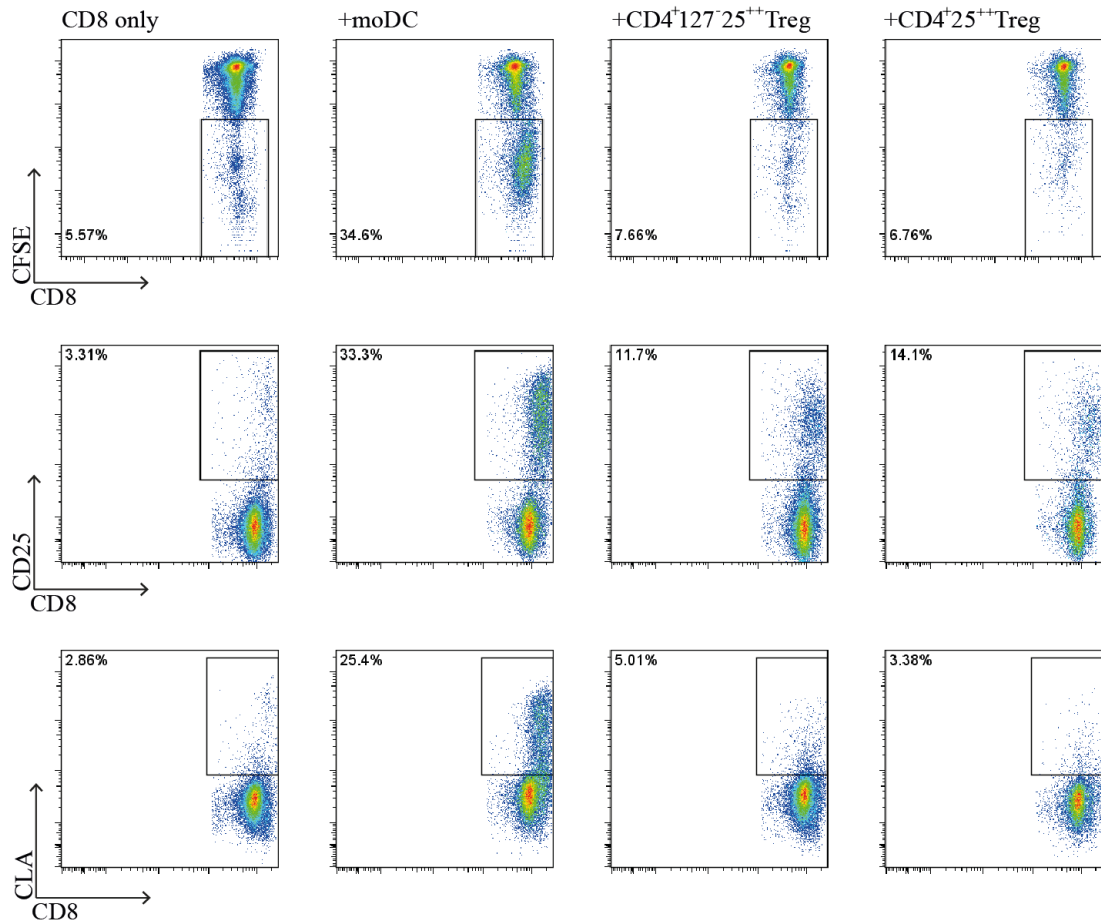
Figure 3.12 Treg suppression of allo-stimulated CD8$^+$ T cell activation and proliferation was comparable between CD4$^+$CD25^{++} and CD4$^+$CD127$^-$CD25^{++} isolation methods.
Prior to plots shown live cells were selected (n=1).

3.3.9 Cryopreservation of Treg has no effect on their phenotype, expansion capacity and suppressive function

In order to ensure that previously cryo-preserved Treg could be used in further experiments the phenotype and suppressive properties were tested prior to and following cryo-preservation. Cryopreservation was shown via several means to have no effect on the ability of Treg to prevent activation and proliferation of allo-stimulated CD8$^+$ T cells. Firstly 3H-TdR incorporation (shown in Figure 3.13) was used to demonstrate that there was no difference in the prevention of allo-stimulated CD8$^+$ T cell proliferation depending on whether the Treg were cryo-preserved or not (p>0.05). To ensure the robustness of this test, matched pairs of Treg with and without cryo-preservation were used in parallel tests.
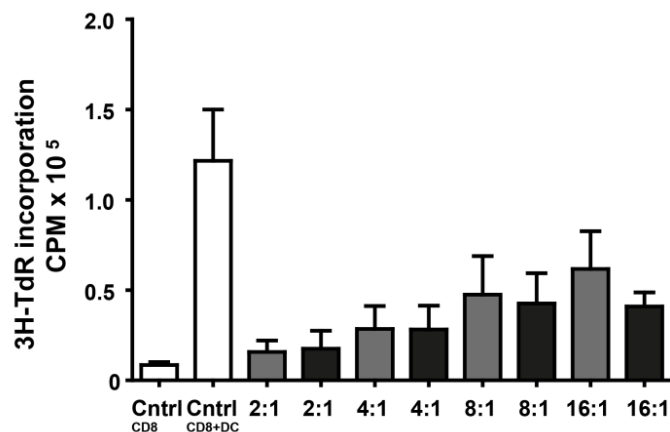
Figure 3.13 Treg dose dependant suppression of CD8$^+$ T cell proliferation is unaffected by cryo-preservation of Treg prior to MLR.
Proliferation was measured using 3H-TdR incorporation by allo-moDC stimulated CD8$^+$ T cells in the presence of freshly expanded (▭) or cryo preserved (▬) Treg. Un-stimulated CD8$^+$ T cells and allo-stimulated CD8$^+$ T cells (▢) served as negative and positive controls. Error bars indicate mean±SEM of 9 individual tests of 3 independent experiments.

Secondly suppression of expression of activation markers and skin homing markers on CD8^{+} T cells by Treg was also unaffected by cryopreservation (Figure 3.14). The skin homing markers CLA and CCR10 were chosen because of their particular relevance to cutaneous GvHD (Tsuchiyama et al., 2009, Faaij et al., 2006). Suppression of the early activation marker CD69 (Figure 3.14a) and the late activation marker CD25 (Figure 3.14b) was comparable between freshly expanded and cryo-preserved Treg at a range of ratios. Treg suppression of CLA expression on the allo-stimulated CD8^{+} T cells was above 85% regardless of whether the Treg were cryo-preserved or not (Figure 3.14c). Cryo-preserved Treg inhibition of CCR10 appeared lower; however, this difference was not statistically significant (Figure 3.14d).
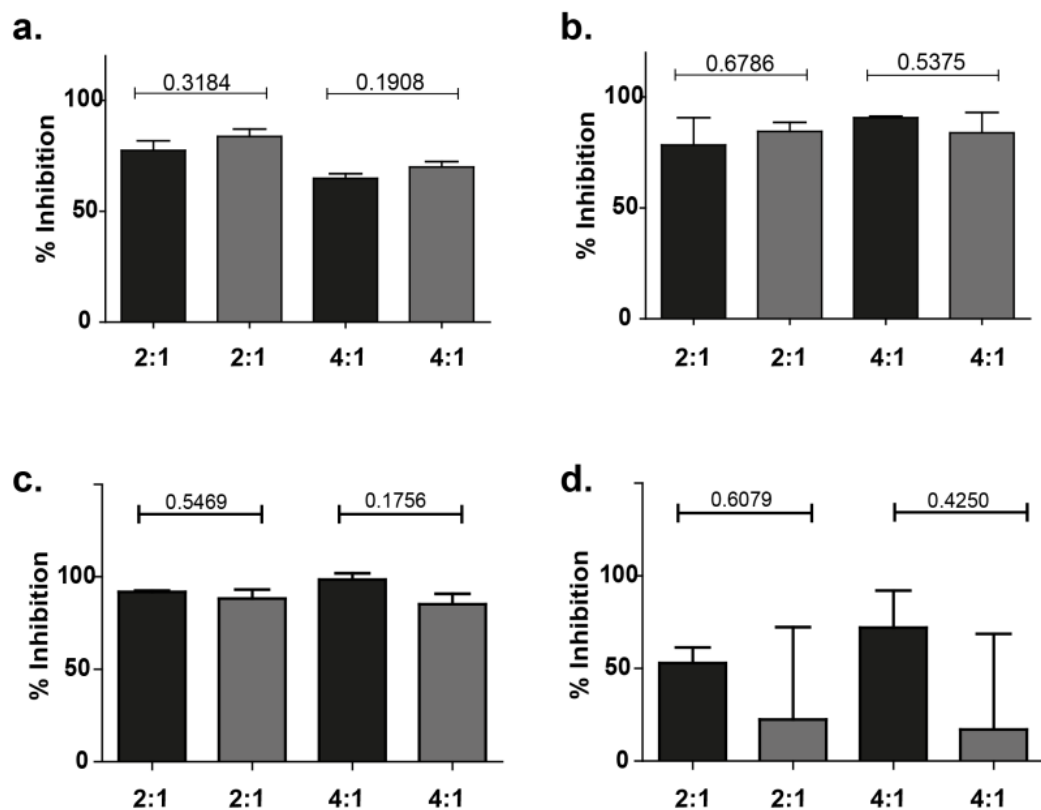
Figure 3.14 Suppression of expression of activation and skin homing on CD8^{+} T cells is unaffected by cryo-preservation of Treg.
Percentage inhibition of activation and skin homing markers by CD8^{+} T cells in the presence of freshly expanded (◼) or cryo preserved (▭) Treg. Inhibition was calculated using un-stimulated CD8^{+} T cells (background levels) and allo-stimulated CD8^{+} T cells (in the absence of Treg) as reference samples. Error bars indicate mean±SEM a) Suppression of CD69 expression on CD8^{+} cells by Treg after 24 hours. b) Suppression of CD25 expression in CD8^{+} cells by Treg after 5 days. c) Suppression of CLA expression on CD8^{+} cells by Treg after 5 days. d) Suppression of CCR10 expression on CD8^{+} cells by Treg after 5 days (n=3).

Detailed phenotypic analysis of Treg was carried out by flow cytometry prior to and following cryopreservation. Foxp3 expression, CTLA-4 expression, LAP (TGFβ1) and IL-10 staining all showed no change in expression following cryo-preservation (Figure 3.15). These markers were chosen due to their importance in both the function and phenotypic identification of Treg.
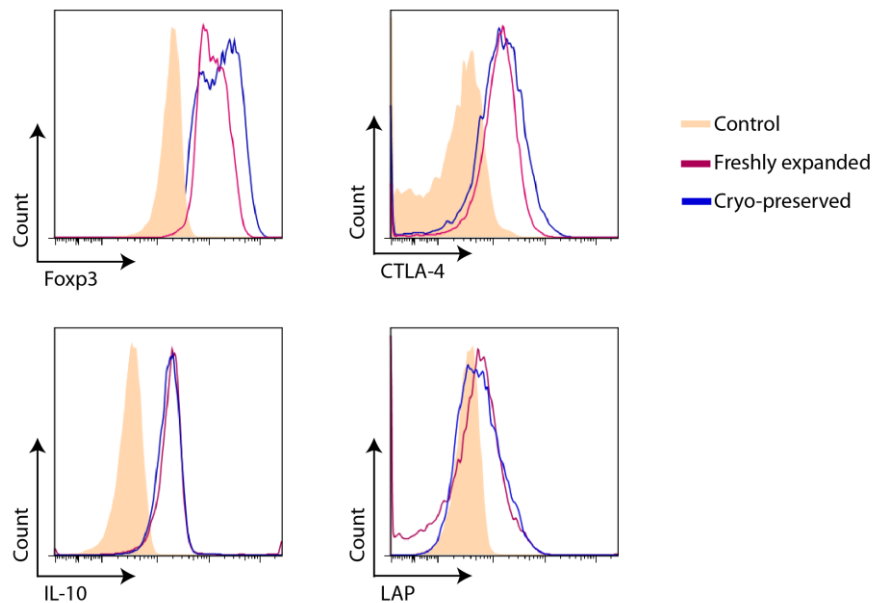
Figure 3.15 Phenotype of Treg following cryo-preservation.
Cells were routinely fixed and stained for both cell surface and intra-cellular markers, Representative histograms are shown (n=3) with freshly expanded (■) cryo-preserved (■) and control (■)

Both the phenotypic and functional comparisons between freshly expanded and cryo-preserved Treg led to the conclusion that cryo-preserved Treg retain their suppressive potency. Therefore it is possible to use previously cryo-preserved Treg for further *in vitro* functional studies.

3.3.10 The use of third party Treg to ameliorate GvH reactions

Recent clinical trials have used third party Treg however the question remains whether the third party Treg are as effective at preventing GvHD as Treg autologous to the donor. *In vitro* functional tests were carried out to investigate the potency of third party Treg in comparison to Treg which were autologous to the CD8$^+$ T cells. The ability of third party Treg to suppress allo-stimulated CD8$^+$ T cell proliferation was measured using 3H-TdR incorporation. Third party Treg were less potent at suppressing proliferation than autologous Treg (p=0.01, 0.03 and 0.01 for 2:1, 4:1 and 16:1 ratios respectively). Although less potent than autologous Treg, the third party Treg were able to significantly reduce proliferation at all ratios when compared to the CD8$^+$ T cells allo-stimulated in the absence of Treg (Figure 3.16).
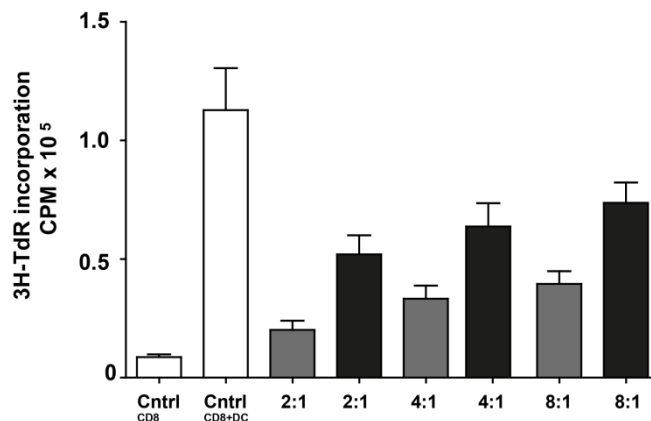
Figure 3.16 Treg dose dependant suppression of CD8$^+$ T cell proliferation was not comparable between autologous and third party Treg.
Proliferation was measured using 3H-TdR incorporation by allo-moDC stimulated CD8$^+$ T cells in the presence of autologous (▥) or third party (●) Treg. Un-stimulated CD8$^+$ T cells and allo-stimulated CD8$^+$ T cells (□) served as negative and positive controls. Error bars indicate mean±SEM of 9 individual tests of 3 independent experiments.

In addition the suppressive capacity of third party Treg was measured by the activation status of the CD8$^+$ T cells after 24 hours and 5 days of allo-stimulation. Levels of CD69 suppression were comparable between autologous and third party Treg (Figure 3.17 p=0.78 and 0.62 for 2:1 and 4:1 respectively). Late activation was assessed via expression of CD25, autologous and third party Treg supressed CD25 expression to the same extent at a 2:1 ratio of CD8:Treg (p=0.23) however, when less Treg were present at the 4:1 ratio the third party Treg were less potent at supressing CD25 expression (p=0.03) although they do show potent expression when compared to allo-stimulation in the absence of Treg.
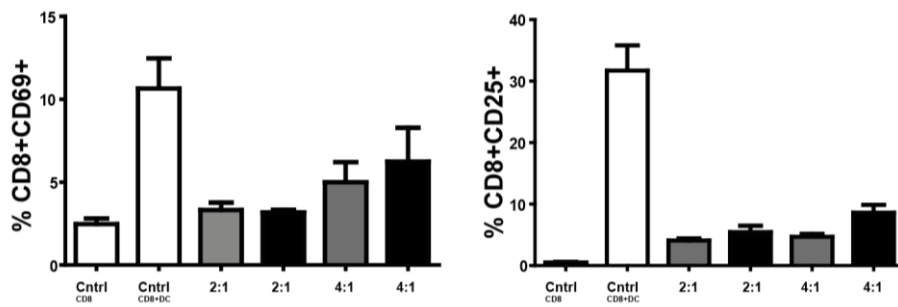
Figure 3.17 Treg dose dependant suppression of CD8⁺ T cell activation by third party Treg.
Activation was measured by flow cytometry for CD69 at 24 hours and CD25 after 5 days of allo-stimulation in the presence of autologous (▬) or third party (■) Treg. Un-stimulated CD8⁺ T cells and allo-stimulated CD8⁺ T cells (□) served as negative and positive controls. Error bars indicate mean±SEM of 4 independent experiments.

Finally the potency of third party Treg was assessed in the skin explant model. Irradiated PBMC were used to stimulate donor PBMC in a 7 day MLR either in the presence or absence of third party Treg, these cells were then incubated with small sections of skin obtained from the stimulator before being fixed and assessed for GvH damage. In the presence of Treg (Figure 3.18C) the severity of damage was greatly reduced compared to when Treg were absent during the MLR phase (Figure 3.18B). This is in line with previously published work using Treg autologous to the PBMC.
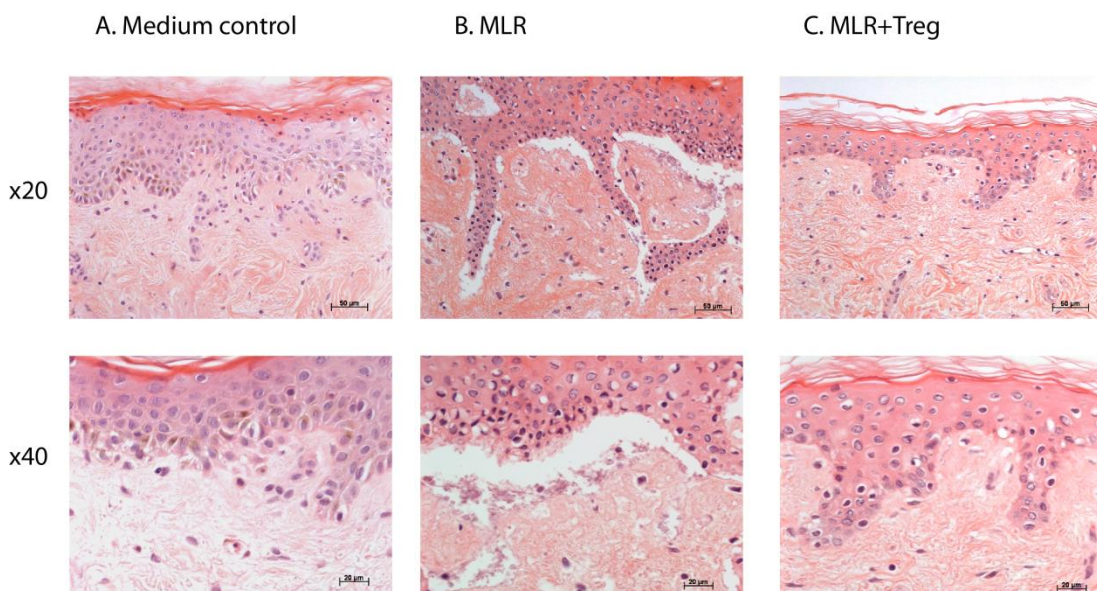
Figure 3.18 Third party Treg are able to prevent cutaneous GvH damage in human skin explant model.
Sections were graded according to the Lerner scale (Lerner et al., 1974). Representative images from 3 independent experiments.

3.4 Discussion

This work demonstrates successful isolation, expansion and cryopreservation of highly purified populations of suppressive regulatory T cells from human peripheral blood samples, and has therefore provided a basis for studies carried out in the following chapters. The isolation and expansion rates seen are consistent with previously published data. Peters *et al* saw a 30 fold expansion in 10 days of expansion culture (Peters et al., 2008). This is a lower fold expansion, but also a shorter expansion time than in this study. Hoffmann *et al* report a much higher Treg expansion of up to 40 000 fold over a period of 3-4 weeks (Hoffmann et al., 2004). In this expansion protocol they used a co-culture containing $CD32^+$ L cells to achieve such high cell numbers. In the absence of the helper cells a lower expansion rate was seen of 2775 fold increase over 4 weeks. The overall fold increase in cell number seen in this study may appear much lower however the cells were expanded for a shorter time period (Hoffmann et al., 2004). Conversely it has been reported that some donor Treg may be suppressive in the absence of activation and expansion; Hagness *et al* reported that in 54% of healthy donors Treg were spontaneously suppressive in a contact dependent APC independent manner (Hagness et al., 2012). Prolonged expansion of Treg was seen to be associated with a reduction in Foxp3 expression and suppressive capacity. It is therefore important to consider the balance between cell number and cell function. An increase in cell number may come at the cost of reduced suppressive function.

Detailed flow cytometry analysis of isolated and expanded Treg was carried out. Freshly isolated Treg were seen to have slightly lower CD4 expression than the $CD4^+CD25^{neg/intermediate}$ population, this could be due to their slightly smaller size prior to expansion (according to forward and side scatter flow cytometry analysis) in line with previous reports (Hoffmann et al., 2004). High levels of Foxp3 expression was seen along with other functional markers including LAP-(TGFβ), CTLA-4 and IL-10. All these markers have previously been associated with Treg suppressive function and confirm the successful isolation and expansion of Treg (Tang and Bluestone, 2008).

Although Foxp3 is considered to be the gold standard identifier and master regulator of Treg development and function it can be transiently expressed in activated conventional T cells (Wang et al., 2007). Assessment of the

methylation status of the Foxp3 locus enables discrimination between Treg and conventional T cells which are transiently expressing Foxp3 (Baron et al., 2007). Therefore further investigation into the methylation status of the Foxp3 locus could provide further insight into the purity and stability of the *ex vivo* expanded Treg used in this study.

The stability of Treg is of great importance when considering the routine use of Treg in the clinic and has been the subject of much debate (Sakaguchi et al., 2013). Under some inflammatory conditions Treg may become unstable and loose Foxp3 expression becoming pathogenic T cells (Zhou et al., 2009, Hansmann et al., 2012). It is thought that Treg induced in the periphery are less stable and therefore more likely to lose Foxp3 expression, ideally only thymus derived natural Treg would be isolated and expanded.

Helios has commonly been used to identify the difference between nTreg and iTreg. However recent reports have drawn attention to the possibility that Helios may not be able to accurately differentiate between the populations (Thornton et al., 2010, Gottschalk et al., 2012, Akimova et al., 2011). In the absence of any other known marker at the time, to differentiate between them, Helios was used in this work to assess the proportion of induced and naturally occurring Treg during isolation and expansion. It was seen that the proportions of Helios$^+$ and Helios$^-$ cells remained consistent throughout isolation and expansion. Helios has been shown, in mouse models, to induce epigenetic silencing of the IL-2 gene locus, thereby preventing Treg production of IL-2 (Baine et al., 2013). Interestingly IL-2 signalling coincided with lower levels of Helios expression and reduced suppressive capacity (Baine et al., 2013). These results would suggest that Helios levels could be down-regulated due to a negative feedback loop during expansion, however no such down-regulation in Helios expression was seen.

Bruder *et al* reported the transmembrane protein neuropilin-1 (Nrp-1) as strongly and constitutively expressed in murine Treg (Bruder et al., 2004). Recent work using mouse models proposed that Nrp-1 is expressed on nTreg at higher levels than on iTreg, therefore potentially differentiating between the two populations (Weiss et al., 2012, Yadav et al., 2012). Unfortunately is has been reported that in humans Nrp-1 is not a marker of Foxp3 Treg, but rather a

general activation marker for human T cells (Milpied et al., 2009). It may still be possible to use Nrp-1 as a marker to differentiate between nTreg and iTreg in the future however more work would be needed to ensure that it is an accurate way of separating the two populations.

There are few reports on the effects of cryopreservation on isolated Treg. However there have been conflicting reports as to the effect of cryopreservation on percentages of Treg within PBMC. Elkrod, in 2009, suggested the percentage of Treg in PBMC were significantly reduced following cryopreservation, however Van Hemelen *et al* provided evidence to the contrary (Elkord, 2009, Van Hemelen et al., 2009). An additional study reported that mRNA expression of Treg associated markers such as Foxp3, CTLA-4 and TGF-β in total PBMC was unchanged following cryopreservation (Kivling et al., 2009). These studies focused on the effects of cryo-preservation on un-isolated Treg and demonstrated that the effects of cryopreservation need to be thoroughly investigated prior to routine use of Treg as an 'off the shelf' reagent for therapeutic use.

Peters *et al* stated that the cryopreservation of isolated Treg reduced their suppressive capacity unless they were expanded after thawing, however they showed that expansion prior to cryopreservation did result in Treg able to suppress autologous CD4$^+$CD25$^-$ proliferation. The results described in this chapter are in line with those published by Peters et al (Peters et al., 2008). In addition the results have demonstrated a comparable suppressive potency on cryopreserved Treg across a much wider spectrum including their ability to modulate activation, proliferation and cytokine production of allo-reactive CD8$^+$ T cells. Taken together the results offer an assurance that cryopreserved Treg can be used in further *in vitro* functional tests enabling reduced cost, reduced workload and increased consistency. Furthermore the results provide the first *in vitro* evidence with particular relevance to their potential use in the prevention of GVHD following allogeneic HSCT. In the context of this work the ability to cryopreserve Treg without any adverse effects on the cells is advantageous as it allows for extra flexibly in experimental design, more efficient use of available resources and the ability to generate a stock supply of Treg.

The use of third party Treg as a therapeutic option has reached clinical trials however questions still remain as to the potency of third party Treg in comparison to autologous Treg. This work has demonstrated that third party Treg are highly suppressive and are able to prevent GvH reaction *in vitro*, however they appear to have less potency than autologous Treg, especially at lower Treg ratios. The successful and safe use of third party Treg to ameliorate GvH has been seen in xenogenic models of GvHD (Parmar et al., 2014) as well as in the clinic (Brunstein et al., 2011). This creates the possibility to generate a stock supply of 'off the shelf' Treg for use in the clinic, potentially improving transplant outcomes and providing a readily available source of Treg for research purposes.

In summary this chapter shows the isolation and expansion of Treg which are able to suppress a range of allo-reactive responses relevant to graft-versus-host disease. In addition the results show that it is possible to cryo-preserve Treg following expansion without adversely affecting their phenotype or suppressive capacity. This work has provided a basis for further assessment of the role of Treg in preventing GvHD following HSCT.

Chapter 4 - Treg modulation of CD8⁺ T cell migration into skin as a mechanism of preventing GVHD

4.1 Introduction

Previous work carried out within our group has demonstrated that 'donor' Treg, when present during priming of effector cells, are able to prevent cutaneous GvH tissue damage (Wang et al., 2009). Work presented in this chapter, aimed to further investigate the mechanism behind this protective effect. It is well reported in the literature that Treg are able to prevent GvHD but what mechanisms might be responsible? Extensive research has been carried out in murine models to model leukocyte migration in GvHD and the effect of Treg may have on this migration, however, currently no such information exists for human GvHD. Murine models have the advantage that experimental GvHD can be designed and performed as study aim desires whereas in the human setting this is not possible. The present study investigated the effect of Treg on CD8⁺ T cell migration in humans using various *in vitro* functional tests including an unique human GvHD *ex vivo* skin explant model.

4.1.1 Donor T cell migration in GvHD

The migration of donor T cells following HSCT is extremely important in the patho-physiology of GvHD as histopathologic diagnosis of GvHD requires the presence of T cells in the target tissue (Lerner et al., 1974). Extensive research has led to the inclusion of effector T cell migration into target tissues as a fourth criterion following the classical Billingham concept requiring 3 prerequisites for GvHD to occur (Sackstein, 1993, Sackstein, 1995). In GvHD the cytotoxic responses against host tissues are mediated by the donor T cells, with the recruitment of other effector cells following the initial damage. The most effective approach to prevent GvHD remains total depletion of T cells from the graft, although this in itself presents alternative problems such as increased incidence of relapse (Blazar et al., 2012). Modulation of allo-reactive T cell trafficking has been identified as means of ameliorating GvHD (Blazar et al., 2012).

In order for T cells to reach their target tissues they must migrate from the vascular system into the tissues via a highly regulated process controlled by various adhesion molecules and chemokines. Typically T cell migration can be

divided into two key processes; firstly extravasation where the cells move from the vessel lumen into the tissue and secondly chemotaxis of cells through the tissue driven by a concentration gradient of chemokines.

An established multistep model has been used to describe leukocyte extravasation (Butcher, 1991, Springer, 1994); firstly cells tether and roll along the endothelial surface (Figure 4.1). Rolling leukocytes then become activated following exposure to chemokines, cytokines and other pro-inflammatory molecules. This causes activation-dependent increase in integrin adhesive capacity, resulting in firm adhesion of the cells. In skin interactions between E-selectin and cutaneous lymphocyte antigen (CLA) result in the T cells rolling along the vascular wall, continuously sampling the endothelium environment. Interactions between a chemokine receptor on the T cell, such as CCR4, and its ligands CCL17/22, being produced by either the endothelium or skin cells, causes activation and up-regulation of integrins on the T cell (such as LFA-1, CD11a/CD18, $\alpha_L\beta_2$) and binding to their ligands (ICAM-1, CD54) on the endothelium resulting in firm adhesion of the T cell (Chong et al., 2004). Transmigration of the cell into the tissue then takes place, but less is known about this process (Wysocki et al., 2005).
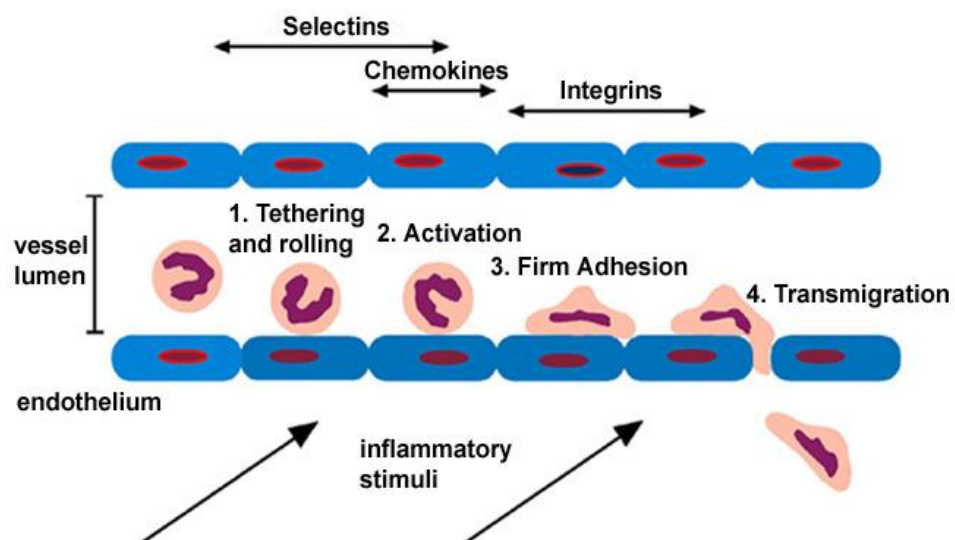
Figure 4.1 Summary of leukocyte extravasation.
Transient interactions between selectins mediate tethering and rolling of cells. When cells are then activated by chemokines leading to firm arrest and then transmigration. Image taken from www.mpi-muenster.mpg.de.

Following extravasation cells then move through the tissue driven by a concentration gradient of chemokines. In order for T cells to infiltrate the skin during GVHD they must complete each stage of migration, therefore blocking

just one stage of this process could be enough to prevent migration into the skin and consequently reduce cutaneous GvHD following HSCT. This provides a possible mechanism for the action of Treg in the prevention of GvHD by interfering with at least one stage of T cell migration. The functions of many of the components in this process are discussed in more detail with particular relevance to GvHD.

4.1.1.1 Selectins and their ligands in GvHD

The initial capture and rolling of leukocytes is mediated by selectins interacting with carbohydrate epitopes of their ligands in a calcium dependent manner (Sperandio, 2006). There are 3 members of the selectin family, L-Selectin (LECAM-1/LAM-1/CD62L), E-Selectin (ELAM-1/CD62E) and P-Selectin (GMP-140/PADGEM) each of which is able to mediate rolling *in vitro* and *in vivo.* It is their different expression patterns which determines which part of the inflammatory response they are involved in (Kansas, 1996). L-selectin is predominantly expressed on neutrophils and monocytes along with other immature hematopoietic cells and upon activation of leukocytes L-selectin is rapidly lost (Kansas, 1996). E-selectin is expressed on endothelium in response to inflammatory responses and in human skin expression can be persistent due to differential polyadenylation of the E-selectin transcript (Chu et al., 1994). In murine models E-selectin has been shown to slow leukocyte rolling and therefore aids leukocyte adhesion in the inflammatory microenviroment (Kunkel and Ley, 1996). P-selectin is expressed by both endothelium and platelets, it is stored in Weibel-Palade bodies and α-granules respectively and these granules rapidly fuse with the cell membrane upon stimulation which exposes P-selectin on the cell surface, P-selectin is then rapidly internalized and either recycled or shed. Like E-selectin, transcription of P-selectin is briefly induced by inflammatory stimulus such as IL-1, LPS or TNFα making it likely to be expressed both early and late in the immune response (Kansas, 1996). Murine studies have shown the importance of selectins in the pathogenesis of GvHD; Lu *et al* demonstrated that the absence of P-selectin in transplanted mice resulted in a reduction in experimental GvHD due to reduced allo-reactive T cell trafficking to inflamed target tissues (Lu et al., 2010).

Skin homing T cells can be identified by their expression of CLA, a carbohydrate epitope predominantly presented on P-selectin glycoprotein

ligand-1 (PSGL-1), which serves as a receptor for vascular selectins (Fuhlbrigge et al., 1997). CLA$^+$ T cells can bind both E- and P- selectins and therefore acting as a homing receptor to skin (Borges et al., 1997). CLA is found on most infiltrating T cells in almost all skin diseases (Kupper and Fuhlbrigge, 2004, Fuhlbrigge et al., 1997). CLA$^+$ T cells also typically express the chemokine receptor CCR4 which can lead to the arrest of rolling lymphocytes. CLA expression allows T cells to interact with E-selectin expressed on endothelial cells which mediates rolling and tethering of the cells but it is its interactions between chemokines and their receptors which cause the activation and diapedesis (Kunkel and Butcher, 2002).

4.1.1.2 Chemokines and chemokine receptors in GvHD

Chemokines are a large family of 8-12kDa proteins which function in the migration of leukocytes (Moser et al., 2004). This section will focus on the function of chemokines as leukocyte chemo-attractants, but it is worth noting that chemokines have also been shown to influence other processes such as hematopoiesis, angiogenesis and immune cell activation (Wysocki et al., 2005). They can be divided into four families based upon the position of N-terminal cysteine residues; most are found in the CC and CXC sub-families with a few in the CX$_3$C and C sub-families (Murphy et al., 2000, Murphy, 2002). Chemokine receptors are a family of 7-transmembrane domain G-protein coupled receptors (GPCRs) which elicit downstream cell signalling. There is a high level of redundancy in the chemokine system with chemokines interacting with several receptors and receptors interacting with several chemokines. The role of a few key chemokine/chemokine receptors will be discussed with relevance to GvHD.

CXCR3 and its ligands

Various mouse models have been used to investigate the role of chemokines in the pathophysiology of GvHD. One of particular interest is CXCR3, an inflammatory chemokine receptor which guides T cell migration by interacting with 3 IFNγ inducible ligands CXCL9 (monokine induced by gamma-IFN, MIG), CXCL10 (interferon-induced protein of 10kDa, IP-10) and CXCL11 (interferon-inducible T-cell alpha chemoattractant, I-TAC) (Kanda et al., 2007). Although all 3 ligands bind to CXCR3 they have been shown to work redundantly, synergistically, collaboratively and antagonistically *in vivo* (Groom

and Luster, 2011). The chemokine receptor CXCR3 is important in gut pathogenesis of GvHD. Using either wild type or CXCR3(-/-) donor cells it was shown that there was reduced pathology and CD8$^+$ T cell infiltration into the gut when the T cells did not express CXCR3 (Duffner et al., 2003). In addition gene expression analysis of cutaneous GvHD in mouse models showed that the ligands CXCL9 and CXCL10 were up-regulated in mouse ears within 1 week of transplantation (Sugerman et al., 2004). A more recent study showed long term use of a CXCR3 blocking antibody in murine models ameliorated GvHD by reducing effector T cell migration into target tissues without adversely affecting the beneficial GvL effect (He et al., 2008).

There is a significant volume of evidence demonstrating the importance of CXCR3 in the development of GvHD in mouse models of HSCT. Piper *et al* showed through serum analysis, T cell phenotyping and immunohistochemistry that CXCL10-CXCR3 interactions cause the recruitment of T cells into the sites of inflammation in human aGvHD (Piper et al., 2007). Interestingly they showed that only CXCL10 (but not CXCL11) –CXCR3 interactions were important in pathogenesis by recruitment of CXCR3$^+$ T cells into the skin. CXCR3 may also play an important role in the pathogenesis of chronic GvHD by causing CD4$^+$ T cell migration to the skin (Croudace et al., 2012). Undoubtedly CXCR3 interactions with its ligands is important in the pathogenesis of GvHD in both mouse models and in humans following transplantation and will therefore be investigated as a potential mechanism for Treg mediated suppression of GvHD.

CCR10 and CCR4 and their ligands

It is thought that CCR4 and CCR10 function redundantly in T cell recruitment into the skin (Xiong et al., 2012). CCR10 interactions with its ligand CCL27 are important chemokine receptor/chemokine interactions for the migration of lymphocytes to the skin (Morales et al., 1999). CCR10 has an additional ligand, CCL28, which is produced by mucosal epithelial cells and is also able to support migration through CCR3 (Pan et al., 2000). Although important in homing to other tissues CCL28 expression will not be discussed further in this section due to the lack of its expression in skin.

Expression of CCL27 is increased with keratinocytes in patients with dermatitis or psoriasis and the majority of skin infiltrating lymphocytes

expressed CCR10 (Homey et al., 2002). CCL27 is secreted and immobilized on the extra-cellular matrix it is in its active form, creating a concentration gradient to guide the T cells through the tissue. Interference of the CCR10-CCL27 pathway also impaired lymphocyte recruitment into skin (Homey et al., 2002). In a cohort of transplant patients those with GvHD, had a higher number of $CD4^+CCR10^+$ in their peripheral blood, and that after resolution of GvHD these numbers reduced back to normal levels, supporting the involvement of CCR10-CCL27 interactions in the pathology of GvHD. Of note CCR10 expression is more common on $CD4^+$ T cells than $CD8^+$ T cells (Faaij et al., 2006). The CCR4-CCL17 pathway is believed to recruit the lymphocytes while the CCR10-CCL27 pathway guides them through the epidermis (Faaij et al., 2006).

4.1.1.3 Integrins in GvHD

Integrins are a family of cell adhesion molecules which are important in the attachment of cells to the extracellular matrix and also in cell-cell interactions. The family contains 24 heterodimeric members consisting of non-covalently associated α and β subunits (Takada et al., 2007). There are resemblances between members of the α subunits and the β subunits but there is no homology between the α and β subunits (Barczyk et al., 2010). With particular importance to T cell migration in GvHD, integrins function in the tethering and rolling of leukocytes. On circulating leukocytes most integrins exist in low affinity states and upon stimulation conformation changes and clustering leads to increased affinity of integrins (Wysocki et al., 2005). Interactions such as those between chemokines and chemokine receptors can elicit "inside-out" signalling which is able to rapidly cause a conformational change in the integrin at the same time as causing clustering (Wysocki et al., 2005). Figure 4.2 contains a representative diagram of the structure of integrins, taken from Barczyk *et al* 2010, showing both the α and β chain domains and arrangement (Barczyk et al., 2010)
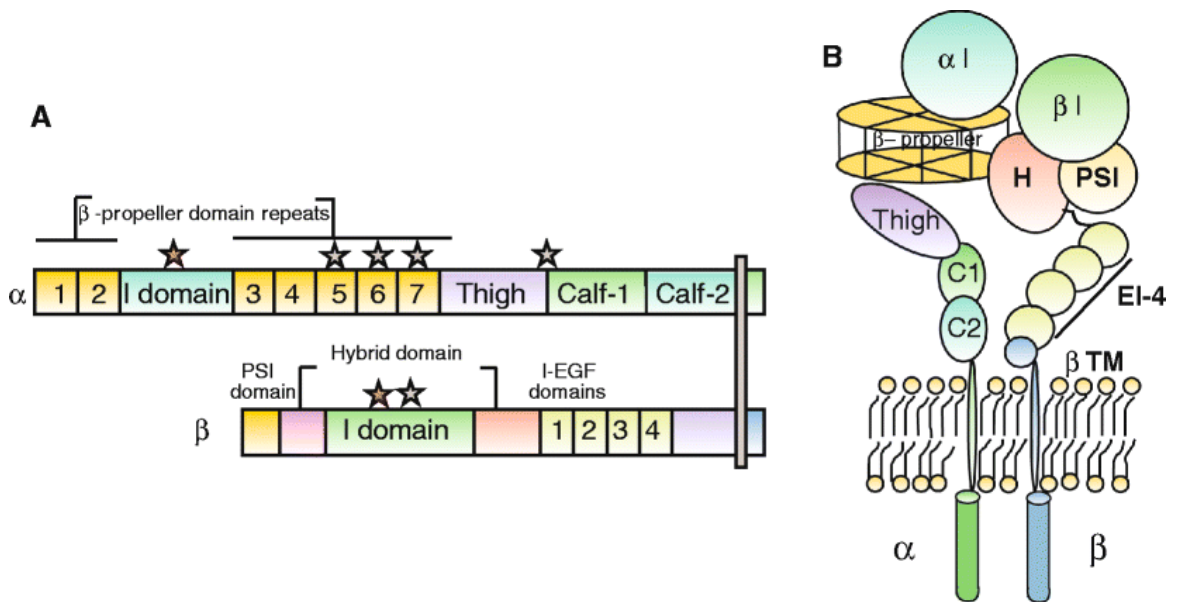
Figure 4.2 Representative diagram of integrin heterodimer.
Half of α subunits contain an αI domain and all integrins contain a βI domain. (**Barczyk et al., 2010**).

The migration of effector CD8^{+} T cells into skin during GvHD is a highly complex process; the effector cells themselves must express the necessary skin homing markers, they then must interact with the selectins and integrins and then transmigrate into the tissue before completing chemotaxis through the tissue. Disruption of this process presents a possible mechanism for the amelioration of GvHD following transplantation.

The specific question asked in this chapter is whether Treg prevention of GvH tissue damage is associated with blocking effector cell migration into tissue. This question will be addressed via examining:

- Association between the number of infiltrating CD8$^+$ T cells in target tissue and the severity of GvH histopathology of the tissue
- Treg modulation of CD8$^+$ T cell infiltration and GvH tissue destruction
- Treg modulation of tissue homing receptor (CLA and CXCR3) expression by effector T cells
- Treg modulation of chemo-attractants (CXCL9, CXCL10 and CXCL11) expression in target tissue
- Treg modulation of migratory functions including CLA-mediated adhesion and CXCR3-mediated chemotaxis of allo-reactive CD8$^+$ T cells
- Treg modulation of production and gene expression of IFNγ, a key inducer of CXCR3 ligands
- Examining PBMC skin homing marker expression in a small clinical cohort

4.2 Methods

General methods used for the work presented in this chapter are included in Chapter 2. Additional techniques are described below; including methods used to measure T-cell adhesion and migration.

4.2.1 *In vitro adhesion assay using the cellix platform*

The cellix platform is a flow based adhesion assay used to measure Treg prevention of CD8$^+$ T cell adhesion to E selectin under flow conditions. In brief the cellix platform consists of a Biochip fitted onto a microscope stage in a temperature controlled environment. The Biochip contains micro channels which are able to mimic *in vivo* capillary flow rates when fluid is passed through them by the nanopump. Images and videos are recorded by a camera attached to the microscope for further analysis (Figure 4.3).
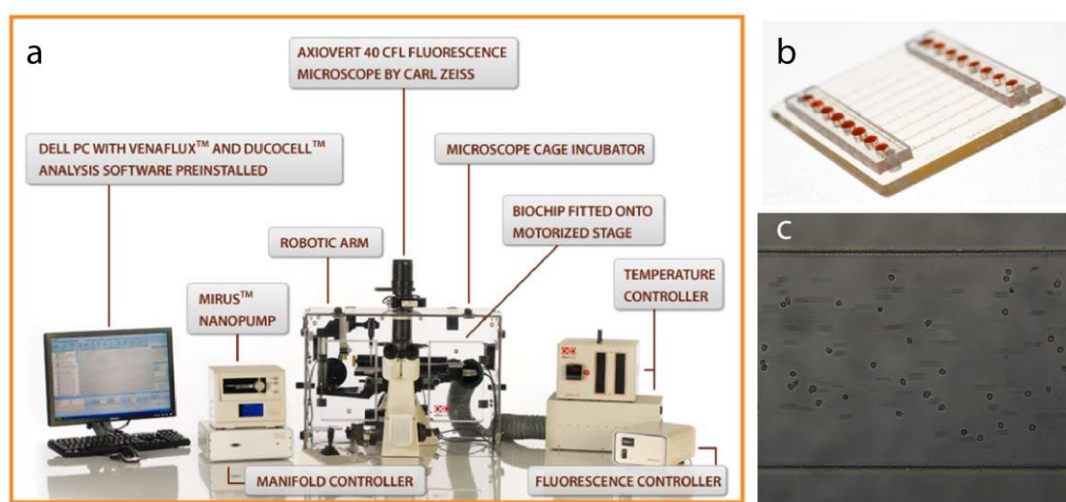
Figure 4.3 Cellix Platform used to measure CD8 adhesion to E-selectin under flow conditions.
A) **labelled image of the cellix platform (image from www.cellixltd.com). B) Image of a Biochip showing micro-channels. C) Still image of cells passing through the micro-channels.**

Biochips were coated over-night at 4ºC with 100µg/ml E selectin (R&D Systems). Non-specific binding was monitored using 1% BSA (Sigma) as a control channel. Mixed lymphocyte reactions (MLR) were set up with allo-stimulated CD8$^+$ T cells either in the presence or absence of autologous Treg as previously described. After 5 days the cells were harvested and subjected to MACS CD4 depletion to remove Treg and any remaining moDC, leaving only the CD8$^+$ cells (Miltenyi Biotech). The CD8$^+$ T cells were then re-suspended at 2x10^{6}cells/ml in RF10 and were passed through the micro channels at 1

dyne/cm^2 mimicking *in vivo* capillary flow rates. Following subsequent washes only CD8$^+$ T cells expressing CLA would remain adhered to the channels. The mean number of cells adhered was analysed by DuoCell software. Flow cytometry was also carried out to assess CLA expression on the CD8$^+$ T cells as previously described (Chapter 2).

4.2.2 Measuring cell migration using the Transwell system

T cell migration towards CXCR3 ligands CXCL9, CXCL10 and CXCL11 was measured using a Transwell chemotaxis assay. Effector CD8$^+$ T cells (MLR cells as previously described depleted for CD4$^+$ Treg and remaining moDC) were loaded into the upper chambers (5x10^5 per well) of 96 well plates with 5µm filters. The lower well contained RPMI with 1% BSA and 18nM of the appropriate chemokine (Figure 4.4). Plates were incubated for 1 hour at 37ºC and then the cells which had migrated to the lower well were collected and counted using Neubauer cell counting chamber as previously described.
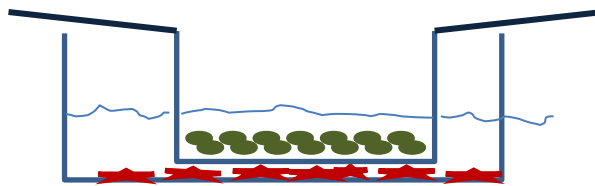
Figure 4.4 Diagrammatic representation of Transwell chemotaxis assay.
CD8$^+$ effector cells (⬤) were loaded into the upper well and appropriate chemokine (★)
into the lower well.

4.2.3 Automated Immunohistochemistry staining using Ventana system

Immunohistochemistry for CD8 (Dako, Cambridge) CXCL10 (PeproTech, Rocky Hill, NJ) and CXCL11 (Santa Cruz Biotechnology, Santa Cruz, CA) was performed on 3µm thick, formalin-fixed and paraffin-embedded skin sections using benchmark autostainer (Benchmart XT; Ventana Medical Systems) with the UltraView and the iView DAB detection kit (Ventana Medical Systems, Roche, Switzerland). Negative controls were stained without the primary antibodies. Cells stained positive or negative for CD8$^+$, CXCL10$^+$ and CXCL11$^+$ cells were manually counted across epidermis and primary dermis. The percentage of positively stained cells was calculated from the total number of cells in the tissue section.

4.2.4 Acknowledgements

Acknowledgment to

1) Dr S Ahmed for IHC staining;

2) Tissue blocks used for IHC staining were generated from both the current study and a previous study by Dr X Wang:(Wang et al., 2009)

3) Dr S Douglass for additional repeats of CD8$^+$ T cell migration assay

4) Dr S Douglass and Dr X Wang for additional repeats of IFNγ staining

4.3 Results

All work presented in this chapter was performed using *ex vivo* expanded Treg with high purity and potent suppressive functionality as shown in the previous chapter. In addition data included in this chapter has been published in the journal Transplantation, 15, 456-64 (2012) *Regulatory T Cells Inhibit CD8$^+$ T-Cell Tissue Invasion in Human Skin Graft-Versus-Host Reactions* (Mavin et al., 2012).

4.3.1 Treg inhibit CD8$^+$ effector T cell infiltration and reduce cutaneous GvH tissue destruction

A unique human GvHD skin explant model was used to assess tissue injury caused by allo-stimulated CD8$^+$ T cells either in the presence or absence of Treg. The severity of cutaneous tissue destruction following the skin explant was scored according to the well-established Lerner grading system (Lerner et al., 1974). Histopathological changes in each section were graded by two independent evaluators.

Control skin sections incubated in either skin explant medium only or with un-activated CD8$^+$ T cells showed minimal tissue injury, all being grade I according to the Lerner scale (Figure 4.5a&b). Incubation with allo-stimulated CD8$^+$ T cells resulted in a high grade of tissue injury, all being graded III or IV (Figure 4.5c). When the CD8$^+$ T cells were allo-stimulated in the presence of Treg this resulted in a significant down-regulation in the severity of tissue injury ($p<0.0001$) with 5 out of the 17 having grade II damage and the remaining 12 only grade I injury (Figure 4.5d). Late addition of the Treg, at the skin explant stage only, showed no inhibition of tissue injury, with all sections having grade II damage or above, suggesting that Treg need to be present during priming of the CD8$^+$ T cells to prevent GvH damage (Figure 4.5e) in line with previous findings (Wang et al., 2009).
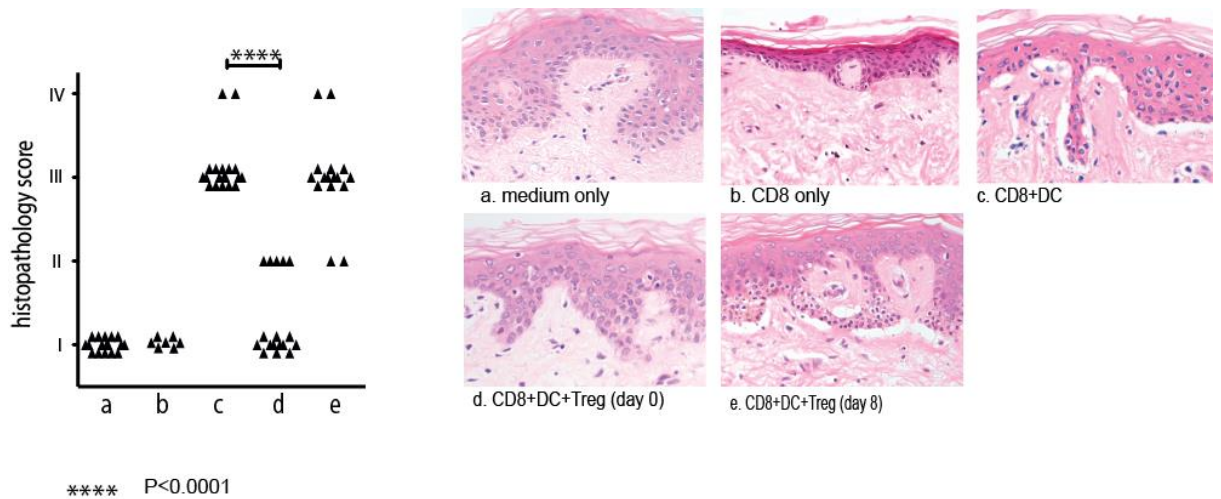
Figure 4.5 Treg reduce the capacity of CD8⁺ T cells to cause a cutaneous GVH response. Cumulative results and representative images of GVH damage of skin sections incubated with either (a) medium, (b) un-stimulated CD8⁺T cells, (c) allo-stimulated CD8⁺ T cells, (d) allo-stimulated CD8⁺ T cells in the presence of Treg and (e) allo-stimulated CD8⁺ T cells with Treg present during the skin explant (effector) phase only. Grading of damage is according to the Lerner scale (Lerner et al., 1974) from 4-11 independent experiments, sections were tested in duplicate where possible.

In addition, the number of infiltrating CD8⁺ T cells was calculated as a percentage of cells in each section after staining by immuno-histochemistry. Control skin sections cultured with medium only showed low numbers of residual CD8⁺ T cells 2.6%±0.77SEM and minimal tissue injury (Figure 4.6a). CD8⁺ T cells allo-stimulated in the absence of Treg showed a high number of infiltrating CD8⁺ T cells, 24.7%±3.66SEM (Figure 4.6b). The presence of Treg during CD8⁺ T cell allo-stimulation resulted in a significant reduction in the number of infiltrating CD8⁺ T cells down to only 11.56%±1.79SEM (p=0.01) (Figure 4.6c). Delayed addition of the Treg, adding them at the skin explant stage rather than the MLR, showed no inhibitory effect on CD8⁺ T cell migration into the skin further demonstrating that Treg need to be present during the initial allo-stimulation for them to be beneficial in protecting against CD8⁺ T cell infiltration (Figure 4.6d).

The grade of histopathological damage and the number of infiltrating CD8⁺ T cells in the skin sections showed a highly significant correlation. As the number of infiltrating CD8⁺ T cells increased the severity of tissue damage also increased with the more CD8⁺ T cells in skin sections showing the higher GvH grade III (Figure 4.7).
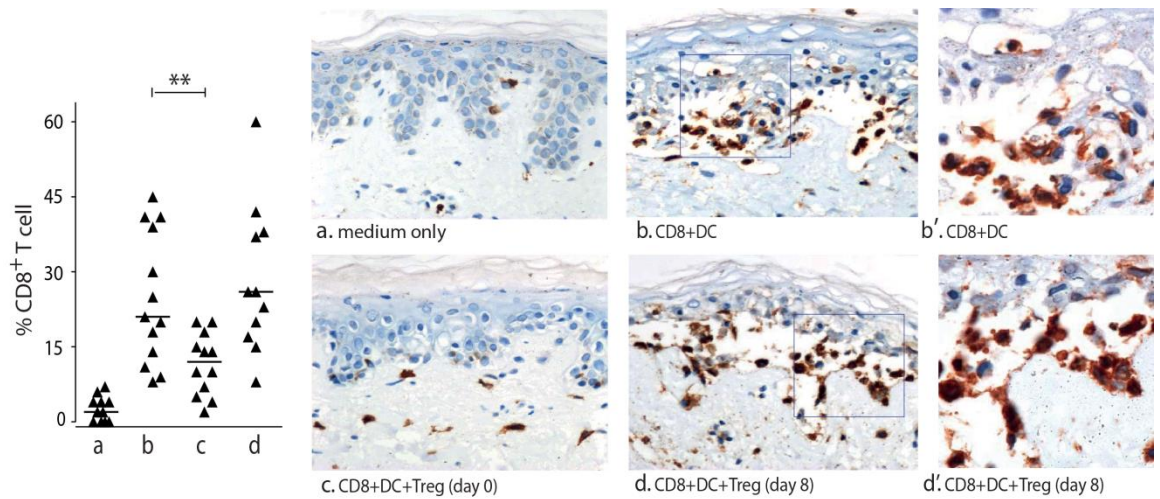
Figure 4.6 Treg prevent tissue infiltration by CD8$^+$ T cells.
Cumulative and representative staining of skin infiltrating CD8$^+$ T cells. Skin sections were incubated with either (a) medium only, (b) allo-stimulated CD8$^+$ T cells, (c) allo-stimulated CD8$^+$ T cells in the presence of Treg and (d) allo-stimulated CD8$^+$ T cells with Treg present during the effector phase only. Horizontal lines indicate median values of 7-11 independent experiments. **$P<0.01$ n=11-13 for each experimental condition.

**** P<0.0001

** P<0.01

* P<0.05
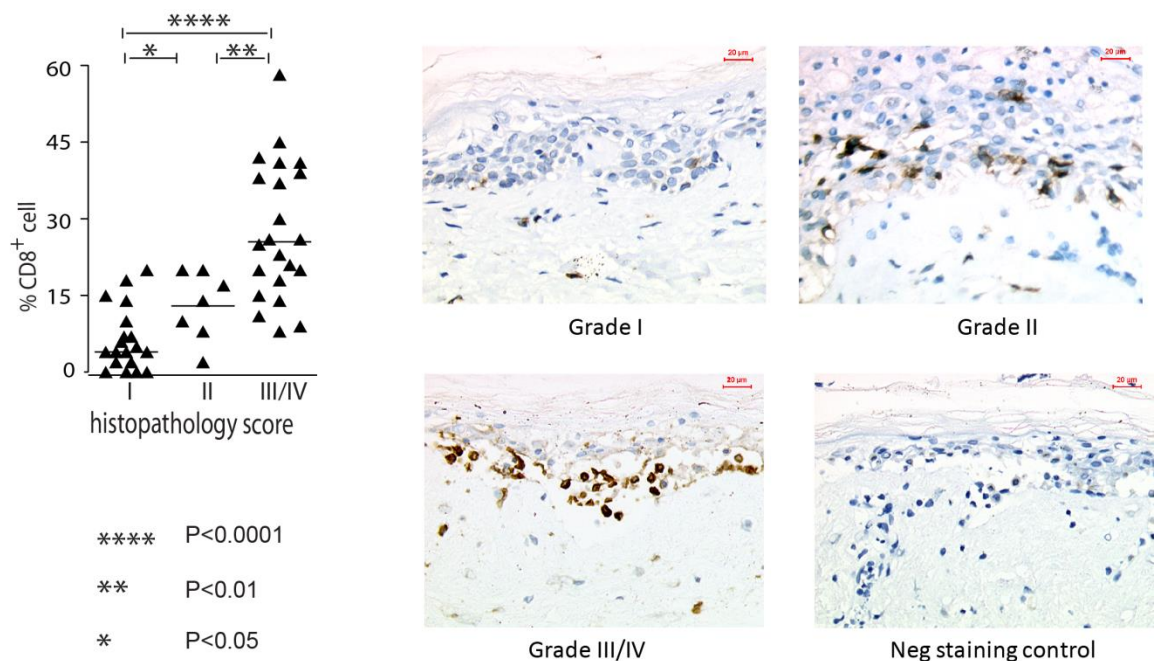
Figure 4.7 Severity of GvH damage increased as the number of infiltrating CD8$^+$ T cells increased.
Cumulative (left) and representative (right) data. The number of infiltrating CD8$^+$ T cells was calculated following IHC and grading of sections was carried out according to the Lerner scale. Negative staining control is shown where no primary antibody was used. n=7-21 for each grade of severity.

4.3.2 Treg reduce expression of skin homing receptors on effector cells

In order for cells to migrate from circulation into the skin they must express the appropriate skin homing receptors, therefore CXCR3 and CLA was measured on the CD8$^+$ T cells following allo-stimulation either in the presence or absence of Treg by multi-parameter flow cytometry. CD8$^+$ T cell surface expression of the chemokine receptor CXCR3 was up regulated from background levels of 28.84%±5.22SEM to 55.98%±4.88SEM upon allo-stimulation (p=0.0021). In the presence of Treg during allo-stimulation CXCR3 expression, although higher than background levels, was significantly reduced to 40.84%±6.44SEM (p=0.0098) when compared to the level of expression in the absence of Treg (Figure 4.8A).

In addition the expression of CLA was also assessed on resting CD8$^+$ T cells and allo-stimulated CD8$^+$ T cells in the presence and absence of Treg and, in concordance with the results seen with CXCR3 expression, CLA was significantly increased upon allo-stimulation and in the presence of Treg this expression was significantly reduced (Figure 4.8B). Mean background expression of CLA was 4.79%±1.47SEM which increased to 31.44%±4.63SEM following allo-stimulation (p=0.0004). Allo-stimulation in the presence of Treg resulted in a highly significant inhibition of CLA expression with only 9.62%±SEM of allo-stimulated CD8$^+$ T cells expressing CLA (p=0.0011).
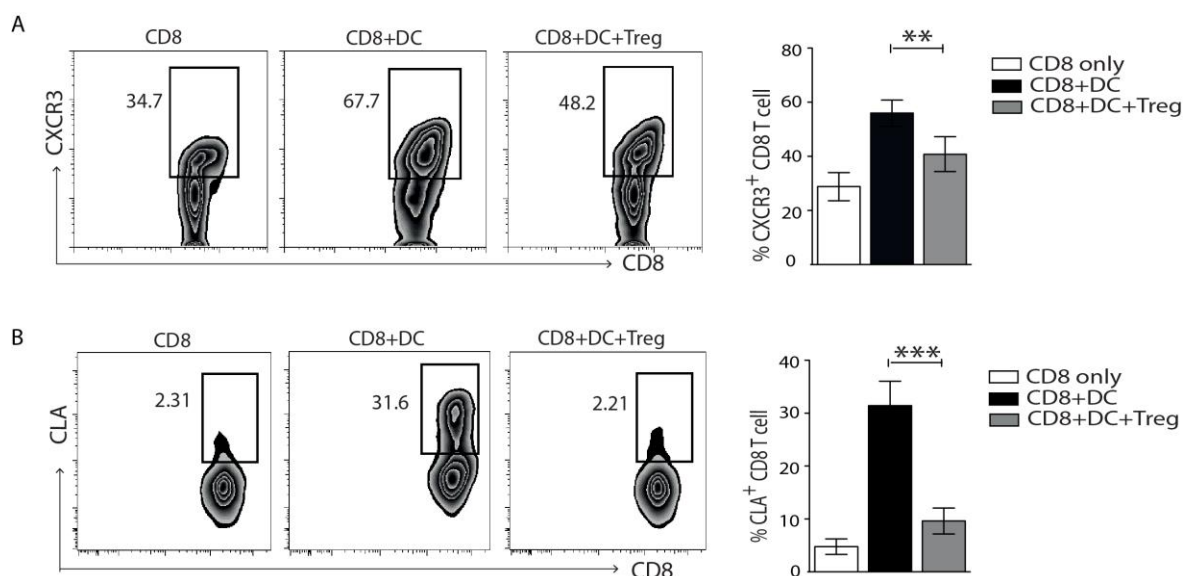
Figure 4.8 Treg reduce skin homing receptor expression.
Representative flow cytometry plots - gated on live CD8$^+$ T cells prior to plots shown- and cumulative graphs showing mean±SEM of skin homing receptor expression on un-stimulated CD8 cells ☐, allo-stimulated CD8 cells ■ and allo-stimulated CD8 cells in the presence of Treg ▨ of A) CXCR3 (n=10) and B) CLA (n=9) respectively. **P<0.01, ***P<0.0001

Next the gene expression of CXCR3 and CLA was assessed using qPCR, with GAPDH used to normalise the Ct values, each sample was assessed in triplicate as normal protocol dictates. The levels of gene expression for both CXCR3 and CLA were not seen to significantly change following allo-stimulation of CD8$^+$ T cells (p=0.16 and 0.08 for CXCR3 and CLA respectively). It was therefore unsurprising that gene expression was not significantly reduced in the presence of Treg (p= 0.08 and 0.87) (Figure 4.9).
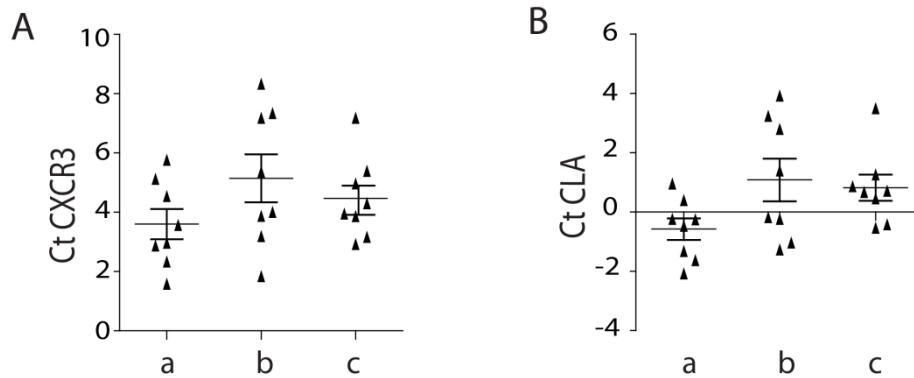
Figure 4.9 Gene expression of A) CXCR3 and B) CLA measured by qPCR and normalized to GAPDH.
a) un-stimulated CD8$^+$ T cells c) allo-stimulated CD8$^+$ T cells and (c) allo-stimulated CD8$^+$ T cells in the presence of Treg. n=8 and error bars indicate mean±SEM.

4.3.3 Treg reduce expression of CXCR3 ligands in the target tissue

Having seen that the presence of Treg during allo-stimulation causes a reduction in cell surface expression of the chemokine receptor CXCR3, the levels of CXCR3 ligands (CXCL10 and CXCL11) were then assessed in the skin sections by immunohistochemistry. Levels of CXCL10$^+$ cells were calculated as a percentage of cells in each section. In skin sections incubated in medium only the background expression of CXCL10 was 40.5%±4.4SEM (Figure 4.3.10A). When allo-stimulated CD8$^+$ T cells were added into the skin explant the number of CXCL10 expressing cells increased to 70.2%±3.26SEM and were significantly reduced to 48.9%±2.76SEM in the presence of Treg during CD8$^+$ T cell priming. Late addition of Treg, in the effector phase only (day 8), did not result in a decrease in CXCL10.
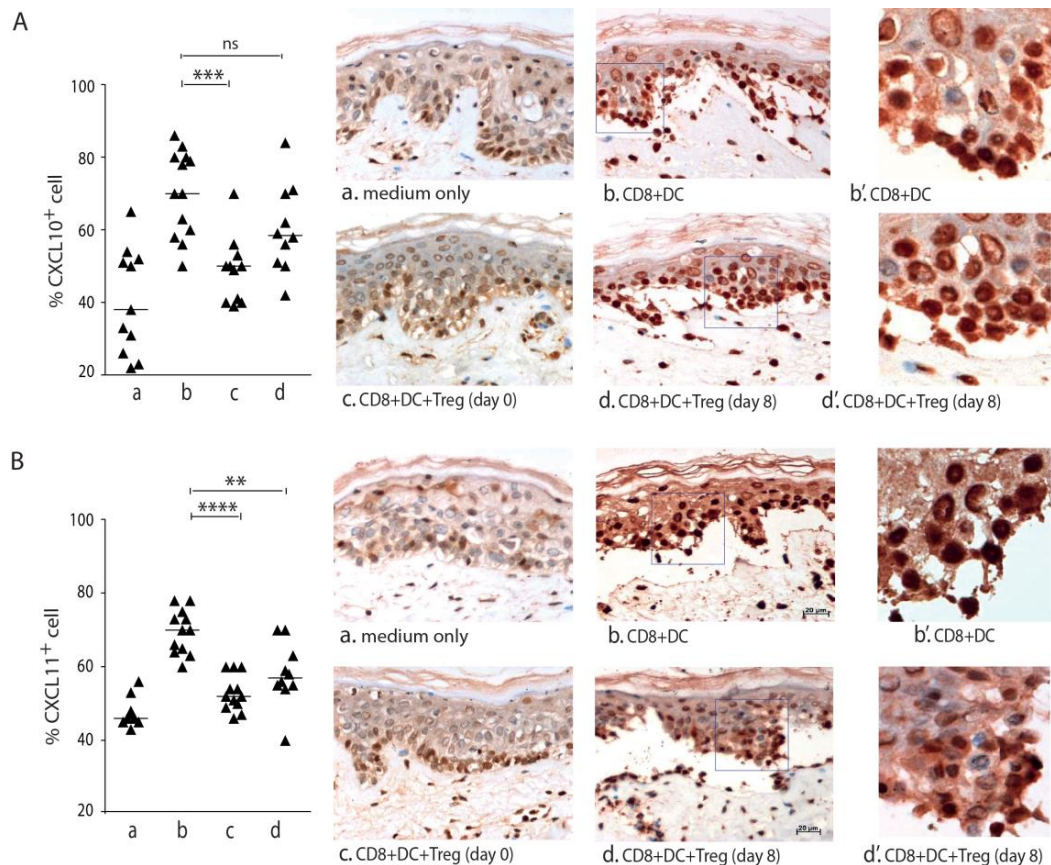
Figure 4.10 Treg reduction in expression of CXCR3 ligands in the skin explant assay. Levels of CXCL10 (A) and CXCL11(B) were measured as a percentage of cells following immuno-histochemistry for (a) medium only, (b) allo-stimulated CD8$^+$ T cells, (c) CD8$^+$ T cells allo-stimulated in the presence of Treg and (d) allo-stimulated CD8$^+$ T cells with addition of Treg at the effector phase only. Cumulative (left) showing overall CXCL10 and CXCL11 levels. Representative (right) staining shows positive staining in relation to histopathological damage with higher magnification inserts.

A similar pattern of CXCL11 expression was seen in the skin sections with the exception of the late addition of Treg. Medium alone background expression was 47.7%±1.36SEM (Figure 4.10B). Incubation with allo-stimulated CD8$^+$ T cells resulted in an increase to 69.6%±1.73SEM of cells expressing CXCL11, and when the CD8$^+$ T cells were allo-stimulated in the presence of Treg there was a highly significant reduction in expression, with only 52.9%±1.42 of cells expressing CXCL11. When Treg were added during the effector phase only, unlike CXCL10 expression, there was a significant reduction in the levels of CXCL11 to only 58%±2.74SEM.

When the percentage of both CXCL10 and CXCL11 expressing cells was correlated with the grade of histological damage in the skin sections there was a highly significant relationship with higher the CXCL10 (Figure 4.11A) or CXCL11 (Figure 4.11B) expressing cells correlating with greater the severity of damage.
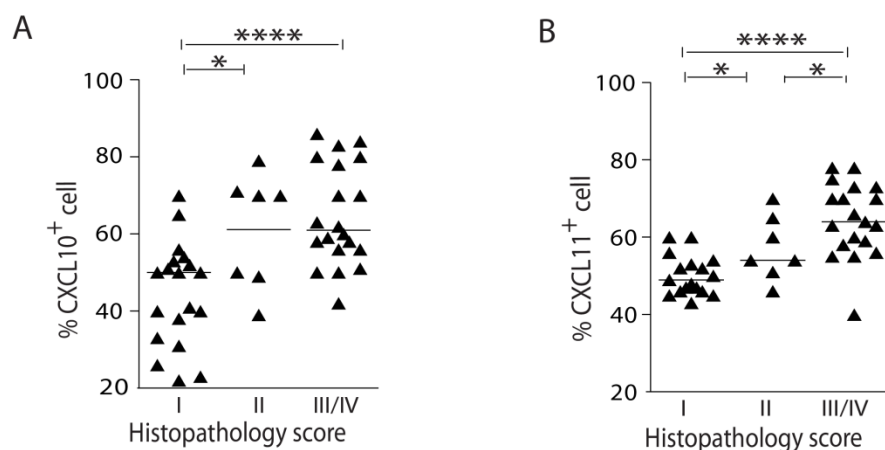
Figure 4.11 Correlation between percentage of cells expressing CXCR3 ligands and severity of tissue damage following skin explant assay.
Data shown is from 7 independent experiments and duplicate sections were used when possible. Horizontal bars indicate median values *p<0.05, ****p<0.0001.

In addition to receptor expression on the effector cells and the presence of ligand in the skin sections skin explant culture supernatants were collected and analysed by CBA flex analysis for levels of secreted CXCL9 and CXCL10. For both chemokines there was a significant decrease in protein concentration in the supernatant when Treg were present during allo-stimulation of CD8 cells (Figure 4.12A). As the severity of damage to the skin increased so did the concentration of CXCL9 and CXCL10 in the culture supernatants (Figure 4.12B).
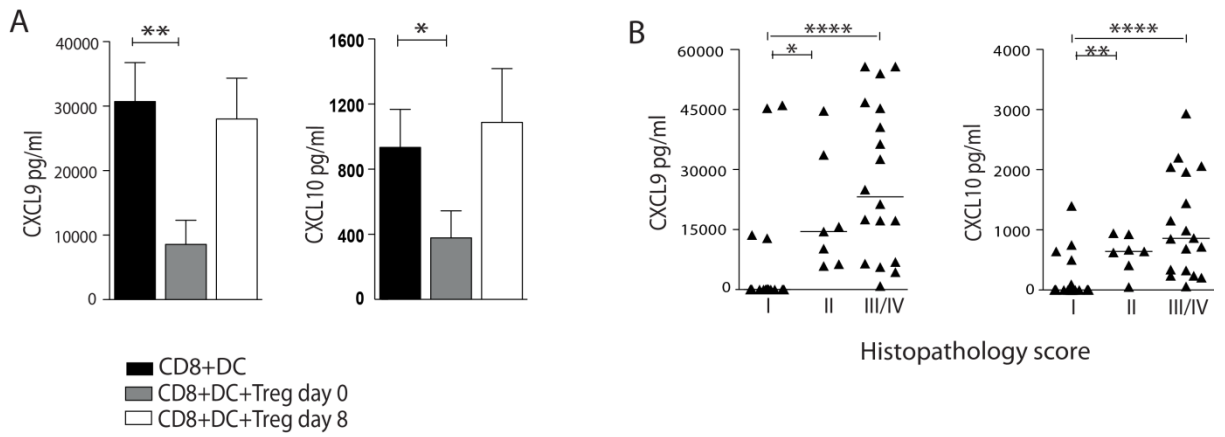
Figure 4.12 The presence of Treg during priming resulted in a reduction in CXCR3 ligands secreted into skin explant supernatant.
This reduction in CXCR3 ligands correlated with the severity of tissue destruction. A skin explant supernatants from skin sections incubated with allo-stimulated CD8^{+} T cells (■), allo-stimulated in the presence of Treg (■) and allo-stimulated with the addition of Treg at the effector stage only (□) were analysed by CBA flex for CXCL9 and CXCL10. B Culture supernatants were grouped according to severity of tissue damage and plotted against concentration of CXCL9 and CXCL10. *P<0.05 **P<0.01, ***P<0.0001 n=7-18.

4.3.4 Treg reduce effector cell adhesion to E selectin under flow conditions

E-selectin is the ligand for CLA and is important in the migration of leukocytes out of the blood vessels and into surrounding tissue (Wysocki et al., 2005). Section 4.3.2 showed that CLA expression on allo-stimulated CD8^{+} T cells was reduced in the presence of Treg. To test the functional importance of this reduction, with particular relevance to cutaneous GvHD, the cellix platform was used to assess CD8^{+} T cell adhesion to E-selectin under flow conditions. The cellix platform is able to mimic *in vivo* flow rates found in capillaries and is therefore a good *in vitro* model of leukocyte tethering.

When unstimulated CD8^{+} T cells were passed through the E-selectin coated micro-channels 5.26±0.67SEM cells adhered per field (Figure 4.13). The number of adherent allo-stimulated CD8^{+} T cells increased to 20.31±1.76SEM (p<0.0001) and when Treg were present during allo-stimulation of the CD8^{+} T cells there was a highly significant reduction in the number of adherent cells per field to 7.41±0.74SEM (p<0.0001) (Representative images (left) and cumulative results (right) Figure 4.13). The results demonstrate that Treg mediated reduction in CLA expression on allo-stimulated CD8^{+} T cells gives rise to a functional reduction in the ability of the CD8^{+} T cells to adhere to E-selectin under flow conditions.
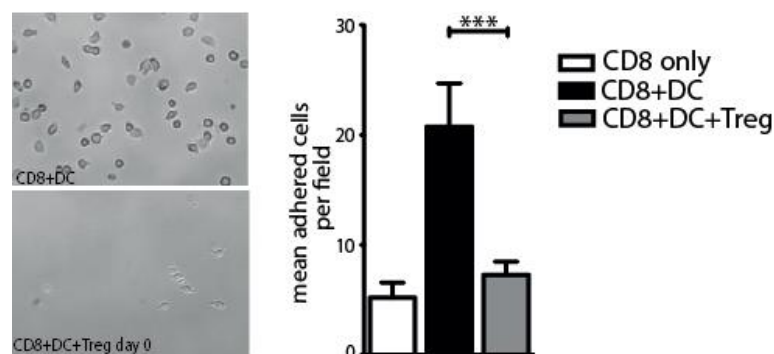
Figure 4.13 Treg mediated suppression of skin homing receptors resulted in a reduced capacity of CD8^{+} T cell adhesion.
Representative still images (left) and cumulative results (right) from 9 individual tests from 3 independent experiments. Un-stimulated (□), allo-stimulated (■) and allo-stimulated in the presence of Treg (▨) CD8^{+} T cells were passed through E-selectin coated micro-channels. Adherant cells, which remained attached when channels were rinsed with medium, were counted using DuoCell software.

4.3.5 Treg reduce effector cell migration towards CXCR3 ligands

The effect of Treg during allo-stimulation upon the migratory capacity of CD8$^+$ T cells was assessed using a Transwell system. In section 4.3.2 allo-stimulation was shown to increase CXCR3 expression of CD8$^+$ T cells, which was suppressed in the presence of Treg. The Transwell assay was used to confirm that this change in CXCR3 expression had a functional implication in the migration of CD8$^+$ T cells. The chemokine ligands for CXCR3 were added into the lower wells and the cells into the upper wells, after 1 hour the number of migrated cells was calculated as a fold increase above background migration (where no chemokine was present in the lower well).

Migration towards CXCL9 by un-stimulated CD8$^+$ T cells was 3.52±0.75SEM fold higher than background migration. When the CD8$^+$ T cells were allo-stimulated migration increased to 6.95±0.96SEM fold above background and in the presence of Treg this fold increase of cell migration was significantly decreased to less than that observed for un-stimulated CD8$^+$ T cells; 2.88±0.57SEM (Figure 4.14).

Migration towards CXCL10 by un-stimulated CD8$^+$ T cells was 2.71±0.23SEM fold higher than background, allo-stimulated CD8$^+$ T cells migrated by a fold increase of 7.17±0.60SEM, compared to only 3.97±0.53 when allo stimulated in the presence of Treg. A similar pattern was seen for the third CXCR3 ligand CXCL11; un-stimulated CD8 only showed a 2.72±0.57 fold increase, allo-stimulated CD8$^+$ T cells a 9.17±1.02SEM fold increase and in the presence of Treg only 4.38±0.90SEM fold increase, a significant reduction.

Although the CXCR3 ligands have been reported to act to work redundantly, synergistically, collaboratively and antagonistically *in vivo* (Groom and Luster, 2011) in this experimental setting they all showed the same pattern of Treg mediated inhibition. In the case of all 3 ligands CD8$^+$ T cell migration was significantly increased upon allo-stimulation. When Treg were present during CD8$^+$ T cell allo-stimulation this increase in migration was significantly impaired confirming an *in vitro* functional effect of the changes observed in CXCR3 expression.
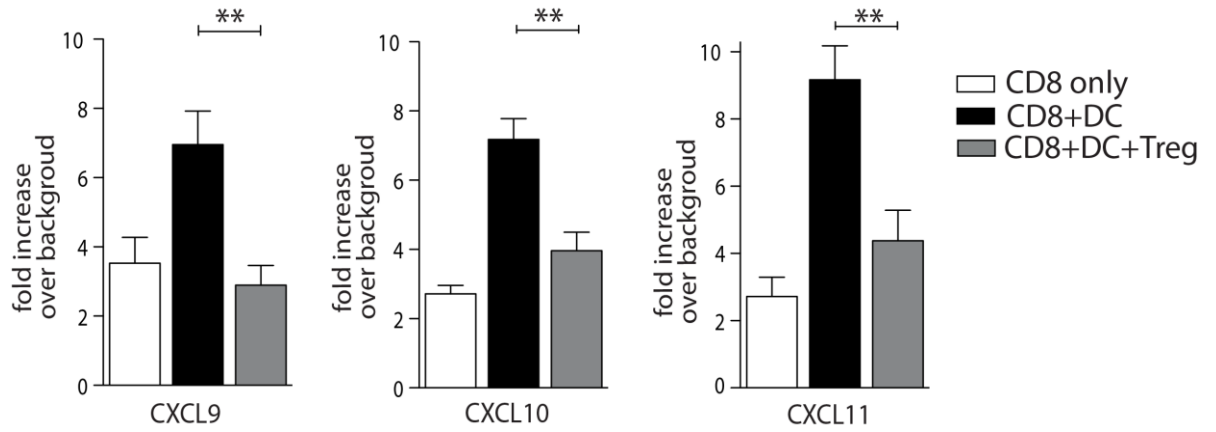
Figure 4.14 The presence of Treg decreased CD8^{+} cell migration.
Migration towards CXCR3 ligands CXCL9 (left), CXCL10 (middle) and CXCL11 (right) was assessed using a Transwell assay Un-stimulated (▢), allo-stimulated (■) and allo-stimulated in the presence of Treg (▥). Migration towards each chemokine was calculated as a fold increase above background migration (when no chemokine was present). ** indicates p<0.01 n=6.

4.3.6 IFNγ production is reduced at all levels

IFNγ is a highly important cytokine in the patho-physiology of GvHD and interestingly it is able to induce expression of CXCL9-11, the ligands for the chemokine receptor CXCR3. The capacity of allo stimulated CD8$^+$ T cells, in the presence or absence of Treg, to produce IFNγ was measured using PMA/Ionomycin stimulation followed by intra-cellular staining. The capacity of the CD8$^+$ T cells to produce IFNγ upon stimulation was significantly down regulated when Treg were present during allo-stimulation from 32.72±4.52SEM to only 14.23±3.22SEM, p=0.0019 (Figure 4.15).
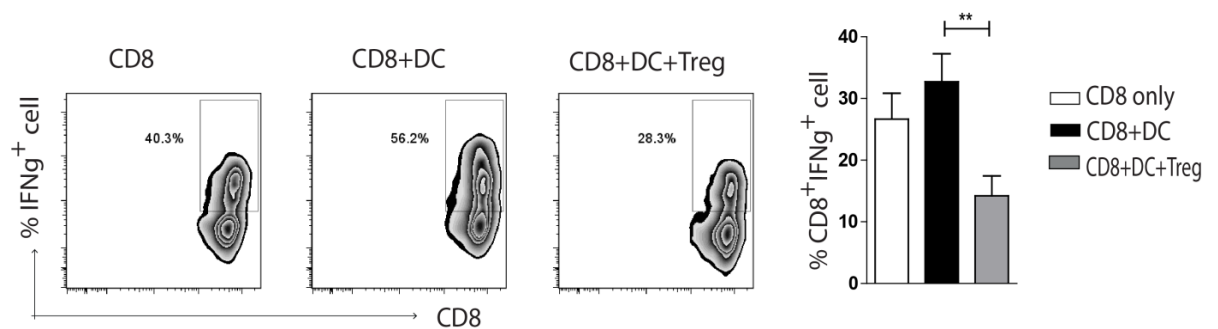
Figure 4.15 The presence of Treg during allo-stimulation resulted in reduced capacity to produce IFNγ.
CD8$^+$ T cells allo-stimulated in the absence ■ or presence ▨ of Treg were stained for their capacity to produce intracellular IFNγ. Representative plots shown were gated on CD8$^+$ T cells prior to assessment of IFNγ; cumulative bar chart shows mean±SEM for 9 independent experiments.

In addition the supernatant from the skin explant cultures were tested for IFNγ levels using CBA flex. A highly significant down regulation of IFNγ secretion was seen when Treg were present during allo-stimulation, p<0.0001 (Figure 4.16A). The levels of IFNγ in the skin explant supernatant was correlated with the severity of histopathological damage seen with lower grades of damage correlates with significantly less IFNγ in the supernatant than medium or high grades (Figure 4.16B).
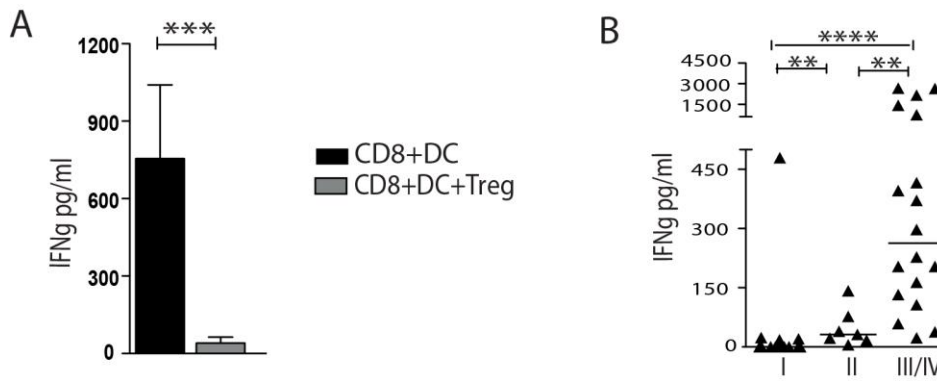
Figure 4.16 IFNγ secreted into skin explant supernatant was measured using CBA flex analysis.
A) supernatant was collected from skin sections cultured with either allo-stimulated CD8^{+} T cells ■ or allo-stimulated in the presence of Treg ▨. B) Correlation between the level of IFNγ secreted into the skin explant supernatant and the pathophysiological grade of damage to the skin.

Levels of IFNγ gene expression were measured with qPCR. Samples were tested in triplicate and normalised to GAPDH. IFNγ expression was calculated as a fold increase in expression relative to un-stimulated CD8^{+} T cells. Expression of IFNγ was significantly reduced in the presence of Treg ($p < 0.0001$) (Figure 4.17).
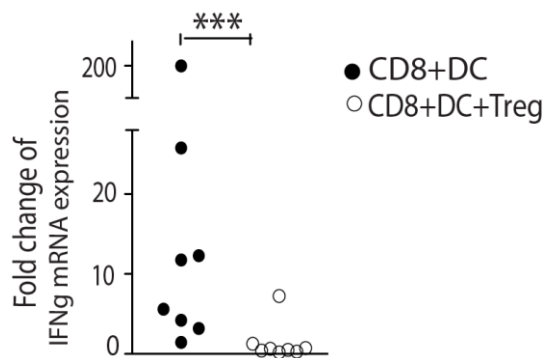
Figure 4.17 Relative IFNγ gene expression measured with qPCR, normalized to GAPDH. Un-stimulated CD8^{+} T cells alone were used as the reference group to calculate relative gene expression, data from 8 independent experiments. ***P<0.0001.

4.3.7 Association between skin homing T cells and cutaneous GvHD in a small clinical cohort

CLA and CCR10 bearing lymphocytes have been implicated in the development of GvHD (Tsuchiyama et al., 2009, Faaij et al., 2006). Following on from the *in vitro* work showing CLA$^+$ CD8$^+$ T cells were potent inducers of cutaneous GvH, T cell populations in PBMC following matched HSCT were assessed for their levels of skin homing markers. This data was then correlated with the GvHD status of the patient following transplantation.

Serial samples of peripheral blood were collected following transplantation as previously described (Chapter 2). Multi-parameter flow cytometry was carried out on the samples and analysed according to the gating strategy shown in Figure 4.18.
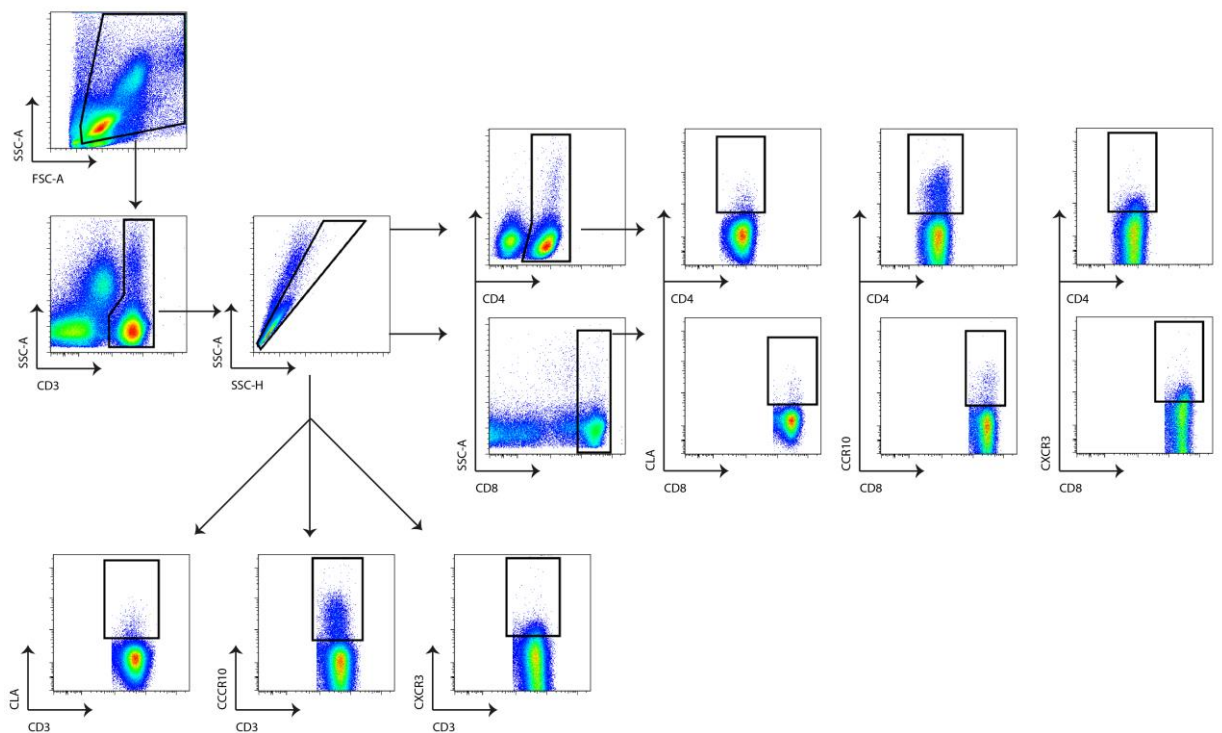
Figure 4.18 Gating strategy used to assess skin homing T cell populations in PBMC.
Flow cytometry was carried out using BD Canto II and analysed using FlowJo (TreeStar).
First live T cells were identified, followed by SSC-A and SSC-H to ensure only single cells
were analysed. Further sequential gating separated CD4$^+$ and CD8$^+$ cells then levels of
CLA, CCR10 and CXCR3 were assessed. Gates were positioned according to appropriate
isotype controls.

The patient cohort (80 samples from 24 donors) was split into those with no GvHD, acute GvHD only and *de novo* chronic GvHD. Patients with both acute and chronic GvHD were not included in this study. The levels of circulating CLA$^+$ CD4$^+$ T cells was seen to be significantly higher in patients with aGvHD than those without up to 100 days post transplantation (Figure 4.19). There was then a decline in levels back down to pre-transplant levels, with a second, however not statistically significant (p=0.0571), peak at 9 months post transplantation. The kinetic pattern of CLA expression on CD8$^+$ T cells was similar but to a much lesser extent. Patients who developed *de novo* cGvHD showed a sharp increase in CD4$^+$CLA$^+$ T cells from as early as day 28 post transplantation which remained significantly high throughout the 12 month period. The CLA expression on CD8$^+$ T cells remained comparable with or without cGvHD at all time points observed.
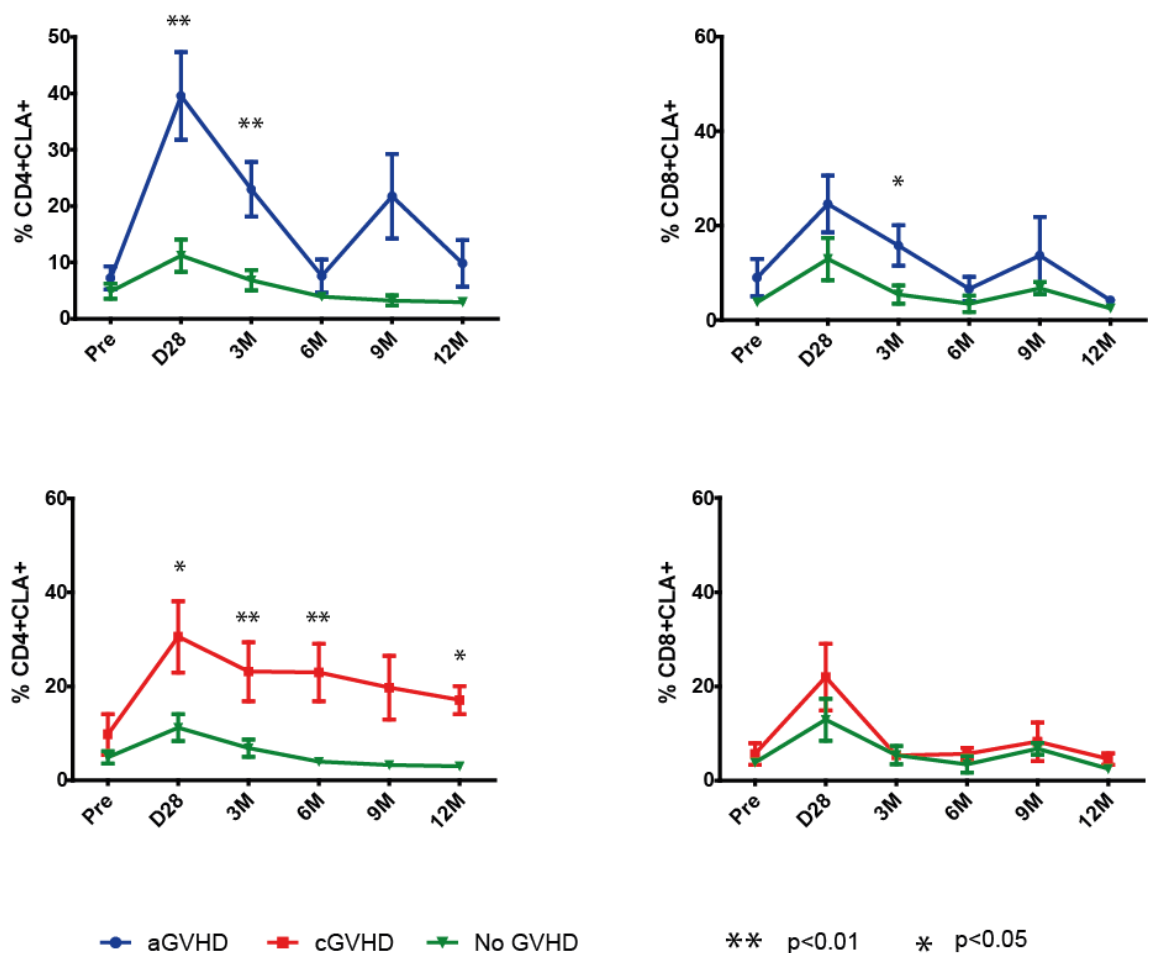
Figure 4.19 Expression of CLA on CD4$^+$ and CD8$^+$ T cells in serial samples following transplantation.
Plots indicate mean±SEM for 3-9 samples at each time point. Statistical significance was assessed by Mann-Whitney U tests.

CCR10 has also shown to be of importance in skin homing in GvHD therefore levels of CCR10 were also investigated on the patient samples. There was a trend showing increased CCR10 expression on both CD4^{+} and CD8^{+} T cells in patients with acute GvHD, however this was not statistically significant (Figure 4.20). CCR10^{+} T cells in patients who developed chronic GvHD were elevated on CD4^{+} T cells, however this difference did not achieve statistical significance.
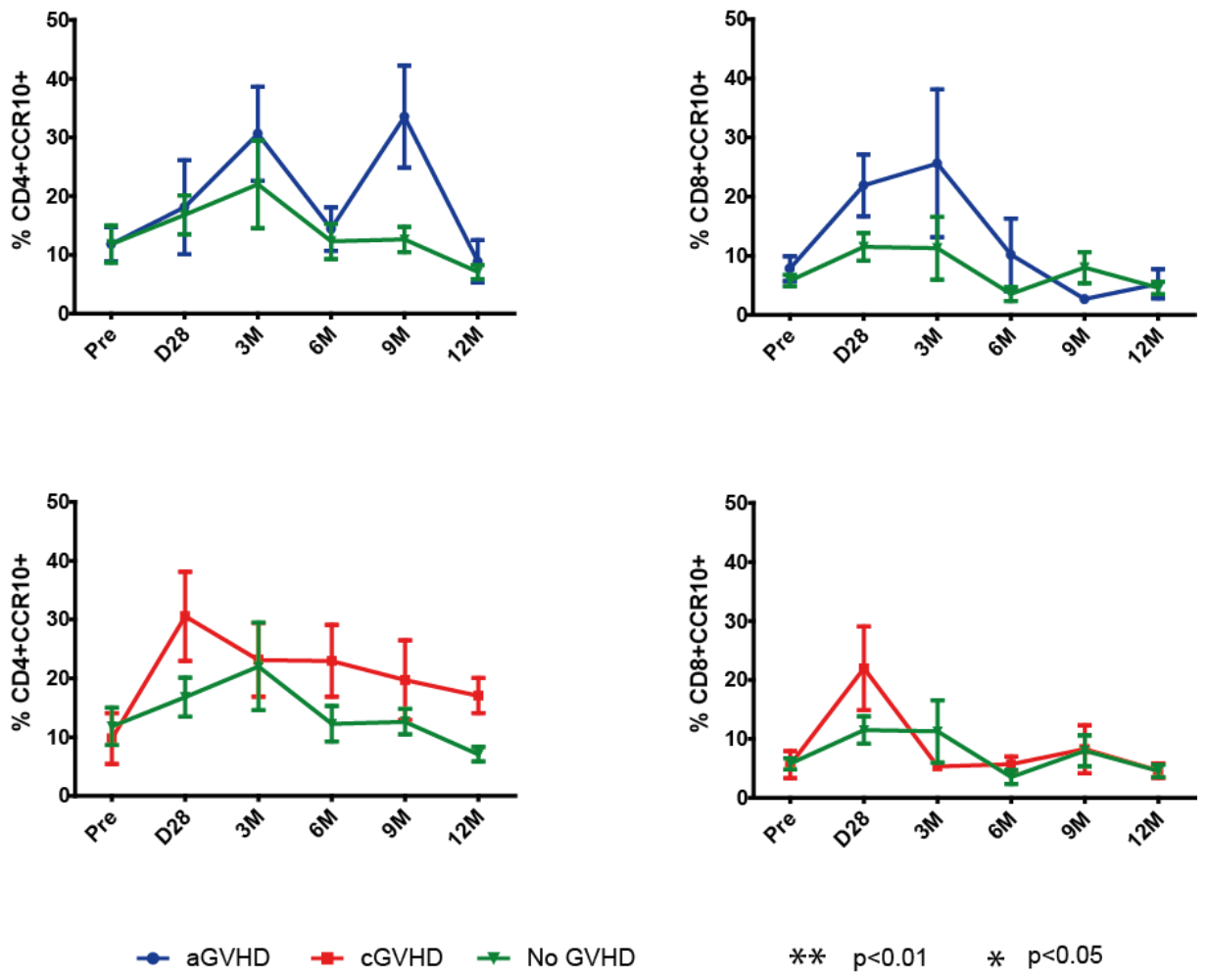
Figure 4.20 Expression of CCR10 on CD4^{+} and CD8^{+} T cells in serial samples following transplantation.
Plots indicate mean ±SEM for 3-9 samples at each time point, Statistical significance was assessed by Mann-Whitney U tests.

The levels of CXCR3 expressing cells within CD4$^+$ and CD8$^+$ T cells from the patient cohort was also assessed (Figure 4.21). A significant decrease in CD4$^+$ T cells expressing CXCR3 was seen in patients who developed aGvHD at 28 days post transplantation (p=0.0095). There was also a trend towards higher CD8$^+$CXCR3$^+$ cells at 3 months post transplantation in patients with aGvHD (p=0.0932) and a statistically significant increase at 12 months post transplantation, p=0.0317. Patients who developed cGvHD had increased levels of CD4$^+$CXCR3$^+$ cells 12 months post transplantation (p=0.0286) and increased CD8$^+$CXCR3$^+$ cells 6 months post transplantation (p=0.0051) when compared to patients who did not develop GvHD.
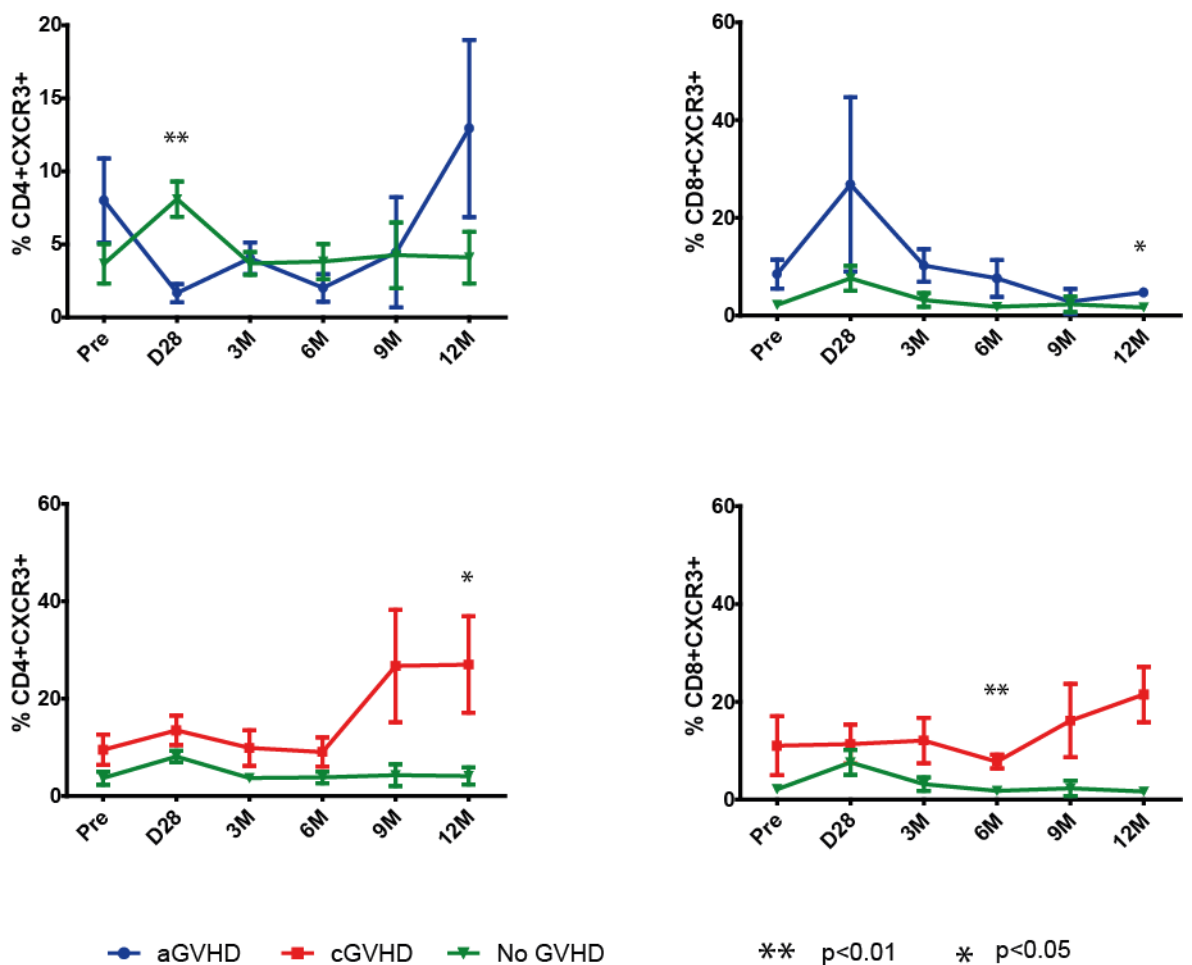
Figure 4.21 Expression of CXCR3 on CD4$^+$ and CD8$^+$ T cells in serial samples following transplantation.
Plots indicate mean ±SEM for 3-9 samples at each time point, Statistical significance was assessed by Mann-Whitney U tests.

In addition to the level of skin homing receptor expression, the Treg content of PBMC was assessed in the patient samples (Figure 4.22). At each time point there was no difference in the number of Treg cells in patients with aGvHD compared to those patients without GvHD. Patients who developed cGvHD did have an increased Treg content at 6 months post transplantation (p=0.0205).
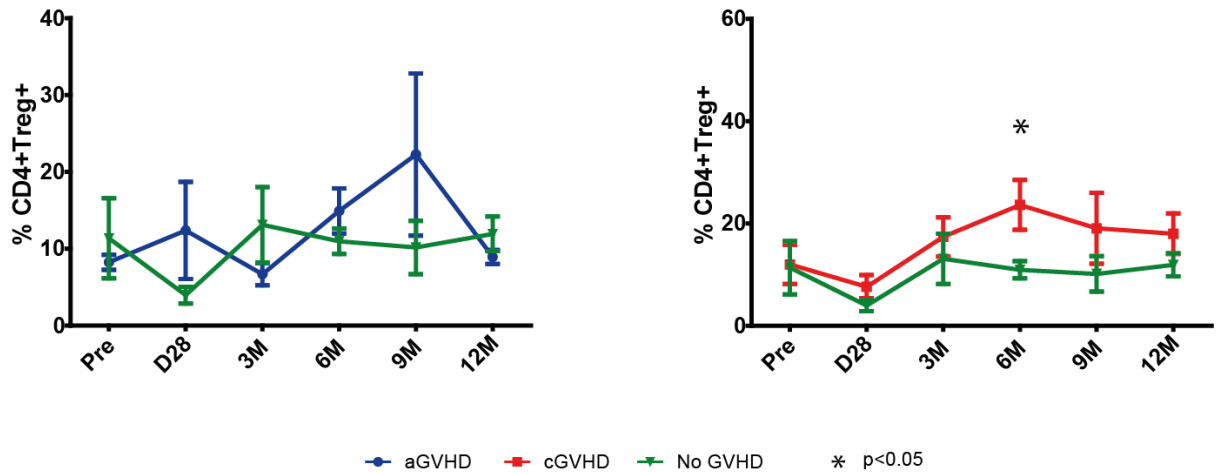
Figure 4.22 Treg content of the CD4+ T cell population in serial samples following transplantation.
Plots indicate mean ±SEM for 3-9 samples at each time point, Statistical significance was assessed by Mann-Whitney U tests.

4.4 Discussion

The beneficial role of Treg in ameliorating GvHD has been convincingly demonstrated using mouse models (Hoffmann et al., 2002, Edinger et al., 2003), *in vitro* studies (Wolf et al., 2007, Rezvani et al., 2006) and early stage clinical trials (Brunstein et al., 2011, Di Ianni et al., 2011). Undoubtedly Treg have an important role in the prevention of GvHD but there are still questions as to the exact mechanism of action. Protection against GvHD has been associated with modulation of various immunological events during the 3 phase pathophysiology of GvHD (Trenado et al., 2006, Wang et al., 2009, Taylor et al., 2004, Ermann et al., 2005) however, until now it was not known if this was also the case in humans. The research conducted in this chapter focuses on the more recently proposed fourth criteria for GvHD pathogenesis which refers to the requirement for effector cells to migrate to the target tissues and plays an important role in the development of GvHD.

Results presented in this chapter provide the first human *in vitro* evidence that Treg are able to prevent cutaneous GvH reaction by blocking the migration of effector T cells into the target tissues. The results have shown that addition of Treg during effector T cell priming resulted in a blockade of effector cell migration into the skin and therefore a reduction in cutaneous histopathological damage. Late addition of Treg, after allo-stimulation, did not cause a reduction in effector cell infiltration or GvH damage verifying that Treg must be present during priming to have the most potent effect, previous work from within our group has also shown late addition of Treg resulted in a loss of their ability to suppress T cell activation, proliferation and cytolytic function (Wang et al., 2009). This is reinforced by work in mouse models where it has been shown that Treg with the capacity to home to secondary lymphoid tissue are more potent inhibitors of GvHD, supporting our evidence that Treg presence during allo-stimulation provides superior protection (Ermann et al., 2005, Taylor et al., 2004).

Interactions between CLA and E-selectin are crucial in the movement of effector cells from the circulation into the skin and are therefore of great interest in the pathophysiology and the potential role of Treg in ameliorating GvHD (Kunkel and Butcher, 2002). The presence of Treg resulted in a highly significant down-regulation of CLA expression on allo-stimulated CD8$^+$ T cells.

The functional effect of this down regulation was seen in the highly significant reduction on the ability of CD8$^+$ effector cells to adhere to the CLA ligand E-selectin under flow conditions. The cellix platform used accurately models the flow conditions and shear forces which are seen *in vivo*, however, unfortunately it is beyond the remit of the skin explant model to conclusively demonstrate the Treg modulation of CD8$^+$ effector cell extravasation by impairing E-selection CLA interactions. The *in vitro* data suggests that *in vivo* initiation of the migration cascade by interactions between CLA and E-selecin could be inhibited in the presence of Treg resulting in the failure of effector cells to extravasate into target tissue.

The molecular pathway by which Treg mediate a reduction in cell surface expression of CLA on CD8$^+$ effector cells remains to be elucidated. To examine whether Treg suppression of CLA involves down-regulation of relevant genes which governs their expression qPCR was carried out, as CLA is a carbohydrate epitope it was the levels of the protein backbone, PSGL-1 mRNA, which were measured. Unexpectedly we found comparable levels of PSGL-1 mRNA expression on CD8$^+$ T cells regardless of allo-antigen stimulation. Consequently the presence of Treg did not result in any changes in PSGL-1 gene expression. CLA is formed via post-translational glycosylation of PSGL-1 by FuncTVII an α(1,3)-fucosyltransferase, found in leukocytes which express CLA (Fuhlbrigge et al., 1997). Therefore it is possible that allo-stimulation, and consequently Treg modulation of allo-stimulation, affects either the function or expression levels of FuncTVII thereby affecting downstream cell surface expression of CLA. Further experiments could examine the levels of FuncTVII mRNA and protein within the CD8$^+$ T cells, potentially providing further insight into the molecular pathways leading to Treg mediated suppression of CLA expression on the surface of allo-stimulated CD8$^+$ T cells.

Further support for the role of Treg in modulation of CD8$^+$ T cell migration as a mechanism for the prevention of GvHD came from the observation that reduced CXCR3 expression on allo-stimulated, in the presence of Treg, CD8$^+$ effector cells lead to a functional impairment of their migration. CXCR3 is a well-known receptor in the recruitment of effector T cells into the skin (Mohan et al., 2005). Early intervention of Treg was seen to impair *in vitro* CD8$^+$ T cell migration towards all three CXCR3 ligands with similar efficiency. Each of the 3

CXCR3 ligands have been shown to transduce different signals, dependent on distinct intracellular domains of CXCR3 therefore it is possible that Treg influence several pathways of signal transduction (Colvin et al., 2004). Further investigations to examine the signal transduction pathways could be carried out to elucidate the effect of Treg on CXCR3 signalling in response to CXCL9-11.

The presence of Treg not only reduced CD8^{+} effector T cell expression of CXCR3 but also the levels of CXCR3 ligands were reduced at the site of tissue damage. As CXCR3 guides activated T cells in to sites of cutaneous inflammation through interactions with its ligands CXCL9-11 reduction in both CXCR3 expression and its ligands is of great interest in the role of Treg in preventing GvHD. Although this study did not investigate levels of CXCR3^{+} infiltrates into the skin explant, Piper *et al* observed large numbers of CXCR3^{+} cells in dermal infiltrates of patients with GvHD (Piper et al., 2007), supporting our observation that Treg mediated reduction of CXCR3 expression on effector T cells and of CXCR3 ligand expression at the target tissue could results in decreased severity of cutaneous damage in the skin explant model.

The three IFNγ inducible ligands of CXCR3 are potent chemo-attractants which are produced by activated keratinocytes in skin (Flier et al., 2001), in particular CXCL10 has already been shown to be increased in the serum of patients with GvHD following transplantation (Piper et al., 2007). The results in this chapter showed a close relationship between the severity of GvH tissue damage in the skin explant model and the levels of CXCL10 and CXCL11 detected by immunohistochemistry, and significantly Treg mediated a reduction in CXCL10 and CXCL11 levels detected in the skin. In addition the levels of CXCL9 and CXCL10 in the skin explant supernatants were lower in the presence of Treg. This Treg mediated reduction in CXCR3 ligands may be attributed to the reduction in IFNγ by the CD8^{+} T cells.

Similarly, to CLA, gene expression was carried out to attempt to clarify the mechanism by which Treg caused a reduction on cell surface expression. Again, surprisingly, there were comparable levels of CXCR3 mRNA present following allo-stimulation and therefore the presence of Treg during allo-stimulation also had no effect on level of CXCR3 mRNA expression, suggesting

control of cell surface expression for CXCR3 must also occur post-transcriptionally.

Additional evidence that CXCR3 expression on CD8+ effector T cells is significant in the pathology of GvHD is provided by a study in murine models using CXCR3-/- donor grafts, they showed a marked reduction in CD8+ T cell infiltration into the gut of recipients associated with improved survival (Duffner et al., 2003). A more recent study used administration of a CXCR3 neutralizing antibody to reduce CD8+ T cell migration into GvHD target organs, ameliorating GvHD without adversely affecting GvL (He et al., 2008). However, the use of chemokine blocking molecules remains a significant challenge in the clinical setting (Proudfoot et al., 2010, Schall and Proudfoot, 2011). The use of Treg may therefore provide an exciting alternative therapeutic option for the prevention of chemokine driven migration of effector cells to the target tissue and therefore amelioration of GvHD.

Treg mediated reduction in the expression of skin homing markers along with reduced IFNγ production by effector cells and a decrease in CXCR3 ligands in the the skin, taken together this suggests that *in vivo* Treg modulation of effector cell migration forms an important role in the mechanisms by which Treg have a beneficial role in the prevention of GvHD following HSCT. Treg modulation of both the CXCR3 and CLA migration pathways shows the suppressive effect that Treg have on distinct but synergistically acting pathways involved in the migration of effector cells to target tissue in GvHD. Treg mediated reduction of CLA levels would interfere with CD8+ T cell rolling which directly impairs effector T cell extravasation whereas a decrease in CXCR3 expression impedes the capacity of the cells to migrate through the tissue following extravasation.

Results presented in this chapter support our proposed mechanism by which Treg are able to prevent cutaneous GvHD following HSCT (Figure 4.23). The presence of Treg during allo-stimulation causes the CD8+ T cells to have lower surface expression of skin homing markers CLA and CXCR3 and also a reduced capacity to produce IFNγ. The reduction in IFNγ production also causes CXCR3 ligands CXCL9-11 to be produced in lower quantities. The combination of less IFNγ, CXCR3, CLA and CXCL9-11 results in a lower

number of infiltrating effector cells into target tissue and lower histopathological damage *in vitro* which in the clinic would result in lower incidence and/or severity of GvHD. In the absence of Treg the effector cells are activated following allo-stimulation, expressing high levels of skin homing markers and producing high levels of IFNγ which in turn leads to the production of CXCL9-11, resulting in more tissue invasion and more severe GVH response.
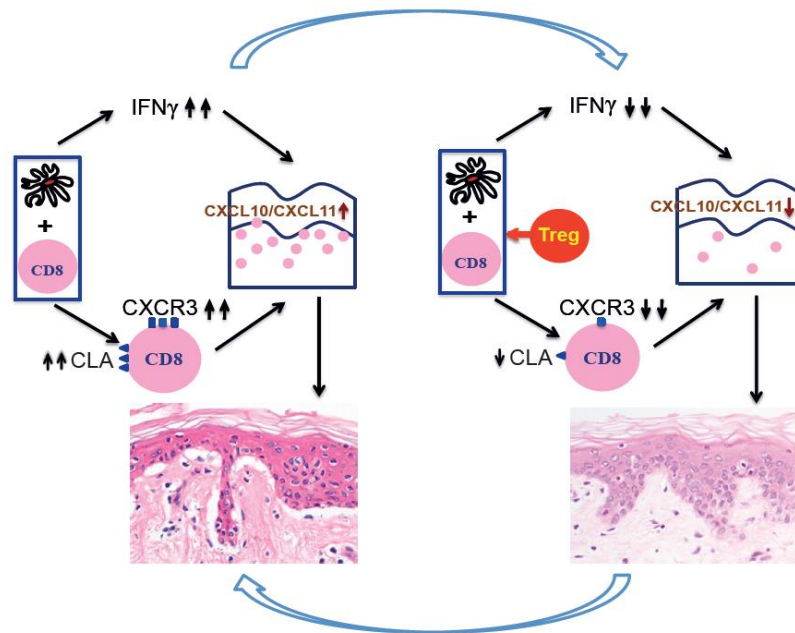
Figure 4.23 Proposed mechanism of Treg action in preventing cutaneous GvHD.

Once the importance of skin homing receptors had been demonstrated *in vitro*, clinical samples were investigated to examine any correlation between skin homing markers on circulating T cells and the GvHD status of the patient. Reports on patient PBMC phenotyping following transplantation are plentiful in the literature, therefore only a small scale patient study was carried out. Results obtained in this study are in concordance with previously reported data. The increase in CLA expression on the circulating $CD4^+$ T cells may be informative as an indicator for acute and chronic GvHD. It has previously been reported that the CLA^+ T cell fraction increases 3-7 days prior to onset of GvHD and could be potentially used as a biomarker (Tsuchiyama et al., 2009) . The expression of CLA^+ on $CD8^+$ T cells was increased in patients with GvHD, *in vitro* CLA^+CD8^+ cells are potent inducers of cutaneous GvHD, therefore it is not unexpected that patients with aGvHD have increased numbers of circulating $CD8^+CLA^+$ cells. This demonstrates the importance of skin homing markers in the clinical setting, supporting the proposed mechanism by which Treg are able to prevent GvHD

by reducing T cell migration into target tissues. Levels of CCR10$^+$ circulating cells were not increased in patients with GvHD, possibly because they had already migrated into the target tissue at the time when the blood samples were collected. Levels of Treg in the peripheral blood of post-transplant patients have been reported to be decreased in aGvHD, but increased in cGvHD (Wolf et al., 2007, Ukena et al., 2011, Zorn et al., 2005) however in this small cohort of patients there was no difference between the groups of patients, possibly due to the small numbers of samples from a heterogeneous cohort of patients.

Taken together the results from this chapter have shown that Treg modulation of skin homing receptor expression on effector cells is an important mechanism for the prevention of GvHD. Also results from a small scale study of patient samples have shown that increasing numbers of circulating T cells expressing skin homing markers can be associated with both acute and chronic GvHD at specific times post transplantation and that in some cases, such as CD4$^+$ T cell expression of CLA and CXCR3 at day 28 post transplant, could be predictive of GvHD.

Chapter 5 - Treg induced impairment of DC function and its impact on GvH reactions

5.1 Introduction

Results presented in Chapter 4 have highlighted two important issues. Firstly, Treg mediated blockade of effector T cell migration into GvHD target tissues contributes to their protective role and is a potential mechanism of action (Mavin et al., 2012). Secondly, Treg mediated protection requires their presence during effector T cell priming. This has led to the hypothesis that Treg modulation of DCs could be a key mechanism by which Treg exert their protective role in GvHD. Results presented in this chapter focused on how Treg conditioning of moDC was able to drive them towards a semi-mature, tolerogenic status. The Treg mediated changes in DC phenotype and function resulted in an impaired ability to stimulate allo-reactive effector T cells and therefore induced less cutaneous GvH tissue damage.

5.1.1 Dendritic cells and in vitro generated DCs

Dendritic cells (DCs) are a population of highly potent and specialised antigen presenting cells which lack expression of lineage markers CD3, CD14, CD19, CD20 and CD56. Since their discovery in 1973 the role of DCs has been increasingly characterised (Steinman and Cohn, 1973). DCs are involved in both the innate and adaptive immune response, and they are responsible for maintaining the balance between tolerance and immunity (Banchereau et al., 2000). Human dendritic cells are a heterogeneous population of cells with several distinct sub-populations, broadly defined as myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).

Due to the difficulties in isolating large numbers of DC from human peripheral blood various methods have been developed to generate DCs *in vitro*. Human DCs can be generated from CD34$^+$ hematopoietic progenitor cells by culturing with GM-CSF and TNFα (Caux et al., 1992). More recently Flt3-L and thrombopoietin have been shown to generate DC from CD34$^+$ progenitors (Chen et al., 2004). However monocyte-derived DCs are most commonly used in aiding immuno-biological research (Wang et al., 2009, Mavin et al., 2012) and development of DC-based immunotherapies for cancers (Schuler-Thurner et al., 2002, Di Nicola et al., 2009), autoimmune diseases (Harry et al., 2010) and

transplantation rejection (Macedo et al., 2013). The method used to generate DCs *in vitro* depends on the experimental requirements; GM-CSF is able to reliably produce large numbers of monocyte derived DCs (moDC) and Flt-3L is able to more accurately mimic in vivo development of DCs (Merad et al., 2013).

In vitro generation of moDCs has been established for 20 years (Sallusto and Lanzavecchia, 1994). Monocytes are available in sufficient numbers to be isolated by immuno-magnetic separation or FACS sorting from peripheral blood. Incubation of isolated monocytes with GM-CSF and IL-4 results in a moDC population similar to a CD1a$^+$ dermal DC (Merad et al., 2013). GM-CSF is currently considered to be a critical factor for DC development under steady state and inflammatory conditions (van de Laar et al., 2012). Intra-cellular signalling from GM-CSF can occur through JAK/STAT, MAPK, PI3K and NFκB to direct pathways inducing differentiation into moDC. Although the role of IL-4 in the differentiation into moDCs was initially unclear (Sallusto and Lanzavecchia, 1994) it is now believed that IL-4 functions to inhibit the growth of macrophages (Menges et al., 2005). These moDC are able to respond to a variety of pathogen-associated molecular patterns (PAMPS) through toll like receptors (TLRs). LPS stimulates moDC maturation through TLR-4 (Anderson et al., 2008), although moDC are also reported to be able to respond to stimulation from TLR7, TLR8 and TLR9 (Lombardi et al., 2009, Hoene et al., 2006). LPS acts through the TLR-4 signal transduction pathway, in either the MyD88 dependent or independent pathways (Kurt-Jones et al., 2000). LPS maturation results in a decrease in antigen processing and an increase in migratory capacity of moDC resulting in DCs able to promote expansion and differentiation of naïve T cells into effector cells (Joffre et al., 2009). moDCs were used throughout this study to stimulate allo-reactive CD8$^+$ T cells or drive naïve CD4$^+$ T cell polarisation.

5.1.2 Tolerogenic DC

It has been well recognised that DCs also have tolerogenic properties. Generation of tolerogenic DCs (TolDC) either *in vitro* or *in vivo* has been uncovered as a potential mechanism for the induction or maintenance of tolerance post transplantation (Ezzelarab and Thomson, 2011). TolDCs have also been designated as alternatively activated DCs (Anderson et al., 2008), modified DCs (Sato et al., 2003a) and regulatory DCs (Sato et al., 2003b).

TolDCs can occur naturally, often in immune-privileged sites such as the lung, eye or intestine (Svajger and Rozman, 2014). Additionally TolDCs can be generated *in vitro* by genetic engineering or controlling culture conditions with cytokines, growth factors and pharmacological agents (Hackstein and Thomson, 2004). The presence of these additional immuno-suppressive signals facilitates the development of TolDCs which are able to actively promote tolerance through the presence of inhibitory signals as well as lacking expression of co-stimulatory signals (Steinman et al., 2003). The key phenotypic features of TolDCs include expression of surface HLA molecules, low expression of co-stimulatory molecules CD80/CD86 and low secretion of IL-12p70 (Morelli and Thomson, 2007).

One important characteristic of TolDC is their ability to migrate towards CCL19 due to their expression of high levels of CCR7, this allows them to home to lymph nodes and exert their tolerogenic effects *in vivo* (Anderson et al., 2008). TolDCs are able to secrete indoleamine 2,3-dioxygenase (IDO) and induce regulatory T cells while promoting the death of effector cells via apoptosis (Morelli and Thomson, 2007). Additionally they express the immuno-suppressive molecules IL-10 and TFGβ which can lead to the induction of peripherally induced Treg (Lan et al., 2006, Wakkach et al., 2003). TolDCs are able to secrete heme oxygenase-1 (HO-1), which prevents maturation of other DCs and has been shown to induce tolerance in solid organ transplantation and prevent rejection (Soares et al., 1998). TolDC are also able to act in a contact dependent manner through the action of PDL-1 (Brown et al., 2003), HLA-G (Carosella et al., 2011) and Fas/FasL amongst others (Griffith et al., 1995).

5.1.3 *Treg interactions with dendritic cells*

Dendritic cells are one of the many cell types known to be influenced by Treg and therefore these interactions are of significant interest when considering the mechanisms by which Treg modulate GvH reactions. There is evidence to suggest that in murine models Treg are able to supress responder T cells indirectly through the modulation of DCs (Onishi et al., 2008, Fallarino et al., 2003). Interactions between human Treg and moDC have also been shown to severely affect the ability of the DC to stimulate and activate CD4^{+} T cells (Misra et al., 2004). More recent studies have demonstrated the ability of Treg to induce a semi-mature phenotype in DCs (Bayry et al., 2007); inducing down-

regulation of co-stimulatory molecules, and reduced capacity to induce allo-stimulation of CD4$^+$ T cells while increasing expression of CCR7 and HLA-DR. Additionally it has been shown that, as well as monocyte derived DC, Treg are able to modulate the function of *ex vivo* sorted myeloid DC (Houot et al., 2006).

Studies have shown that this semi-mature phenotype induced by Treg to be modulated through several different mechanisms. Firstly, activated Tregs express high levels of Lymphocyte-activation gene-3 (LAG3) which is related to the CD4 protein and binds HLA-class II molecules with a high affinity (Huang et al., 2004). LAG-3 is required for maximum Treg suppression, when it binds to class II molecules LAG-3 causes ERK mediated recruitment of SHP1 resulting in prevention of immature DC maturation and poor immuno-stimulatory capacity of human CD4$^+$ T cells and murine CD8$^+$ T cells (Bayry et al., 2007, Liang et al., 2008). Secondly, interactions between the co-inhibitory receptor CTLA-4 constitutively expressed on the Treg, and its ligands CD80/CD86 expressed on the DC, are able to modulate the action of the DCs (Vignali et al., 2008). CTLA-4 is able to capture its CD80/86 ligands from other cells via trans-endocytosis and degrade them, resulting in reduced capacity for co-stimulation (Qureshi et al., 2011). *In vitro* blockade of CTLA-4 does not appear to reduce Treg function, however *in vivo* CTLA-4 blockade nullifies Treg mediated suppression in several mouse models of inflammation (Tang and Bluestone, 2008). Thirdly, Treg have been shown to promote secretion of IDO by DCs (Grohmann et al., 2003). IDO is an enzyme commonly associated with pregnancy and immune evasion that acts by local depletion of tryptophan (Mellor and Munn, 2004). The deprivation of tryptophan leads to starvation of T cells, increasing apoptosis and preventing further local T cell proliferation. This Treg promotion of IDO expression in DCs has been shown to depend on interactions between CTLA-4 and CD80/86 (Fallarino et al., 2003). Although CTLA-4 and IDO provide important mechanisms of Treg modulation of DC, it has been shown through blocking CTLA-4 and IDO that these interactions are not functioning in the generation of semi-mature Treg conditioned moDC (Amarnath et al., 2010).

In addition IL-10 has been shown to play a significant role in Treg modulation of DC function in a mouse model through a MARCH1 dependent mechanism (Chattopadhyay and Shevach, 2013). MARCH1 is an E3 ubiquitin ligase found to degrade CD86 and class II expression on the cell surface by

ubiquitination and internalisation (Tze et al., 2011). However, it remains unknown if Treg are also able to modulate human DC functions through increasing expression of MARCH1 via IL-10.

It is not only regulation of DCs by Treg which is important in the maintenance of tolerance but also DC regulation of Treg. Figure 5.1 shows a mechanism proposed by Darrasse-Jeze *et al* by which Treg and DC are involved in a regulatory feedback loop (Darrasse-Jeze et al., 2009). Previously it had been shown that the peripheral Treg population expanded following treatment with Fms-like tyrosine kinase 3 ligand (Flt-3L) (Swee et al., 2009), and that loss of Treg increases DC numbers in a Flt-3L dependent manner (Liu et al., 2009). Treg are also able to regulate DC through the action of TGFβ and IL-10, but conversely DC are also able to regulate Treg in the presence of TGFβ (Sela et al., 2011). This illustrates the highly complex bi-directional regulation between Treg and DC, which results in the maintenance of tolerance.
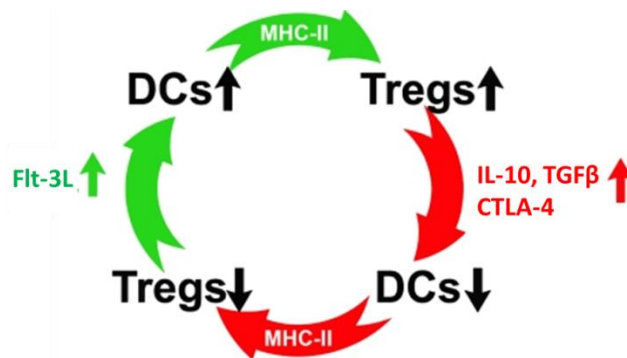
Figure 5.1 Homeostatic feedback loop between Treg and DCs (Darrasse-Jeze et al., 2009).

5.1.4 Regulatory DC and Treg conditioned DC in GvHD

DCs have a critical role in the pathophysiology of aGvHD as previously discussed. Various interventions specifically targeting DCs for the prevention and/or treatment of GvHD have been summarised in a recent review (Stenger et al., 2012). Despite advances in the understanding of the key features and the role of regulatory DCs in modulating autoimmunity, little is known in the current literature with regard to their effect on CD8^{+} T cell mediated allo-reactive immune responses, particularly in the context of GVHD in humans. Observations from one group have reported that administration of IL-10 and

TGFβ treated DCs protected mice from aGvHD and leukemia relapse (Sato et al., 2003b) as well as decreased incidence and severity of cutaneous cGvHD (Fujita et al., 2007). To date, no convincing evidence is available demonstrating whether Treg treated DCs are able to ameliorate human GvHD, although one report has demonstrated the GvHD protection by Treg treated DC's in a xenogenic GvHD model (Amarnath et al., 2010).

This chapter aimed to address whether Treg treatment of DC reduced CD8$^+$ T cell activation, proliferation and subsequent GvH damage. This question will be addressed via examining the following;

- The phenotype and morphology of Treg treated moDC.
- The ability of Treg treated moDC to induce allo-reactive CD8$^+$ T cell activation and proliferation.
- The capacity of CD8$^+$ T cells stimulated by Treg treated moDC to induce GvH tissue damage reaction in the skin explant model.
- Changes in naïve T cell polarisation induced by Treg treated moDC.

5.2 Methods

The experimental outline for results presented in this chapter as illustrated in Figure 5.2. The majority of methods are presented in Chapter 2; additional methods used only in this chapter are described below. Unless otherwise stated all data presented in this chapter was generated using a 3:1 ratio of Treg:moDC.
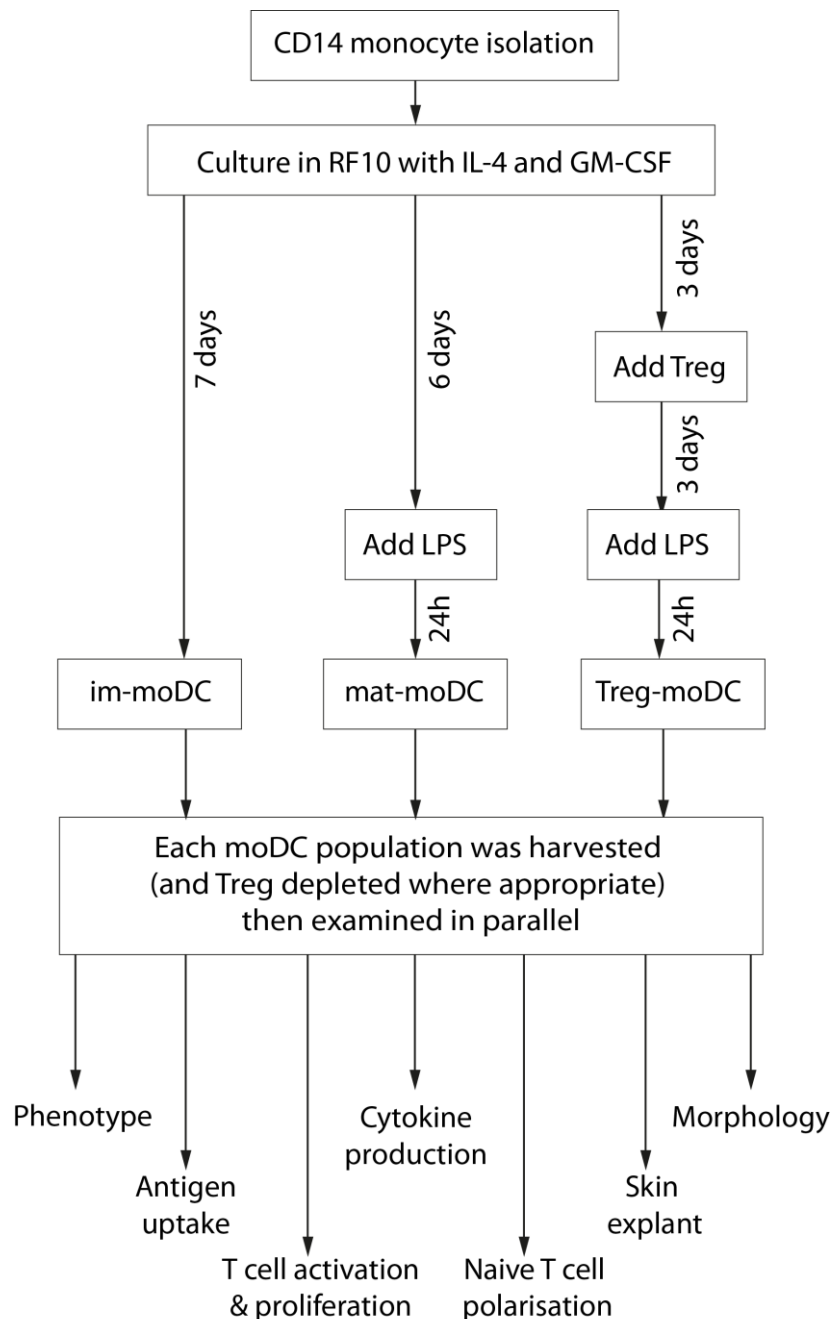
Figure 5.2 Summary of the experimental outline for results presented in this chapter. Phenotype was assessed by flow cytometry, antigen uptake by FITC dextran and morphology by cytopsin. T cell activation, proliferation, cytokine secretion, polarisation and the skin explant were all assessed following MLRs using third party responder cells.

5.2.1 Generation of moDC

Immature and mature moDC were generated according to standard protocols with IL-4 and GM-CSF. Treg conditioned moDC were generated by the addition of (unless otherwise stated) 3 Treg per 1 moDC on day 3 of generation, summarised in Figure 3.3. Prior to functional and phenotypic analysis Treg were separated from the Treg-moDC by cell sorting. For cell sorting Treg were identified by positive expression of CD3 and the Treg-moDC negative CD3 expression.

5.2.2 Antigen uptake assay

Antigen processing was measured by FITC-dextran uptake (Sigma-Aldrich). 5×10^4 immature, mature or Treg treated moDC were incubated for 1 hour at 37°C or 4°C in RF10 with 1mg/ml FITC dextran uptake. Cells were then extensively washed in cold FACS buffer and then the amount of FITC dextran was analysed by flow cytometry. Dead cells were excluded from analysis based on positive staining for either 7AAD or DAPI.

5.2.3 Assessment of cell morphology

Cytospin was used to assess the morphology of the moDC, 1×10^4 immature, mature and Treg conditioned moDC (following the removal of Treg) were spun in a cytospin centrifuge at 400g for 4 minutes onto coated microscope slides. Slides were then left to air dry before fixation in methanol for 15 seconds. Cells were then stained with geimsa red and blue for 10 and 45 seconds then rinsed in distilled water. Images were then taken using Axio imager.

5.2.4 Naïve T cell polarisation

Naïve CD4$^+$ T cells were isolated from PBMC using RoboSep automated CD4$^+$ selection kit (StemCell technologies). Naïve CD4$^+$ cells were incubated with either immature, mature or Treg-moDC for 6 days in RF10 at a ratio of 10 naïve cells per 1 moDC. Cells were then rested in RF10 supplemented with 25IU/ml IL-2 for a further 4 days. After resting, cells were either stained for IC levels of cytokines following PMA/Ionomysin stimulation, stored as cell pellets for RNA extraction or stimulated at a 1:1 ratio with CD3/CD28 T cell expandor beads for 24 hours and the culture supernatants were stored for CBA flex analysis of cytokine secretion.

5.3 Results

5.3.1 Generation of Treg-moDC

Immature, mature and Treg conditioned moDC (Treg-moDC) were successfully generated by culturing CD14$^+$ monocytes in RF10 supplemented with GM-CSF and IL-4 for 7 days. CD14$^+$ monocytes were successfully isolated from PBMC by miltenyi MACS technology to a high purity (Figure 5.3a). Monocyte and DC status were closely monitored by their expression of appropriate cell surface molecules. Following 6 days of culture the monocytes lost expression of CD14 and gained CD1a as expected indicating their differentiation into immature moDC (Figure 5.3b). DC maturation was induced by the addition of LPS for a further 24 hours. DC maturation status was confirmed by expression of DC maturation markers CD83/CD40, increased expression of co-stimulatory molecules CD80/CD86 and expression of the antigen presenting molecule HLA-DR.
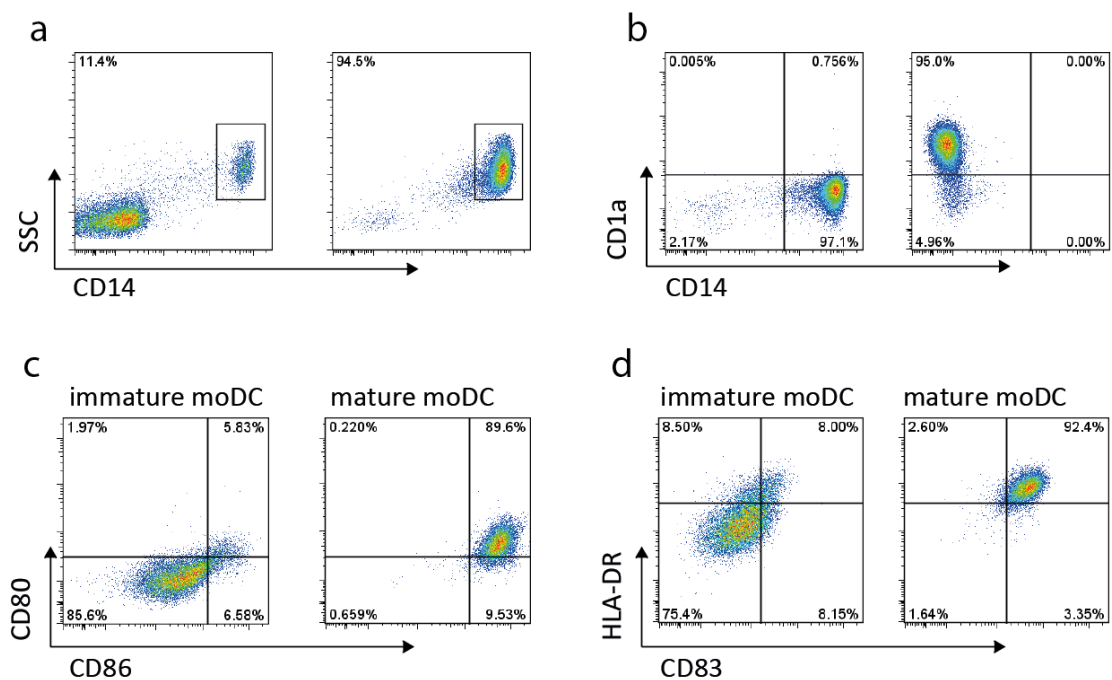
Figure 5.3 Steps in the generation of Treg-moDC.
a) Isolation of CD14$^+$ monocytes from PBMC, left prior to isolation and right post isolation. b) On D0 cells were CD14$^+$CD1a$^-$ (left) and following culture cells had lost CD14 and gained CD1a (right). c) and d) DC maturation was confirmed by expression of CD80, CD86, CD83 and HLA-DR. Representative data shown (n=10-22).

Timing of Treg addition

To identify the most appropriate time window to add Treg into the DC culture the phenotype profiles of Treg-conditioned moDC generated by adding Treg on either day 3 or day 6 were compared. LPS maturation was carried out on day 6 for both conditions. By the time Treg were added on day 3 of moDC culture over 80% of cells had already lost expression of CD14 and gained expression of CD1a suggesting the changes observed are due to modulation of moDC maturation rather than due to prevention of the monocytes differentiating into moDC. Adding Treg on day 3 resulted in a population of moDC which had decreased expression of CD83, DC86 and CD40 (Figure 5.4). This suggests these cells may have been resistant to LPS maturation. On the other hand, adding Treg on day 6 resulted in a population of moDC which expressed high levels of CD83, HLA-DR, CD86 and CD40 suggesting that these cells were not LPS maturation resistant and Treg need to be present for a longer time period to effectively condition the moDC.
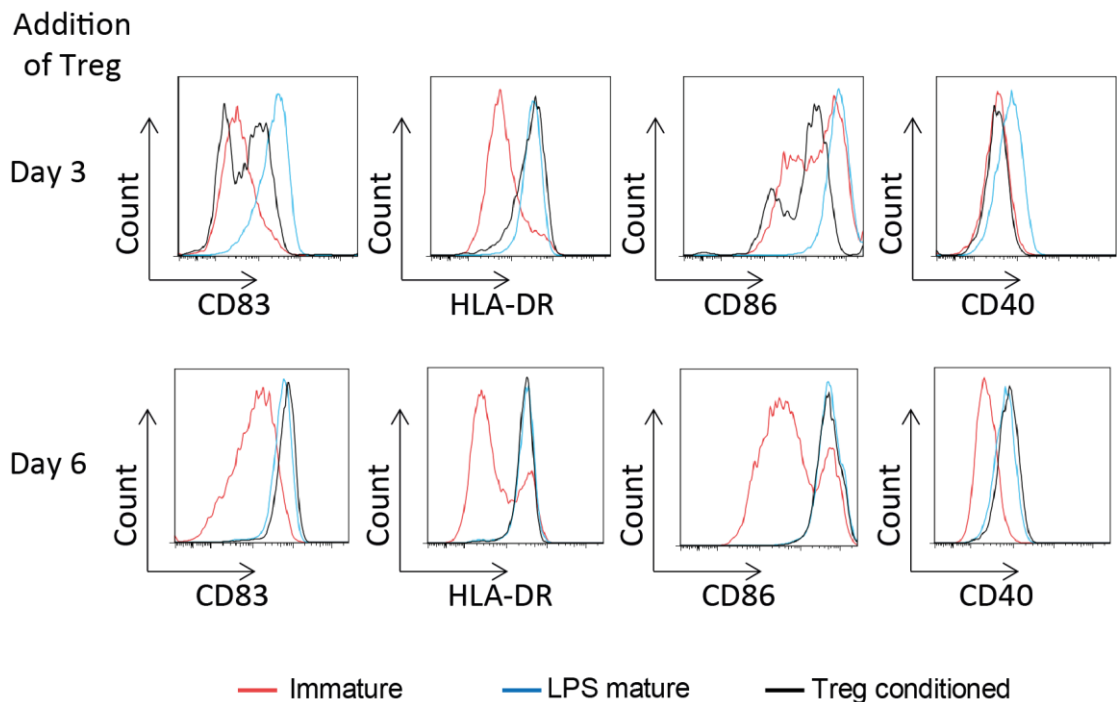
Figure 5.4 Late addition of Treg does not prevent LPS maturation of moDC.
Representative histograms show expression of maturation markers, measured by flow cytometry. Immature (▬) mature (▬) and day 6 Treg conditioned (▬). Data representative of 2 independent experiments.

Titration of Treg dose

Based on day 3 addition of Treg to the DC culture we next compared the phenotypic profile of DCs treated with titrated numbers of Treg ranging from 3:1 to 0.5:1 for DC:Treg ratios. Untreated moDC served as a control. A clear dose response was observed (Figure 5.5). A 3:1 ratio resulted in the greatest inhibition of maturation, with maturation marker MFI values similar to that of immature moDC. In addition, this ratio closely mimicked *in vivo* conditions therefore the ratio of 3 Treg :1 moDC was used throughout this work.
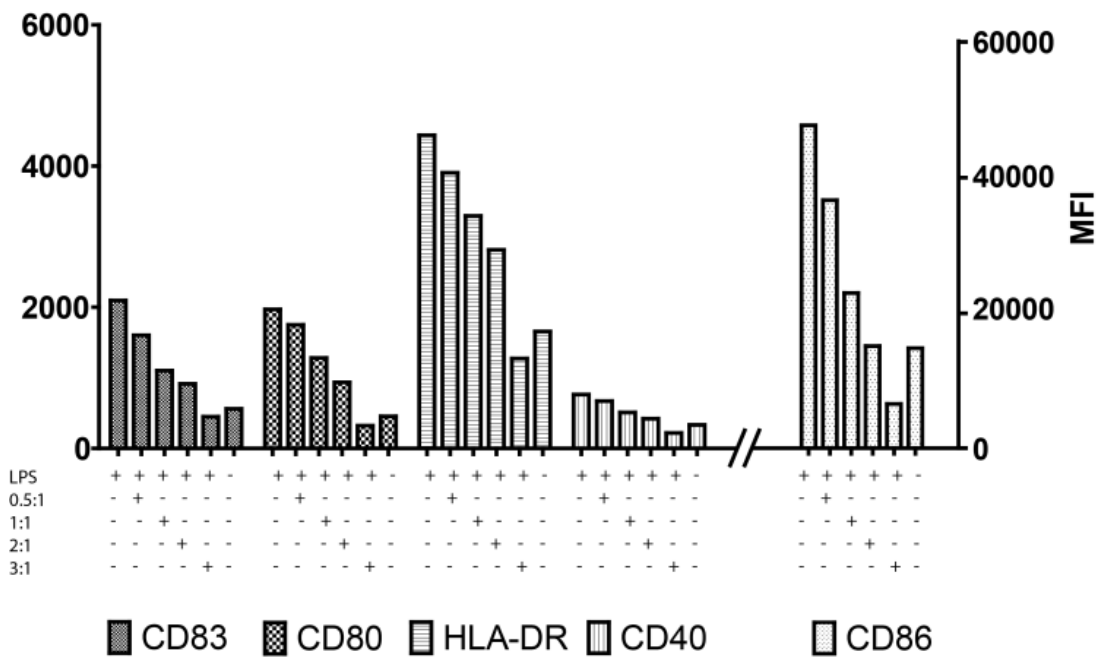
Figure 5.5 Treg prevent moDC maturation in a dose dependent manner.
Treg were added on day 3 at a range of ratios and MFI of maturation markers was assessed by flow cytometry. Bars indicate mean values from 2 independent experiments.

Removal of Treg from DC cultures

In order to carry out investigations into the functional capacities of the Treg conditioned moDC it was important to remove the Treg ensuring the functional results obtained were solely due to Treg mediated changes in moDC properties without a direct Treg effect on the T cells or DC-T cell interactions. Initially CD3 MACS separation was performed however the efficiency of the Treg depletion was poor (data not shown). Therefore cell sorting was used to remove Treg. Cells were stained with anti-CD3 APC and then sorted based on FSC and SSC along with negative expression of the T cell marker CD3, the gating strategy used for sorting is shown in Figure 5.6A. It was also important to ensure the process of sorting did not adversely affect the Treg conditioned moDC therefore detailed phenotypic analysis prior to and after sorting was carried out. Expression of CD80, CD83, HLA-DR and CD86 was unchanged following cell sorting (Figure 5.6B)
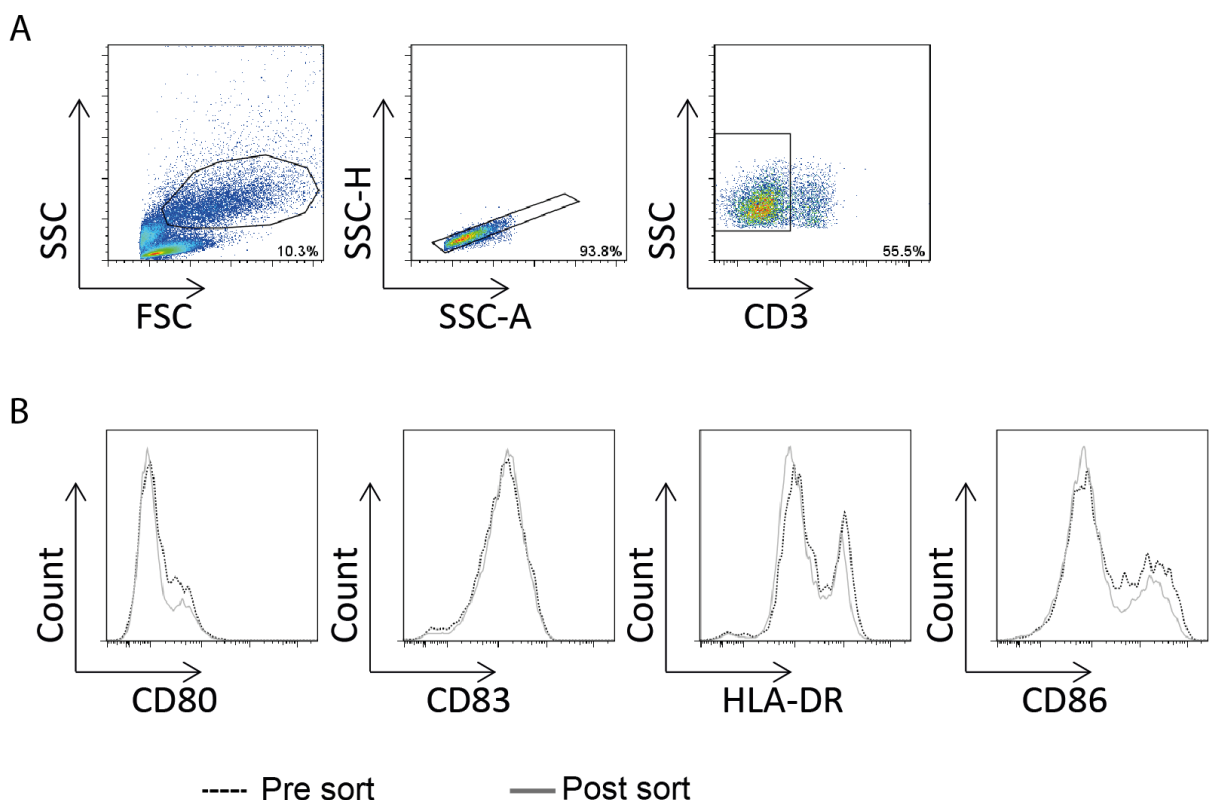
Figure 5.6 Cell sorting to separate Treg-moDC from the remaining Treg.
A) Gating strategy for cell sorting, moDC were selected based on FSC SSC profile, and then single cells (SSC-A vs SSC-H) followed by cells not expressing CD3. B) Representative Histograms of cell surface maturation markers on Treg-moDC pre-sorting (▬ ▬ ▪) and post sorting (▬▬▬) (n=10).

Data from this pilot study was used as the basis for the experiments performed in this chapter.

5.3.2 Treg conditioned DC exhibit immature phenotype

The effect of Treg conditioning on the LPS maturation of moDC was assessed by flow cytometry and analysed using mean fluorescence intensity of several maturation markers. Both immature and un-treated mature moDC were used as comparisons throughout. As expected, immature moDC expressed low levels of CD80, CD83, HLA-DR, CD86 and CD40 on their cell surface. Upon maturation with LPS expression of all of these markers significantly increased confirming that the moDC had a mature phenotype (Figure 5.7).

The phenotype of the Treg conditioned moDC was semi-mature, levels of CD80, CD83 and CD86 were much lower than that of the mature moDC however levels were also significantly higher than the immature moDC. Levels of CD40 on the Treg conditioned moDC were not significantly different from that of the immature moDC (p=0.0890) but it did show a trend towards higher CD40 expression on Treg-moDC. Levels of HLA-DR on the Treg-moDC were similar to that of the mature moDC (p=0.6357).

Overall the addition of Treg on day 3 of moDC generation resulted in a significant change in the phenotype of the moDC following exposure to LPS, as assessed by flow cytometry. The cells appeared to have a semi-mature moDC phenotype, consistent with previous publications (Misra et al., 2004, Bayry et al., 2007).
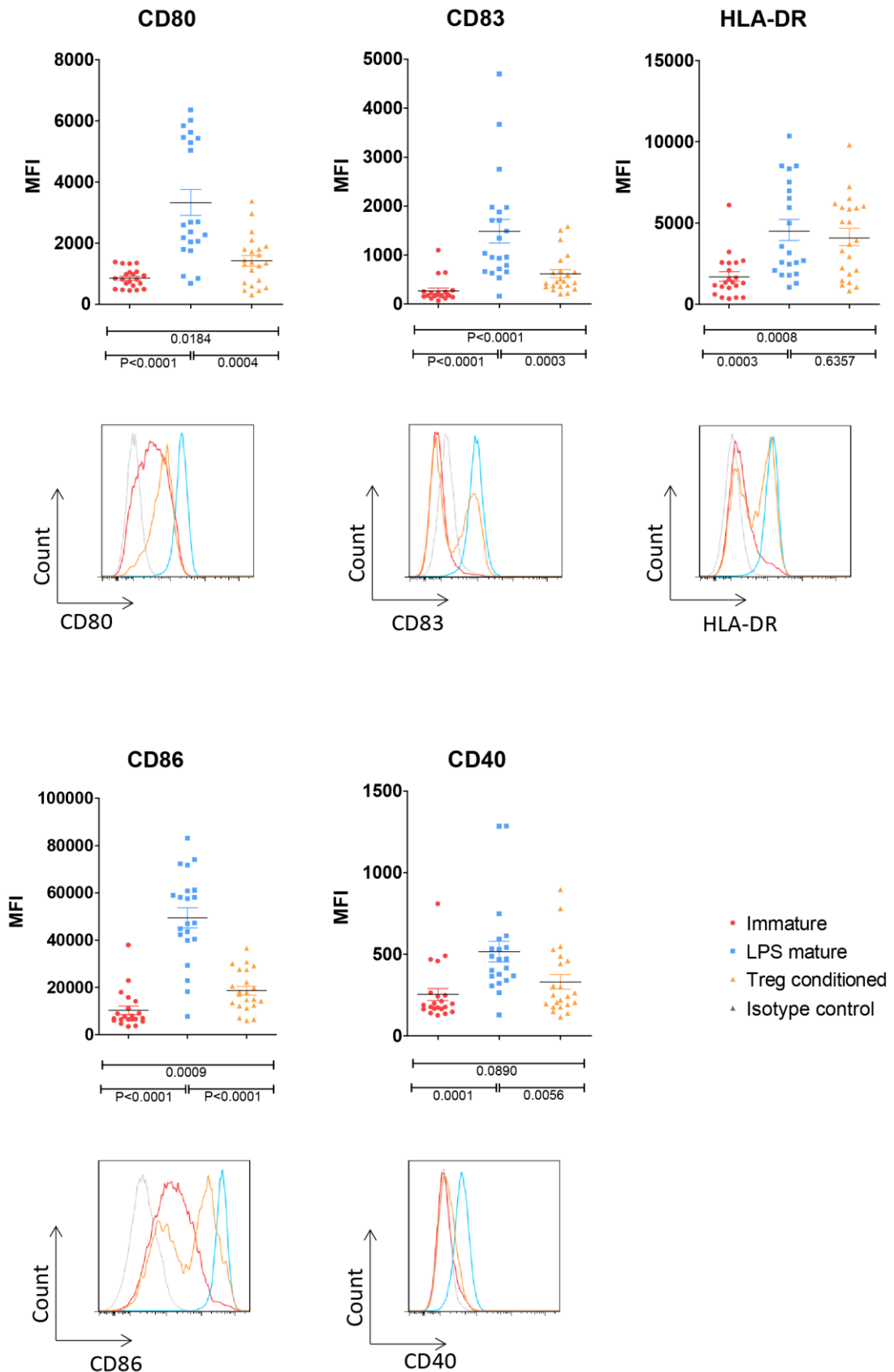
Figure 5.7 Treg modulation of moDC maturation.
Cumulative (Top) and representative (Bottom) expression of surface markers CD80, CD83, HLA-DR, CD40 and CD86, determined by flow cytometry on immature, mature and Treg-moDC. Graph shows mean ±SEM for n=20-22. Mann-Whitney U values are shown.

Following the finding that early addition of Treg was important the next question was to assess if the effect of Treg on moDC maturation was contact dependent. Previous work has suggested that Treg modulation of DC is contact dependent however due to the complex nature of Treg and DC interactions the requirement for contact between the moDC and Treg was further assessed using Transwell assays. moDC were generated in the lower well and Treg added to the upper well on day 3. When there was no direct cell-cell contact between the moDC and Treg expression of CD80, CD83, CD86, HLA-DR and CD40 increased to a level similar to that of moDC matured in the absence of Treg (Figure 5.8). When there was direct cell-cell contact Treg conditioned moDC retained an immature-like phenotype following LPS maturation. This showed that in this experimental setting Treg modulation of moDC maturation occurred in a contact dependent manner and future experiments were carried out with direct cell-cell contact between the moDC and Treg.
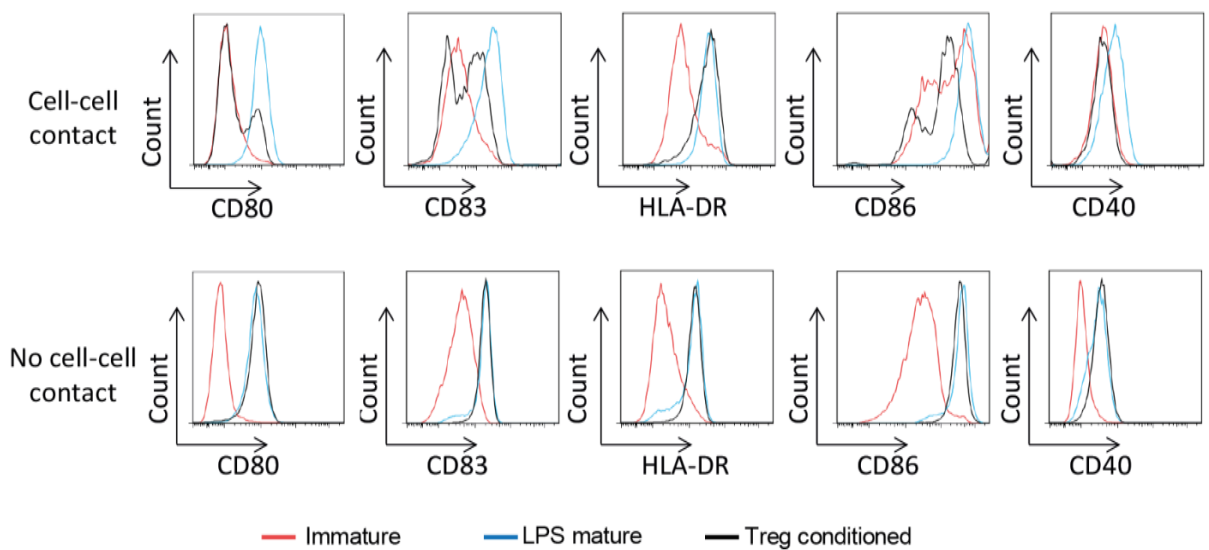
Figure 5.8 Treg need direct cell-cell contact to influence moDC maturation in response to LPS.
moDC were Treg conditioned with direct cell-cell contact (top panel) and in a Transwell system without direct cell-cell contact (bottom panel). Representative histograms show expression of maturation markers, measured by flow cytometry. Immature DC (▬) mature DC (▬) and Treg conditioned DC (▬). Data representative of 2 independent experiments.

5.3.3 Treg conditioned moDC express markers associated with immune modulatory function

The cell surface expression of TGFβ is an effective mechanism employed by various cell types with immuno-modulatory properties. Levels of LAP-TGFβ expressed on the cell surface of the moDC were measured by flow cytometry. LAP-TGFβ is the latent form of TGFβ which can be activated by proteases, a pH change or through the action of integrins (Tran, 2012). Expression levels of LAP-TGFβ were similar on the immature and mature moDC, however the Treg conditioned moDC had increased levels of cell surface TGFβ when compared to the mature moDC (p=0.0396). In addition Treg conditioned moDC showed a trend towards higher expression of CCR7 when compared to the mature moDC, although this was not statistically significant.
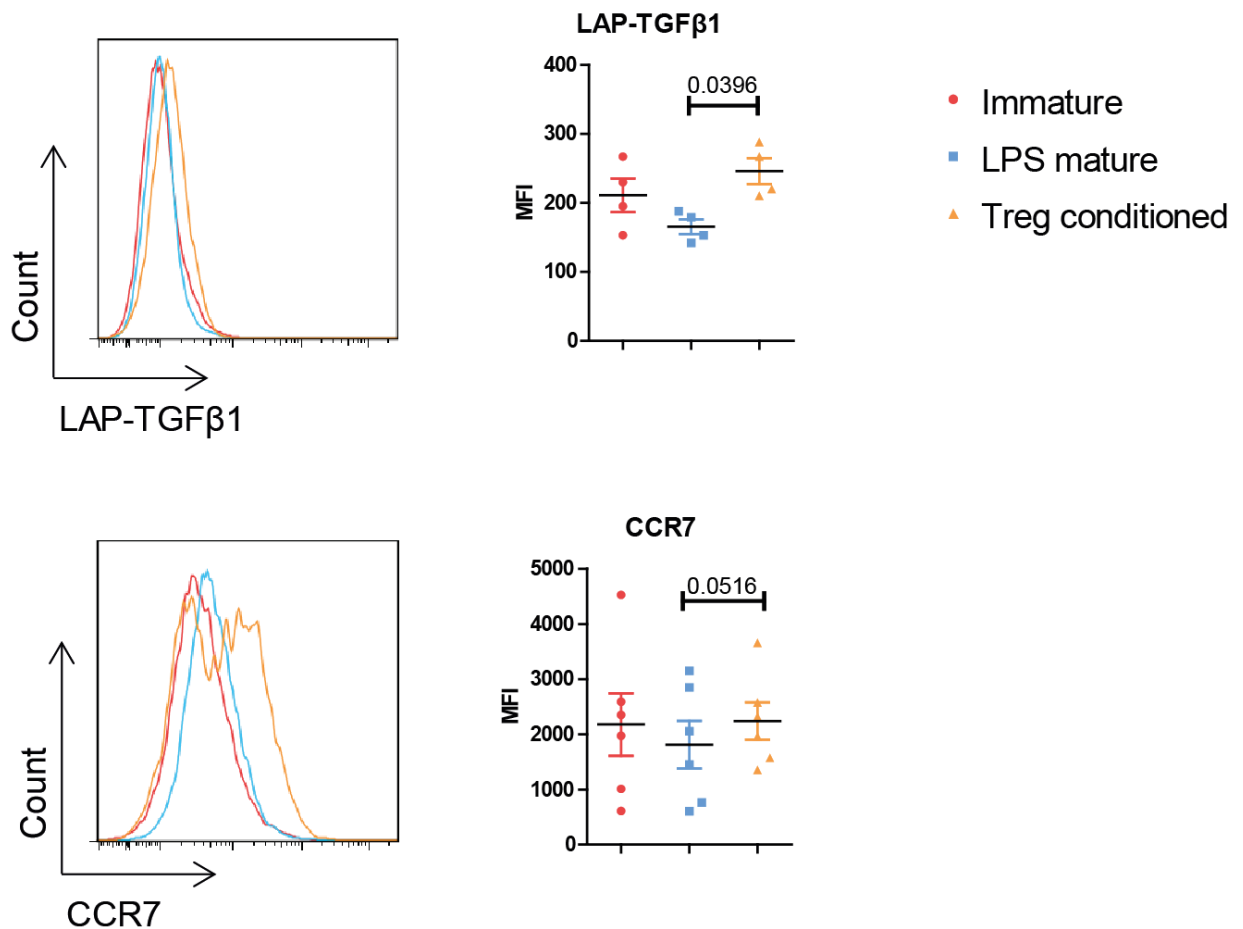
Figure 5.9 Treg moDC express increased levels of LAP-TGFβ and CCR7 on their cell surface.
Left– representative histogram and right- cumulative levels of expression analysed by flow cytometry. Live cells were selected prior to histograms shown. N=4-6. Paired t test values shown

5.3.4 Treg conditioned moDC secrete lower levels of cytokines

Mature DCs produce high levels of the pro-inflammatory cytokines IL-6 and IL-12p70 (Lutz and Schuler, 2002) therefore levels of IL-6, IL-12p70 and additionally the anti-inflammatory cytokine IL-10 were measured in the culture supernatants following generation of moDC and Treg-moDC.

Levels of IL-6 were low in the supernatant collected from immature moDC; 143pg/ml±102SEM (Figure 5.10A). The levels of IL-6 secreted by Treg-moDC were higher than that those of the immature moDC, but significantly lower than that secreted by non-Treg treated mature moDC, 702pg/ml±444SEM and 12703pg/ml±4208SEM respectively (p=0.02, Mann-Whitney U test). The IL-6 detected in the Treg-moDC was assumed to originate from the moDC rather than the Treg as IL-6 levels were below the detectable range in the Treg alone control culture.

Compared to mature moDC, the Treg conditioned moDC produced significantly lower IL-10, 141pg/ml±77.7SEM and 5.43pg/ml±2.16SEM, respectively (p=0.0317) (Figure 5.10B). The levels of IL-10 produced by the Treg-moDC was comparable to that produced by immature moDC (6.72pg/ml±3.55SEM) and the levels of IL-10 detected for Treg only were very low (0.48pg/ml±0.18SEM), therefore it can be assumed that the majority of the IL-10 present in the Treg-moDC culture was derived from the Treg-moDC.

Treg-moDC produced 4.65pg/ml±4.27SEM of IL-12p70 which was significantly less than mature moDC (530pg/ml±242SEM) (p=0.0079, Mann-Whitney U test). IL-12p70 was not detectable when Treg were cultured alone and immature DC only produced 0.56pg/ml±0.56SEM (Figure 5.10C).
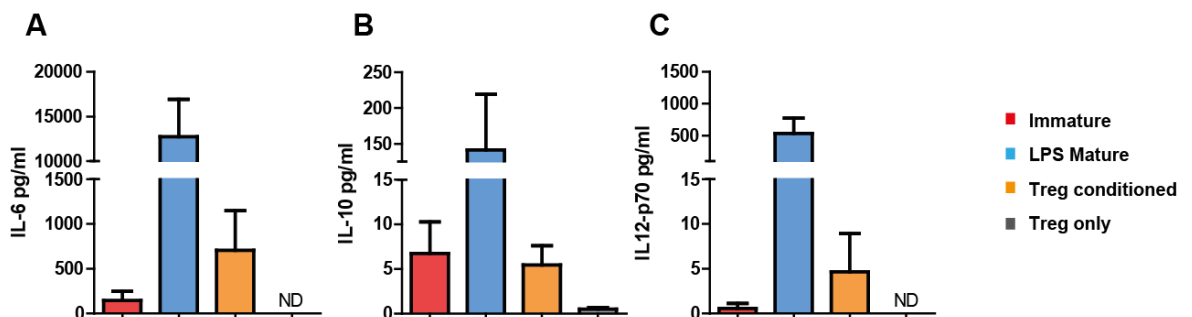
Figure 5.10 Treg-moDC produce lower levels of pro-inflammatory and anti-inflammatory cytokines.
Cell culture supernatants were collected on day 7 of moDC generation and analysed by CBA flex. Data acquired on FACS Canto II and analysed with FCAP array. Error bars indicate SEM, n=5 independent experiments. ND not detectable.

5.3.5 Treg conditioned moDC have a morphology similar to immature moDC

Further to assessment of cell surface maturation markers on the moDCs cytopsins with giemsa staining were carried out to assess the morphology of the cells. The Treg treated moDC appeared to have a morphology which was similar to the immature moDC rather than the mature moDC (Figure 5.11). Therefore this data supports that obtained from flow cytometry for maturation markers.
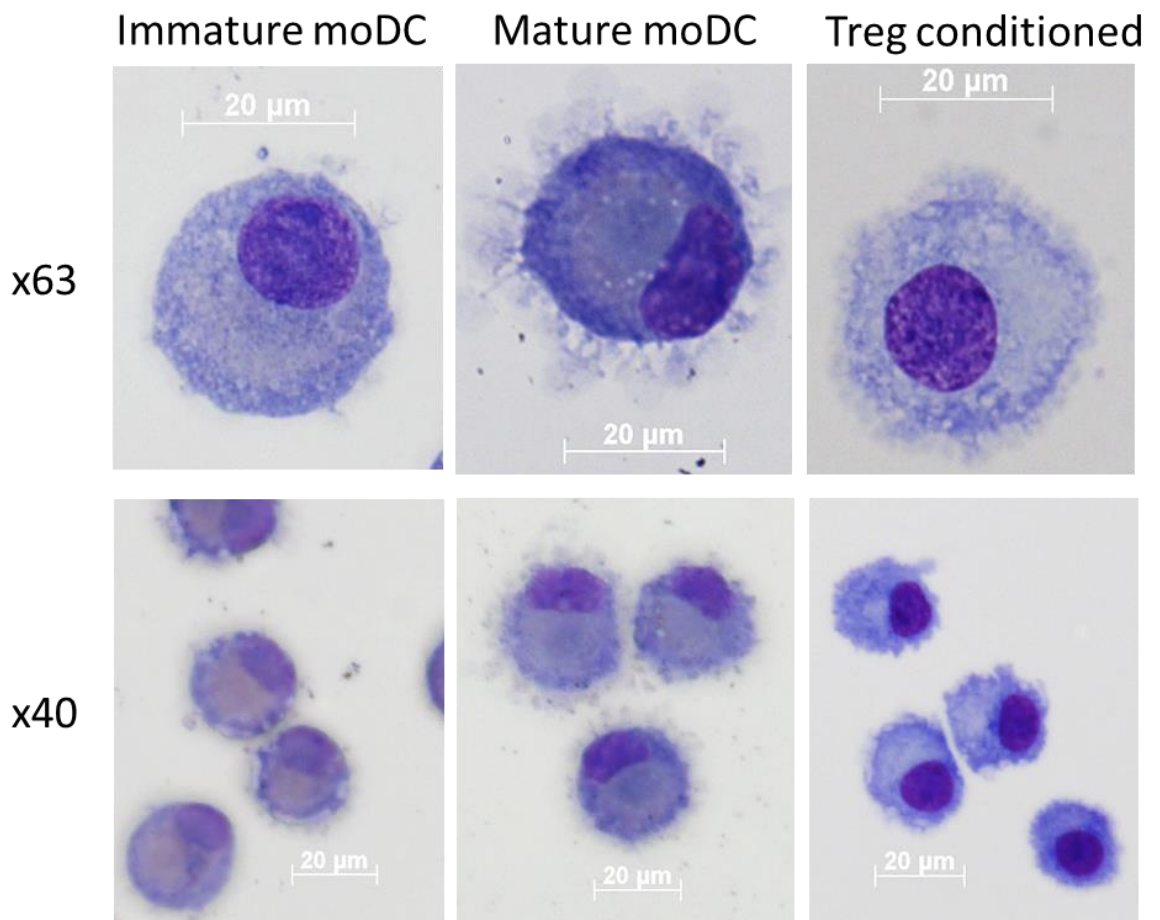
Figure 5.11 Treg treated moDC retain an immature-like morphology following treatment with LPS.
Immature, mature and Treg conditioned moDC were stained with giemsa red and blue, images representative of 5 independent experiments.

5.3.6 Treg conditioning results in impaired antigen processing by moDC

Once it was established that Treg conditioning affected both the phenotype and morphology of moDC further testing was carried out to investigate the effect Treg had upon the functional properties of moDC. FITC dextran uptake was used to measure the antigen processing capacity of the moDC. Unsurprisingly immature moDC took up the greatest amount of FITC dextran, which was significantly higher than that shown for mature and Treg conditioned moDC (p= 0.0038 and 0.0063 for mature and Treg conditioned moDC respectively). Interestingly, immature moDC also lost their ability to uptake FITC dextran when treated with Treg (p=0.0474) (Figure 5.12).

Although the Treg conditioned moDC had what appeared to be an immature phenotype and morphology, the amount of FITC dextran uptake was not significantly different from that of mature moDC. As antigen processing is typically carried out by immature moDC Treg treated immature moDC were included as an additional control. These cells were generated as the normal Treg treated moDC but without the addition of LPS for the final 24 hours of culture. These cells, when analysed by flow cytometry, appeared to be immature according to levels of maturation marker expression but again had reduced FITC dextran uptake compared to untreated immature moDC. This provides evidence that the presence of Treg results in a reduced capacity of moDC to process antigen rather than just arresting maturation.
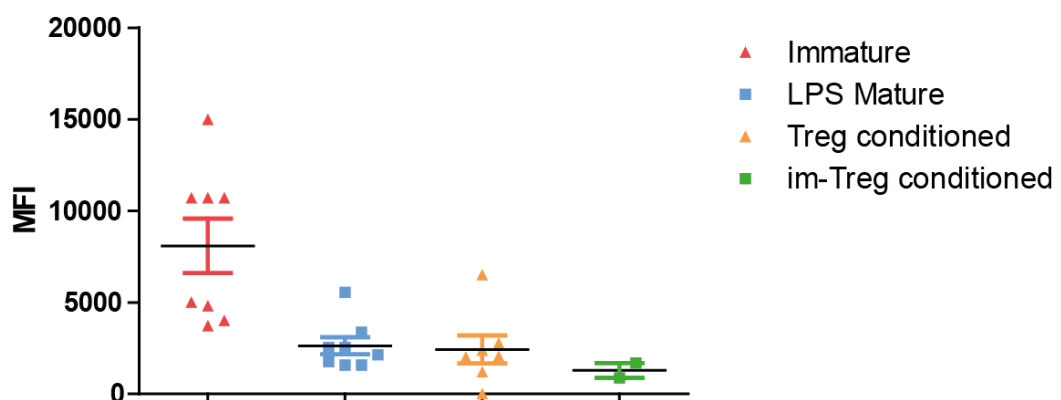
Figure 5.12 Treg treated moDC have impaired FITC dextran uptake.
Antigen processing was calculated by the amount of FITC dextran take up by immature (▲), mature (■), Treg conditioned (▲) and Treg conditioned immature (■) moDC, measured by flow cytometry. N=3-8 for each condition.

5.3.7 Treg conditioned moDC exhibit a reduced capacity to stimulate allogeneic CD8⁺ T cell activation and proliferation

Following transplantation the allo-stimulation and proliferation of donor effector cells is crucial for the development of GvHD. Therefore the capacity of Treg conditioned moDC to induce allogeneic CD8$^+$ T cell activation and proliferation was investigated. CD8$^+$ T cell activation was measured by levels of CD25 expression on the cell surface after 5 days of allo-stimulation. Unstimulated CD8$^+$ T cells showed minimal CD25 expression (0.73%±0.12SEM). Stimulation of the CD8$^+$ T cells with immature moDC resulted in 30%±4.54SEM of cells becoming activated, and stimulation with mature moDC resulted in the highest levels of CD8$^+$ T cell activation with 40%±2.17SEM of cells expressing CD25. Following stimulation with Treg conditioned moDC only 26%±3.19SEM of cells expressed CD25, a significant decrease compared to mature moDC (p=0.0090). As expected the CD8$^+$ T cells which were allo-stimulated with mature moDC in the presence of Treg showed low levels of activation (only 6%) as reported in Chapters 3 and 4 and this was significantly lower than the Treg conditioned moDC (p=0.0085) (Figure 5.13).
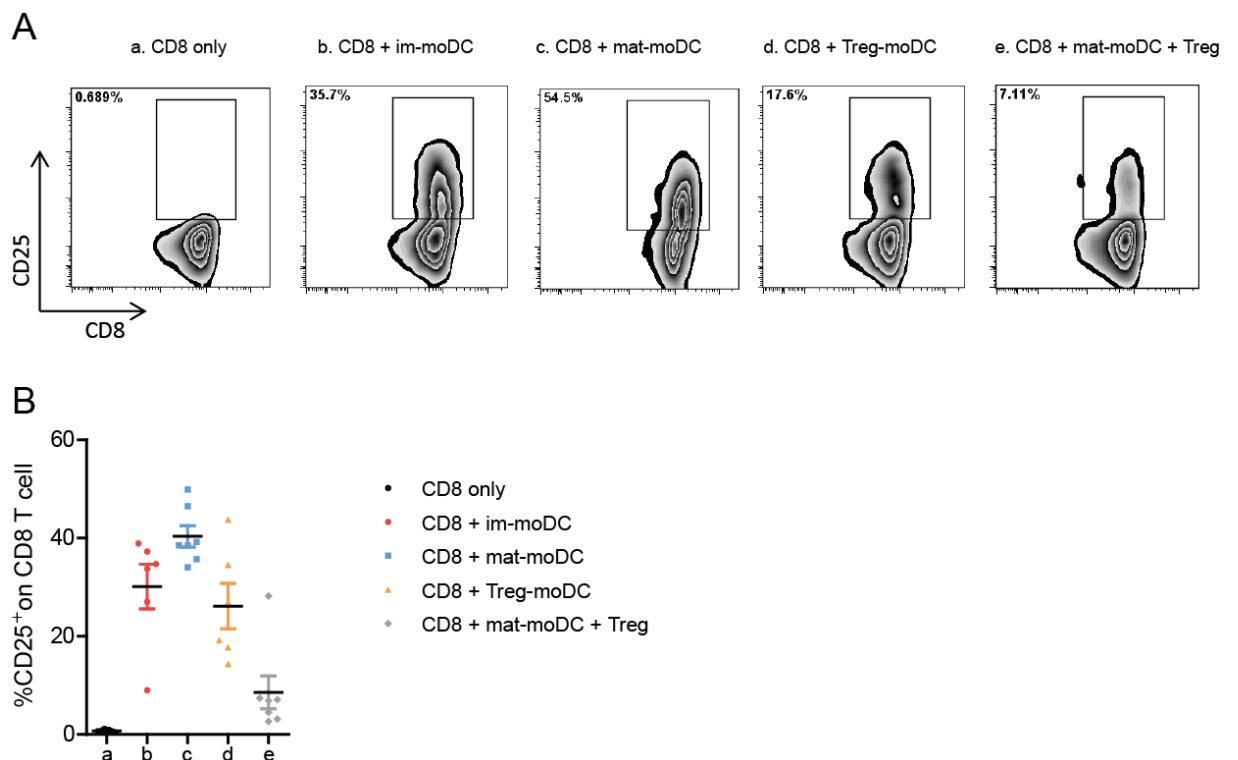
Figure 5.13 Treg-moDC have a reduced capacity to induce allo-stimulated CD8+ T cell activation.
A) Representative plots and B) cumulative data (a) unstimulated CD8+ T cells, CD8+ T cells stimulated with (b) immature (▲), (c) mature (■), (d) Treg conditioned (▲) and (e) mature moDC in the presence of Treg (■), measured by flow cytometry. Treg conditioned moDC (Treg-moDC).

The levels of CD8^{+} T cell proliferation was assessed by CFSE dilution over 5 days. Unstimulated CD8^{+} T cells showed background levels of proliferation of only 0.3%±0.09SEM. Upon stimulation with immature and mature moDC proliferation levels were 20%±5.12SEM and 30%±5.02SEM respectively. The Treg treated moDC resulted in only 23%±3.85SEM of CD8^{+} T cells proliferating, a significant reduction compared to the untreated mature moDC (p=0.0467) (Figure 5.14). Again the CD8^{+} T cells allo-stimulated in the presence of Treg showed significantly less proliferation (5.06%±1.23SEM) than the CD8^{+} T cells stimulated by Treg conditioned moDC (p=0.0085).
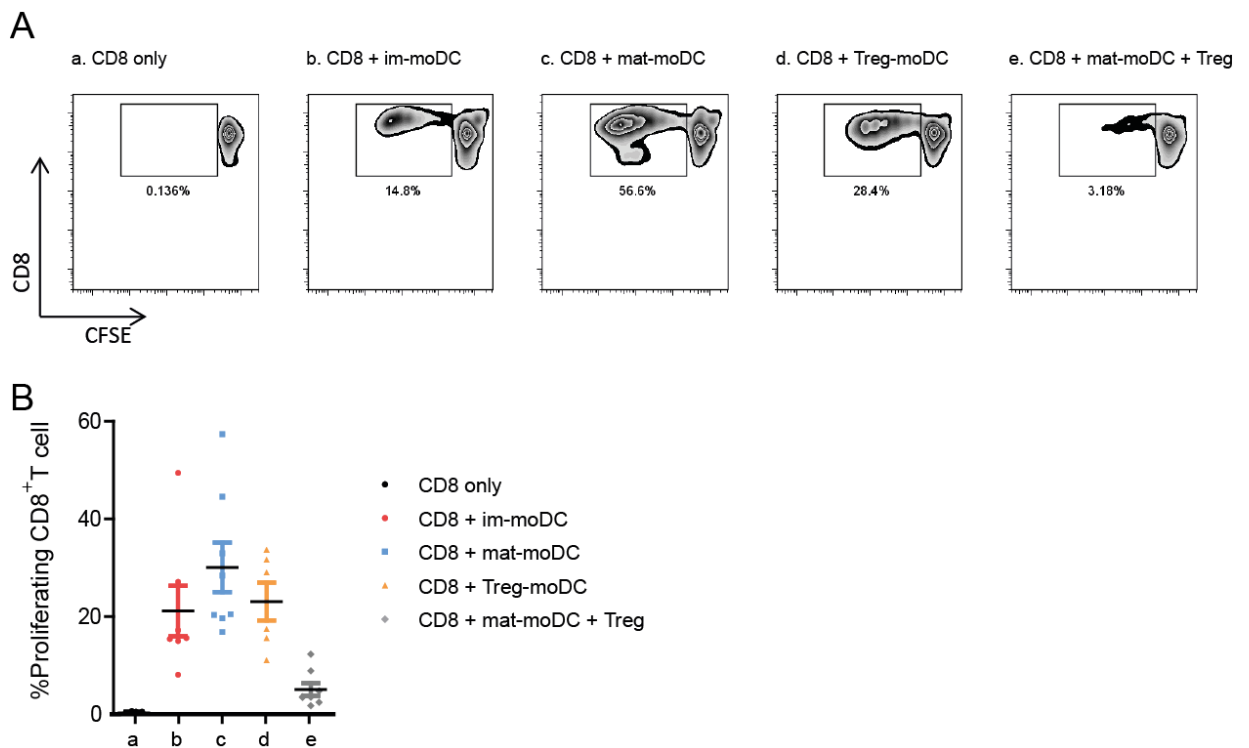
Figure 5.14 Treg-moDC have a reduced capacity to induce allo-stimulated CD8^{+} T cell proliferation.
A) representative and B) cumulative data for (a) unstimulated CD8^{+} T cells, CD8^{+} T cells stimulated with (b) immature (▲), (c) mature (■), (d) Treg conditioned (▲) and (e) mature moDC in the presence of Treg (■), measured by flow cytometry. Previously to plots shown, gates were applied on live CD8^{+} cells. Treg conditioned moDC (Treg-moDC).

Increased CD8^{+} T cell activation is associated with increased CD8^{+} T cell proliferation for immature, mature and Treg conditioned moDC. Treg treatment of moDC results in an impaired capacity to induce CD8^{+} activation and proliferation which is an important factor in the pathogenesis of aGvHD induction following HSCT.

5.3.8 *Treg conditioning of moDC prior to MLR results in reduced GvH severity in the skin explant model*

To examine the capacity of Treg conditioned moDC to induce allo-stimulated effector CD8$^+$ T cell mediated GvH reactions an *in vitro* human skin explant model was used. This model has been used for studying GvHD in humans for 20 years and is considered to closely mimic the type of skin lesions observed in clinical GvHD.

As reported in Chapter 4 skin sections incubated in medium only, with unstimulated third party CD8$^+$ T cells or CD8$^+$ T cells stimulated in the presence of Treg have the lowest levels of GvH damage, only being grade I (Figure 5.15 a, b and f). When immature or mature moDC were used to allo-stimulate the CD8$^+$ T cells the majority of skin sections exhibited grade III damage (Figure 5.15 c and d). Interestingly when the CD8$^+$ T effector cells were allo-stimulated with Treg conditioned moDC grade I tissue injury was observed in 2 out of 3 independent experiments, and grade II in the other (Figure 5.15e) – this was a significant reduction in the level of damage induced by untreated mature moDC (p=0.038) and not significantly different to the CD8$^+$ cells allo-stimulated in the presence of Treg (p=0.423).
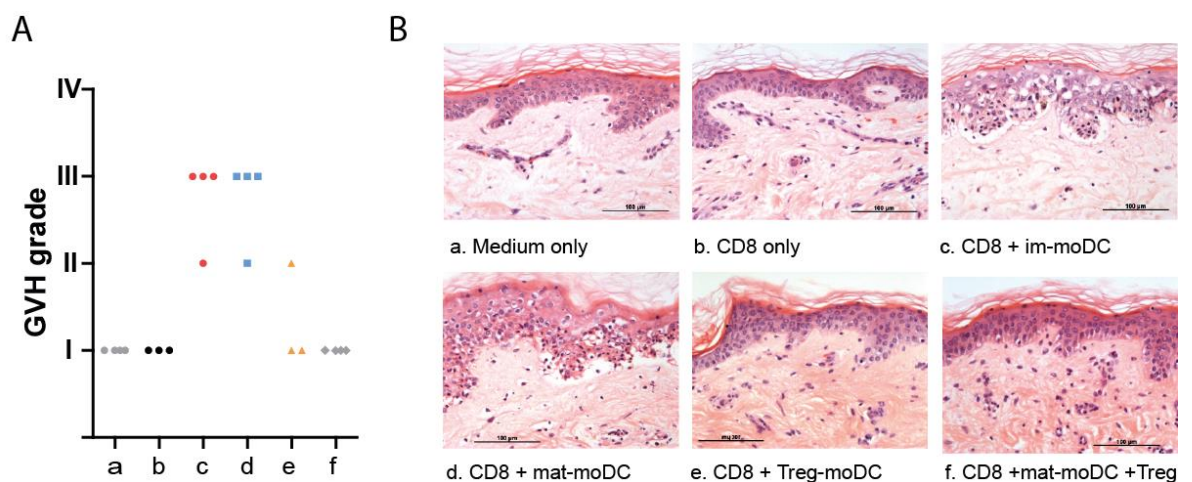
Figure 5.15 Treg-moDC have a reduced capacity to induce allo-stimulated CD8+ T cell mediated cutaneous GvH damage.
A) Cumulative and B) Representative data for skin sections incubated with (a) medium alone (●), (b) unstimulated CD8$^+$ T cells (●), CD8$^+$ T cells stimulated with (c) immature (●), (d) mature (■), (e) Treg conditioned (▲) and (f) mature moDC in the presence of Treg (◆), sections were graded according to the Lerner scale as previously discussed (Lerner et al., 1974). Treg conditioned moDC (Treg-moDC).

5.3.9 Treg impair moDC function partially through a MARCH1 dependent mechanism

A recent publication provided evidence that in a mouse model Treg impairment of DC function occurred through a IL-10/MARCH1 dependent mechanism (Chattopadhyay and Shevach, 2013). Therefore the levels of CD86 and MARCH1 gene expression were assessed to investigate if Treg modulation of moDC was also occurring via this mechanism.

As previously discussed, the expression of CD86 was increased, following LPS treatment, on the cell surface of mature moDC and this increase was impaired in the Treg conditioned moDC (Figure 5.16A and section 5.3.2). CD86 gene expression was seen to correlate with the cell surface protein expression levels, with significantly more CD86 mRNA detectable in the mature moDC (Figure 5.16B). Based on the nature of MARCH1 regulation of CD86 expression in that higher MARCH1 expression leads to lower CD86 expression, it was expected that if regulation was occurring via a MARCH1 dependent manner that high levels of CD86 would associate with low levels of MARCH1 mRNA. In the immature moDC high levels of MARCH1 were detected when CD86 was low and in mature moDC when CD86 expression was high MARCH1 expression was low as expected (Figure 5.16C). Interestingly the MARCH1 expression did not mirror CD86 expression exactly. In the Treg conditioned moDC levels of CD86 protein and mRNA were decreased but the MARCH1 expression was significantly higher than the mature moDC, suggesting the decreased expression of CD86 may be occurring via a partially MARCH1 dependent mechanism.
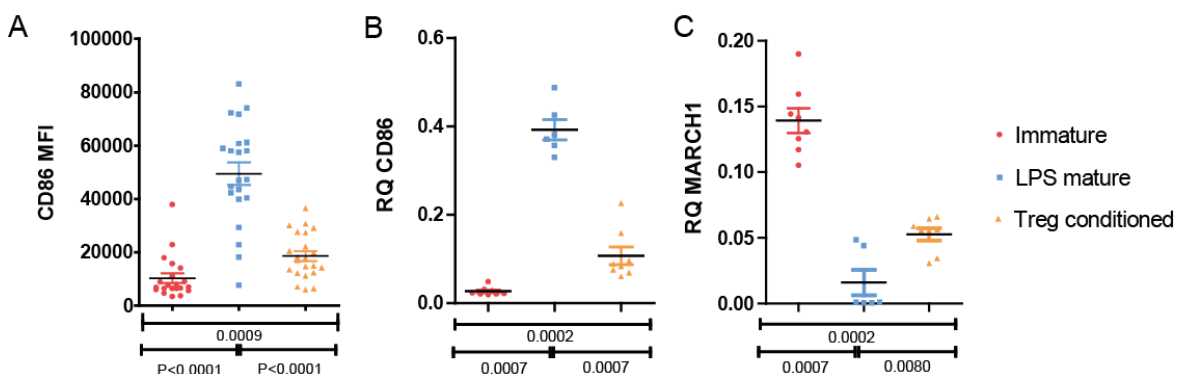
Figure 5.16 Treg impair moDC function through a MARCH1 independent mechanism.
A) Protein expression of CD86. Gene expression of B) CD86 and C) MARCH1 was assessed by qPCR and normalised to GAPDH. cDNA was generated by random hexamer priming from RNA isolated from immature (●), mature (■), and Treg-conditioned (▲) moDC and analysed by qPCR. Duplicate wells were assessed from 4 independent experiments and analysed by Mann-Whitney U tests.

5.3.10 Naïve T cell polarisation by Treg conditioned moDC

The ability of Treg-moDC to polarise naïve T cells was assessed using re-stimulation assays to measure cytokine production associated with T cell polarisation. Naïve T cells were stimulated for 6 days then rested for a further 4 days. The polarised cells were then stained for intracellular levels of the typical Th1 and Th2 cytokines, IFNγ and IL-4 following PMA and ionomycin stimulation. The capacity of the Treg-moDC polarised naïve CD4 cells to produce IFNγ (15.5%±4.64SEM) was not statistically different to that of naïve T cells polarised by mature moDC (20.8%±2.78SEM); however although not significant, there was a trend towards lower levels of IFNγ producing cells (Figure 5.17A). The capacity of the polarised cells to produce IL-4 was significantly higher when the naïve cells were polarised with the Treg-moDC (4.35%±0.37SEM) compared to that by mat-moDC (2.5%±0.30SEM) suggesting increased presence of Th2 cells (Figure 5.17A&B). Levels of IL-10 and IL-17a were also tested; unfortunately they failed to yield meaningful results due to technical difficulties (data not shown).
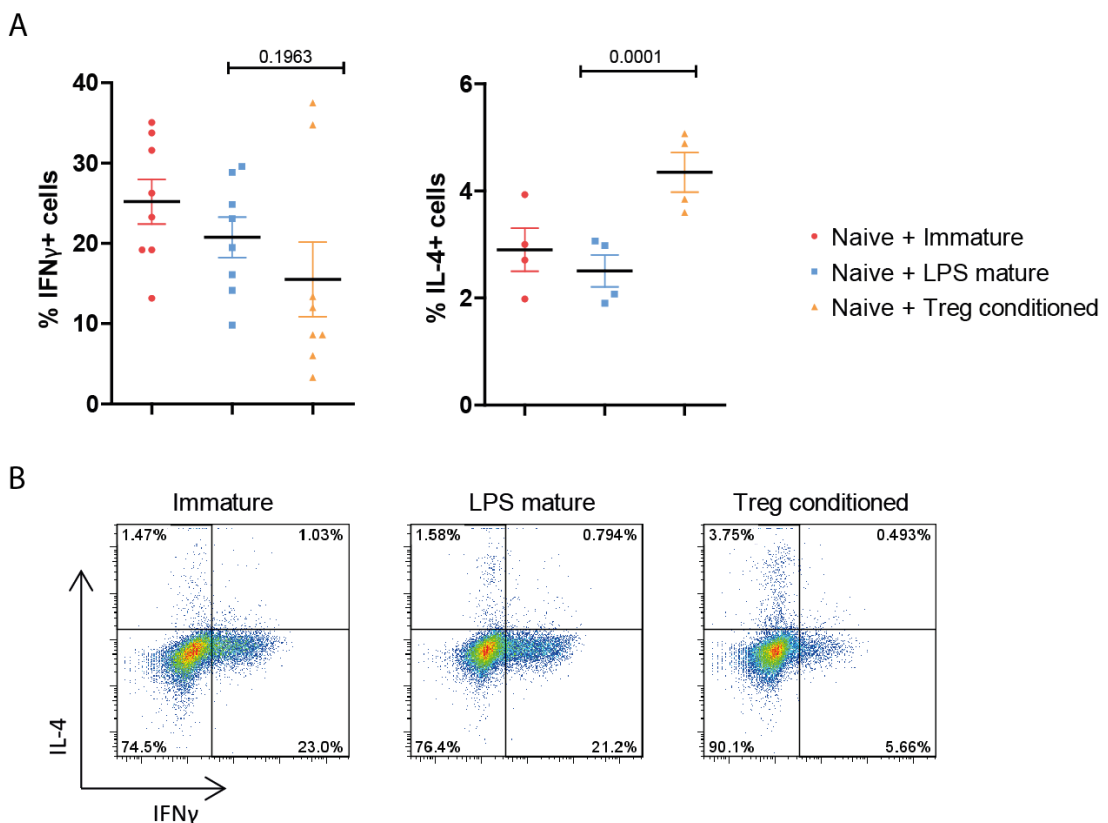
Figure 5.17 Cytokine production following naïve CD4^{+} T cell polarisation.
The capacity to produce Th1 and Th2 cytokines is altered when naïve CD4^{+} cells are polarised by Treg-moDC. A cumulative and B representative data for naïve T cell polarisation. Naïve CD4^{+} T cells were stimulated with immature (●), mature (■), and Treg-conditioned (▲) moDC for 6 days then rested for a further 4 days before staining for levels of IC IFNγ and IL-4. Data generated from 1-2 repeats of 4 independent experiments.

In addition to IC staining (following PMA/Ionomysin stimulation) to detect the capacity of the polarised naïve cells to produce Th1/Th2 type cytokines, the secretion of cytokines into the culture supernatant was measured using CBA flex. After 6 days stimulation and 4 days resting 1×10^5 cells were re-stimulated with CD3/CD28 beads at a 1:1 bead to T cell ratio for 24 hours and the supernatant was collected. The results of CBA flex are shown in Figure 5.18. Secreted levels of IFNγ were significantly lower when the naïve cells were stimulated with Treg-moDC, 2163pg/ml±378SEM compared to when stimulated with mature moDC, 3182pg/ml±37SEM (p=0.021). Levels of IL-4, were not detectable when the naïve cells were stimulated with mat-moDC, but when polarised by Treg-moDC 0.18pg/ml±0.1SEM was detected. IL-17a was also not detectable when cells were polarised by mat-moDC, however it was present at low levels in those polarised by Treg-DC, 1.44pg/ml±0.84SEM. Due to the low levels of IL-4 and IL-17a detected during this assay it was not possible to draw firm conclusions. Levels of IL-10 secreted by the polarised cells were increased when the cells were polarised by Treg-moDC compared to the mat-moDC (770pg/ml±252SEM compared to 175pg/ml±45.5SEM).
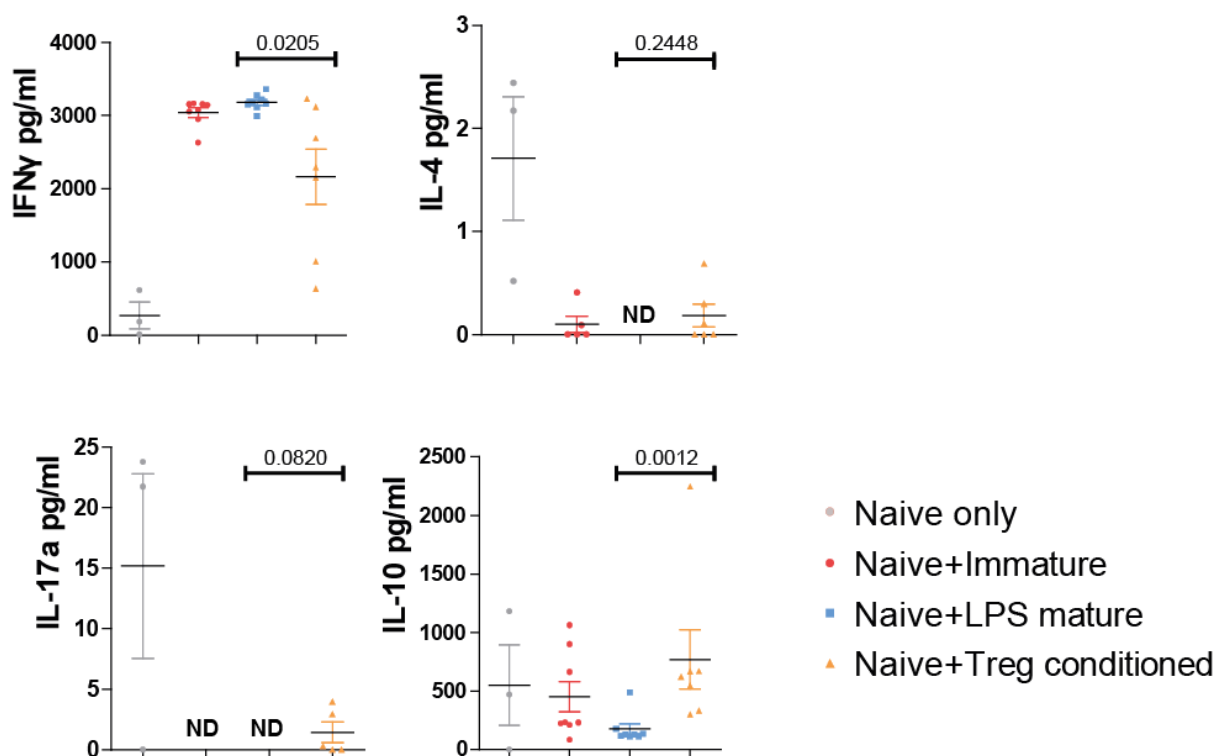
Figure 5.18 CBA analysis of secreted cytokines after re-stimulation assay.
Data was collected on Canto II and analysed using FCAP array software. Mann-Whitney U tests values are shown. Data generated from 1-2 repeats of 3 independent experiments. ND=not detectable.

Due to the increased levels of the regulatory cytokine IL-10 in the polarisation culture supernatant the expression of intracellular Foxp3 was assessed to examine polarisation towards a Treg phenotype. Naïve cells polarised by mature moDC had a mean Foxp3 expression of 9.7%±2.07SEM whereas the Treg-moDC polarised cells had a significantly higher expression of Foxp3 with 15.0%±2.15SEM (Figure 5.19).
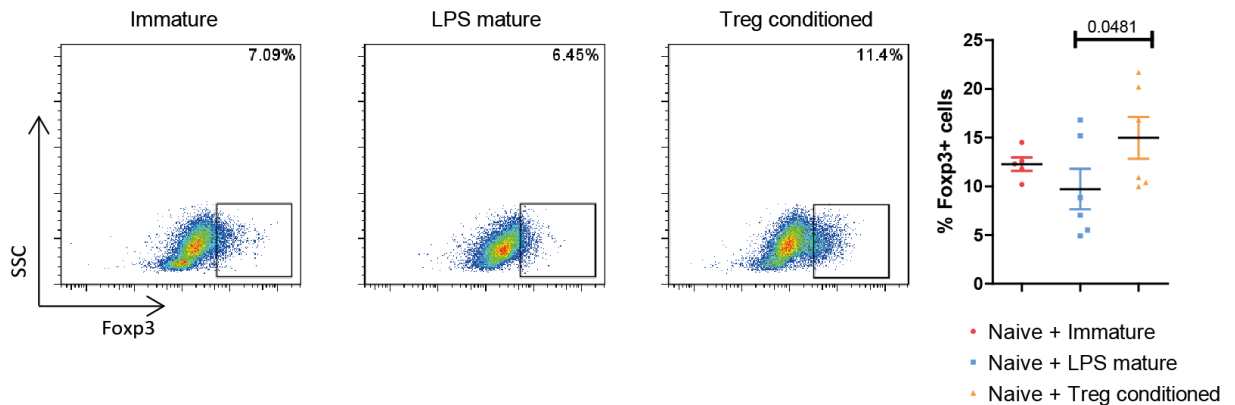
Figure 5.19 Treg conditioned moDC show a trend towards increased polarisation towards Foxp3 Treg phenotype.
Left-representative and right-cumulative plots, data obtained from 2 repeats of 4 independent experiments. Cells were harvested and stained for Foxp3 expression after re-stimulation assays. Prior to plots, gates were applied to select lymphocytes.

In addition to examination of the cytokine profile to assess naïve T cell polarisation the expression of master transcription factors were measured using qPCR. Levels of TBX21 (Th1), GATA-3 (Th2) and Foxp3 (Treg) mRNA were all unchanged regardless of the moDC used to polarise the naïve cells (Figure 5.20). RORC, the transcription factor associated with Th17 cells was up-regulated in cells polarised by Treg-moDC.
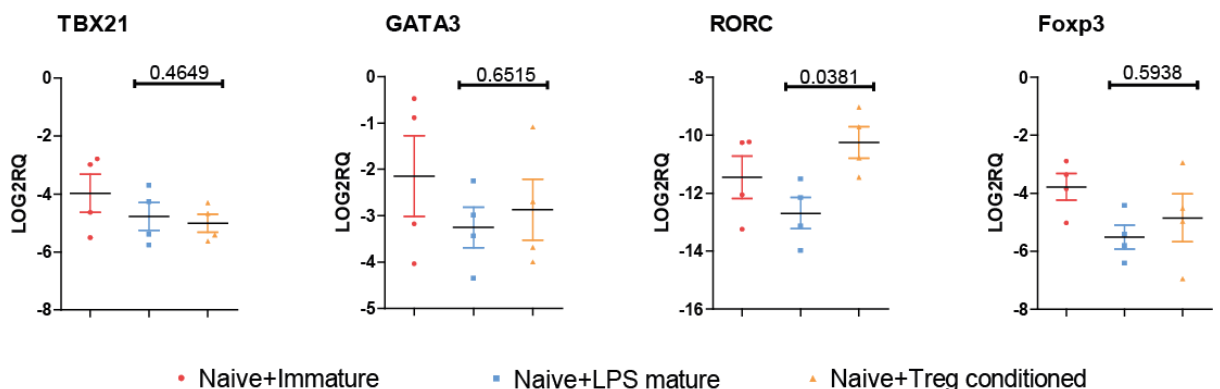
Figure 5.20 Gene expression of Th1, Th2, Th17 and Treg transcription factors.
mRNA was isolated after naive T cell polarisation by im-moDC, mat-moDC and Treg-moDC. Gene expression was measured by qPCR and normalised to GAPDH. Paired t test values shown, n=4.

5.4 Discussion

Accumulating evidence has shown the potential of modified DCs such as TolDC or regulatory DC for therapeutic use (Ezzelarab and Thomson, 2011, Stenger et al., 2012, Harry et al., 2010). However the studies carried out in this chapter were not intended to generate a new population of regulatory DCs to aid in the prevention or treatment of GvHD. Rather this was a proof of principle study which aimed to further investigate Treg mediated impairment of DC function and its impact on the amelioration of GvH reactions. Data presented in this chapter shows the first multi-parameter functional analysis of the effects of Treg conditioning on moDC including assessment of their phenotype, morphology, cytokine production, antigen uptake and ability to polarise naïve T cells. Furthermore the impact of Treg mediated changes in moDC on allo-reactive CD8$^+$ T cell induced GvHD target tissue damage was also examined, for the first time using an *in vitro* human GvH skin explant model.

Treg conditioned moDC (Treg-moDC) were generated based on a previously published protocol (Bayry et al., 2007) with further refinement to confirm the optimal conditions such as the timing and dose of Treg. The successful generation of Treg-moDC gave assurance as to the reliability and reproducibility of the results reported in this chapter.

Results presented in this chapter have shown that for Treg to drive moDC towards a tolerogenic status direct cell-cell contact was required. Contact dependent regulation of DCs has also been reported in *ex vivo* mouse models where Treg form aggregates on/around dendritic cells, preventing their maturation (Onishi et al., 2008). The formation of these aggregates was dependent on leukocyte function-associated antigen-1 (LFA-1) expression by Treg cells; however the actual inhibition of maturation was dependent on CTLA-4 as well as LFA-1. Interestingly Onishi *et al* also reported that Treg were able to out-compete naïve T cells to form these aggregates, which suggests *in vivo* Treg are also able to outcompete the naïve T cells, blocking their access to DCs thereby preventing their activation.

Data presented in this chapter suggests that the regulation of DC phenotype function may occur, at least partially, in a MARCH1 dependent mechanism. This is supported by data from a recent publication that reported antigen specific

iTreg were able to modulate DC function through an IL-10/MARCH 1 dependent mechanism (Chattopadhyay and Shevach, 2013). The maturation marker CD83 promotes MHC class II and CD86 expression by blocking IL-10 driven MARCH1-dependent ubiquitination and degradation of MHC class II and CD86 (Tze et al., 2011). Further investigation would be required to confirm the requirement for Treg derived IL-10 in this human *in vitro* model.

In this study the *in vitro* generated Treg-moDC have shown remarkably consistent phenotypic and functional profiles. Treg-DC expressed lower levels of maturation and co-stimulation molecules (CD83, CD40, CD80 and CD86) and comparably high levels of HLA-DR when compared to mature moDC. This finding suggests that Treg-moDC acquired a phenotype profile that overlaped the features of immature and mature DCs. This partially resembles the features of semi-mature DC, a new concept proposed by Lutz *et al* based on data obtained from murine bone marrow derived DC (Lutz and Schuler, 2002), which challenges the bimodal concept of DC status with an immature tolerogenic status and a mature immunogenic status. The partially mature status indicated by high level expression of HLA-class II and co-stimulation molecules was required to establish tolerance (Lutz and Schuler, 2002). Interestingly the levels of maturation and co-stimulation markers expressed on Treg-DC were not only significantly lower than those expressed on mature moDC but also significantly higher than those on immature moDC, suggesting the maturation status of Treg-moDC was distinctively different from both immature and mature DC, which has added to the theory of a the semi-mature DC. The high level of HLA-DR expression on Treg-moDC allows Treg-moDC to engage the T cells but a low expression of co-stimulatory molecules would give rise to a reduced or failed second signal resulting in T cell anergy/apoptosis (Alegre et al., 2001).

Treg-moDC also expressed an increased level of CCR7 which is a chemokine receptor responding to the ligands CCL19 and CCL21 (Forster et al., 2008). CCR7 expression is important for tolerance induction *in vivo* due to its ability to guide the cells with immuno-modulatory properties, such as TolDC and Treg, into lymph nodes (Garrod et al., 2006, Forster et al., 2008). However the increased CCR7 expression on Treg-moDC was not likely to have contributed to the data reported in this chapter, due to *in vitro* nature of the experiments. On the other hand, the observed increase in expression of LAP-

TGFβ on Treg-moDC may have played an important role in the promotion of tolerance. TGFβ is able to promote tolerance via the induction of Foxp3 in naïve T cells (Chen et al., 2003).

The phenotype and functional properties of Treg-moDC closely resemble that of TolDC (Svajger and Rozman, 2014). It is worth noting that TolDC are not a distinct DC subset, identifiable by specific markers, but rather a functional DC status induced by the biological environment, such as inhibitory cytokines or immunosuppressive drugs (Hackstein and Thomson, 2004). TolDC exert either a modulatory or inhibitory function towards immune responses. The data presented in this chapter have shown that Treg-moDC fit the description of TolDC. The key point is that most TolDC induced by cytokines (Svajger and Rozman, 2014) or pharmacological agents (Harry et al., 2010) were generated for their potential therapeutic use (Ezzelarab and Thomson, 2011, Stenger et al., 2012) whilst this work was conducted to underpin the hypothesis that Treg induced development of TolDC plays a key role in Treg mediated protection from GvHD.

Treg-moDC and im-moDC share similar properties, however they are not interchangeable. Immature moDC have been shown to induce T cell tolerance *in vitro* (Lutz and Schuler, 2002). However immature DC are unstable, upon stimulation with LPS or other endogenous TLR-4 ligands such as heat shock proteins 60 and 70 (Ohashi et al., 2000, Vabulas et al., 2002) they mature, increasing expression of maturation makers and co-stimulatory molecules, hence switching their immune tolerogenic properties to immune-stimulatory properties. Data presented in this chapter has shown that Treg-moDC were resistant to LPS maturation and retain their low levels of co-stimulatory molecules and maturation markers following LPS stimulation which is consistent with the properties of Treg-moDC generated in previous studies (Misra et al., 2004, Bayry et al., 2007). LPS stimulation of TLR-4 requires interactions between several molecules including LPS binding protein (LBP), CD14, MD-2 and TLR-4 (Lu et al., 2008). TLR-4 signal transduction occurs through MyD88 dependent and independent pathways. These findings could be strengthened by exploring the molecular pathways underlying Treg mediated resistance to LPS maturation.

Along with decreased expression of co-stimulatory molecules and increased expression of immuno-modulatory molecules Treg-moDC were shown to secrete low levels of the pro-inflammatory cytokines IL-6 and IL-12p70. This was in line with the cytokine secretion of semi-mature DC derived from mouse bone marrow and from human TolDC (Lutz and Schuler, 2002, Anderson et al., 2008). Unexpectedly the IL-10 levels secreted by Treg-moDC were significantly lower than that of mature moDC. This could be due to the timing of cell culture supernatant collection. Measuring DC cytokines following resting and re-stimulation should be performed to confirm the current findings.

In addition to modulation of moDC phenotype and morphology, Treg conditioning also impaired the functional capacity of moDC. Antigen uptake in Treg-moDC (both those which had received LPS stimulation those which had not received LPS stimulation) was significantly decreased when compared to the non-Treg treated immature moDC, suggesting that Treg are not solely arresting the maturation of moDC but also actively regulating the functions of the moDC. Similarly LPS stimulated Treg-moDC exhibited a significantly diminished capacity to stimulate activation and proliferation of CD8$^+$ effector T cells when compared to the non-Treg treated mature moDC, although this effect was not as potent as when Treg were present during the allo-stimulation. Previous publications have shown that Treg-moDC had reduced ability to stimulate both autologous and allogeneic CD4$^+$ T cell proliferation (Misra et al., 2004, Bayry et al., 2007). Treg-moDC were also less able to stimulate allo-reactive CD8$^+$ T cell proliferation in a humanised mouse model of GvHD (Amarnath et al., 2010). This data provides the first evidence of Treg modulation of immature moDC antigen uptake and impairment of efficient allo-stimulation of human CD8$^+$ effector T cells by Treg-moDC.

Furthermore the results have shown, for the first time, that allo-reactive CD8$^+$ effector T cells stimulated by Treg-moDC induced significantly less severe cutaneous GvH damage than those stimulated by non-Treg treated mature moDC. This observation was supported by the recent humanised mouse study in which Treg-moDC had limited capacity to induce T cell proliferation and therefore protected against xenogenic GvHD (Amarnath et al., 2010).

The observed Treg-moDC protection against GvHD target tissue damage was further strengthened by the findings that Treg-moDC skewed naïve T cell polarisation from Th1 towards Th2 accompanied with a trend towards increased Treg lineage. Th1 type responses are dominant in the pathophysiology of aGvHD (Ferrara et al., 2009) and therefore Treg modulation of DC resulting in skewing of naïve T cells away from a Th1 phenotype may contribute to Treg prevention of GvHD. It is well established that TolDC are able to induce Treg in the periphery as a mechanism for maintaining peripheral tolerance (Sela et al., 2011, Svajger and Rozman, 2014) and therefore may further contribute to protection against GvHD. In addition the Treg-moDC showed a trend towards increased naïve T cell differentiation into a Th17 type phenotype with significantly increased RORC mRNA, however the IL-17a secretion data was inconclusive. Th17 cells have been shown to be sufficient but not necessary to induce lethal GvHD, although IL-17 producing cells can also be suppressive (Carlson et al., 2009, Coghill et al., 2011, Pesenacker et al., 2013).

Due to difficulties in isolating sufficient numbers of DC from peripheral blood moDC were used in this study. Ideally for clinical significance these results need to be confirmed using relevant sub-sets of DC sorted directly from human blood or tissues. CD1c$^+$ dermal DC are the most prominent population of human DC and their role as potent stimulators as naïve T cells (Collin et al., 2013) would make them interesting to study in this skin explant model following Treg conditioning.

The potent effect Treg have been shown to have on the functional capacity of moDC provides insight into the mechanism by which Treg are able to prevent GvHD post HSCT. Questions still remain as to the signalling pathways which are involved in the Treg modulation of DCs. TolDC have been shown to have down-regulated components of the NF-κB signalling pathway caused by Dex and VitD3 (Matasic et al., 1999, Xing et al., 2002) therefore it would be interesting to further investigate the NF-κB signalling pathway in Treg conditioned moDC.

Data presented in this chapter provides the first comprehensive phenotypic and functional profiling of Treg-moDC in relation to human GvH reactions. The magnitude of GvH modulation appears to be less potent when only the moDC

are Treg conditioned than when Treg modulation is persistent over the entire allo-stimulatory process. This suggests that the most effective Treg modulation is likely to require the multidimensional effects of Treg including induction of tolerogenic DCs and direct suppression of the effector T cells including blocking of effector T cell migration.

Chapter 6 – *In vitro* and *in vivo* investigation into the potential impact of Treg on GvL effects

6.1 Introduction

In vivo murine models and early clinical trials have reported Treg protection against GvHD without compromising GvL (Edinger et al., 2003, Di Ianni et al., 2011, Brunstein et al., 2011). A large volume of *in vitro* research, including our own, has demonstrated beyond doubt that Treg potently down-regulate GvH responses (Wang et al., 2009, Mavin et al., 2012, Edinger and Hoffmann, 2011) however, a certain level of GvH responses are required to achieve the beneficial GvL effect (Kolb, 2008). Increased frequency of Treg was also shown to correlate with CML relapse after HSCT (Nadal et al., 2007). Due to the conflicting nature of the current literature available and the fact that detrimental GvHD and beneficial GvL share a common immuno-biology the research presented in this chapter was conducted to evaluate the potential impact of Treg on GvL effects. This was carried out using *in vitro* cellular assays and assessment of Treg content in patient peripheral blood and occurrence of relapse post transplantation.

6.1.1 *Graft-versus-leukaemia; historical perspectives*

The beneficial GvL effect was first described when it was observed that patients who suffered with GvHD incurred a lower incidence of relapse (Weiden et al., 1979). Disparities in major or minor histocompatibility antigens between the donor and the recipient not only lead to GvHD but also to tumour eradication. The importance of the GvL effect became more evident when T cell depletion was introduced to prevent GvHD, patients who received T cell depleted grafts had an increased risk of leukaemia relapse compared to those who received un-depleted grafts (Horowitz et al., 1990). The effectiveness of GvL was further recognised by the observation that with donor lymphocyte infusion following a relapse post HSCT could induce remission. This lymphocyte infusion is able to induce remission, most potently in chronic myelogenous leukaemia (CML) patients, and also to a lesser extent in those patients with acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) amongst others (Kolb, 2008). Consequently the current goal of HSCT is to allow for sufficient GvL effect without permitting the development of GvHD; this is

particularly important in the case of non-myeloablative conditioning regimens which rely on the GvL activity of the graft to eradicate tumour cells (Martino et al., 2002).

6.1.2 Natural Killer cells and allo-reactive CD8^{+} T cells in the graft-versus-leukaemia effect

The GvL effect is mainly mediated by allo-reactive donor CD8^{+} T cells and NK cells. The allo-reactive CD8^{+} T cells mediated GvL, is allo-antigen specific via adaptive immunity whereas the NK cells act through an innate non-antigen specific mechanism.

Both natural killer (NK) cells and allo-reactive CD8^{+} cytotoxic T lymphocytes (CTL) are important in the protection against pathogens and transformed cells (Caligiuri, 2008, Vivier et al., 2011). Both populations mediate cytotoxicity through the release of granzyme and perforin, activation of death receptor pathways (Fas/TRAIL) along with the production of a range of cytokines. However, the control of cytotoxicity occurs in a very different manner. Regulation of NK function is a balance between activation and inhibition signals caused by the binding of various ligands to NK cell surface receptors (Caligiuri, 2008). Inhibitory killer immunoglobulin-like receptors (KIR) expressed on the surface on NK cells recognise MHC class I molecules, allowing the NK cells to recognise 'self'. Engagement of the inhibitory KIR with their ligands occurs at a higher affinity than the activation receptor, meaning that the inhibitory signal will predominate (Hsu and Dupont, 2005). When a cell is altered by infection or leukaemic transformation it may cause an alteration in expression of MHC class I molecules which can be recognised by the diverse repertoire of NK cells (Caligiuri, 2008). In this case the absence of an inhibitory signal and the presence of a ligand in an activation KIR can result in NK mediated cytotoxicity. It has long been established that NK cells are beneficial in HSCT, promoting engraftment and amplifying the GvL effect through 'missing self' recognition (Ruggeri et al., 2006). A study by Ruggeri *et al* reported a highly significant survival advantage following HSCT if the donor was NK-allo-reactive, meaning the donor possessed a KIR ligand which was missing in the recipient (Ruggeri et al., 2007). This NK-allo-reactivity means donor NK cells would become activated due to the 'missing self' expression of a MHC class I molecule and mediate cytotoxicity, lysing the host leukaemia cells.

On the other hand, donor-derived allo-reactive CD8$^+$ CTL mediate the GvL effect in an allo-antigen specific manner by recognising the disparate HLA class I antigens expressed on residual host leukemic cells then initiating allo-reactive immune responses. The ability of donor CD8$^+$ CTL to eliminate recipient leukemic cells is mediated mainly by activated DCs of recipient origin (Chakraverty et al., 2006, Merad et al., 2013) although donor derived DCs could also contribute via indirect allo-antigen presentation (Shlomchik, 2007). In HLA matched HSCT it is the differences in miHA between donor and recipient which are responsible for the GvL effects (Bleakley and Riddell, 2004).

6.1.3 Treg and leukaemia relapse in HSCT

There are a number of publications associating Treg and leukaemia relapse, however the majority of the research has been conducted in relation to the role of Treg in tumour immunity in an autologous system (Ersvaer et al., 2010, Ustun et al., 2011). Research with regard to the effect of Treg on GvL in the HSCT setting was mainly carried out in murine models. Early studies investigating the infusion of Treg in the prevention of GvHD reported that Treg were able to prevent GvHD without affecting GvL in mice (Edinger et al., 2003, Trenado et al., 2003). In the human setting there is limited information available, the early stage clinical trials which administrated Treg to high risk HSCT patients revealed no change in relapse/GvL compared to historical cohorts of patients who did not receive Treg infusions (Brunstein et al., 2011, Di Ianni et al., 2011). However, there are also opposing results showing that higher Treg content post HSCT in patient peripheral blood is associated with a higher incidence of CML relapse (Nadal et al., 2007). Furthermore, a small scale clinical trial studying the safety and efficacy of Treg depleted DLI has demonstrated that Treg depletion of the graft improved the GvL effect of DLI which was associated with improved survival (Maury et al., 2010). Undoubtedly more investigation is required into the effect Treg may have on the GvL effect following an allogeneic HSCT.

The specific questions asked in this chapter aimed to further investigate whether Treg were able to impair GvL reactions and therefore impact on leukaemia relapse. This was addressed by examining and analysing the following:

- The effect of Treg on NK cell functions *in vitro.*

- The impact of Treg on allo-reactive CTL cytotoxicity *in vitro*.
- The Treg content in patient peripheral blood at various time points post-transplant
- The association between Treg content in post-transplant patient peripheral blood and the clinical outcome of leukaemia relapse.

6.2 Methods

6.2.1 Maintenance and generation of target cells

To examine the non-allo-specific cytotoxicity mediated by NK cells and the allo-reactive CTL mediated cytolytic functions K562 cell line and PHA blasts were used as respective target cells. The PHA blasts were autologous to the moDC used to prime the CD8⁺ CTLs. The K562 cell line was maintained in general purpose RF10 media; cells were regularly split to maintain log phase expansion for use in assays. PHA blasts were generated by culturing PBMC at 1x10^6 cells/ml in RF10 with 5μg/ml PHA and 50IU/ml IL-2 for 6 days.

6.2.2 Generation of effector cells

NK effector cells, generated by negative enrichment, were cultured in RF10 with 100IU/ml IL-2 overnight either in the presence or absence of 1:1 ratio of Treg. Prior to functional testing Treg were removed by CD3 MACS depletion. NK cells were then assessed for their functional capacities using IC staining for IFNγ production, NKG2D cell surface expression and co-culture with K562 target cells (Figure 6.1A). CTL effector cells were generated using a priming MLR. CD8 cells were allo-stimulated either in the presence or absence of Treg for 6 days at a ratio of 4 CD8 to 1 Treg, unstimulated CD8 cells served as a control. Treg were depleted with CD4 MACS depletion prior to functional testing. Primed CTL were then assessed for levels of perforin and granzyme expression along with their ability to degranulate and induce target cell death (Figure 6.1B).

6.2.3 Assessment of target cell death and effector cell degranulation

To assess the functional abilities of NK cells they were added to 96 well U-bottomed plates with a range of K562 ratios in RF10. If required CD107a antibody (a marker for degranulation) was added at this point. Plates were then spun down to ensure cell-cell contact between K562 and NK cells, and then incubated at 37ºC for 2 hours to measure degranulation and 3 hours to measure target cell death. Both degranulation and cell death were measured by flow cytometry. Gating strategies are shown in section 6.3.1.The ability of CTL to degraunlate and cause target cell death was also assessed by flow cytometry. Briefly CD8⁺ T cells either unstimulated, allo-stimulated or allo-stimulated in the presence of Treg were cultured in a 96 well plate with a range

of ratios of PHA blasts, and spun down. The co-cultures were incubated for 2 hours in RF10 supplemented with 25IU/ml IL-2. Gating strategies and assessment of target cell death and effector cell degranulation are discussed in section 6.3.1.
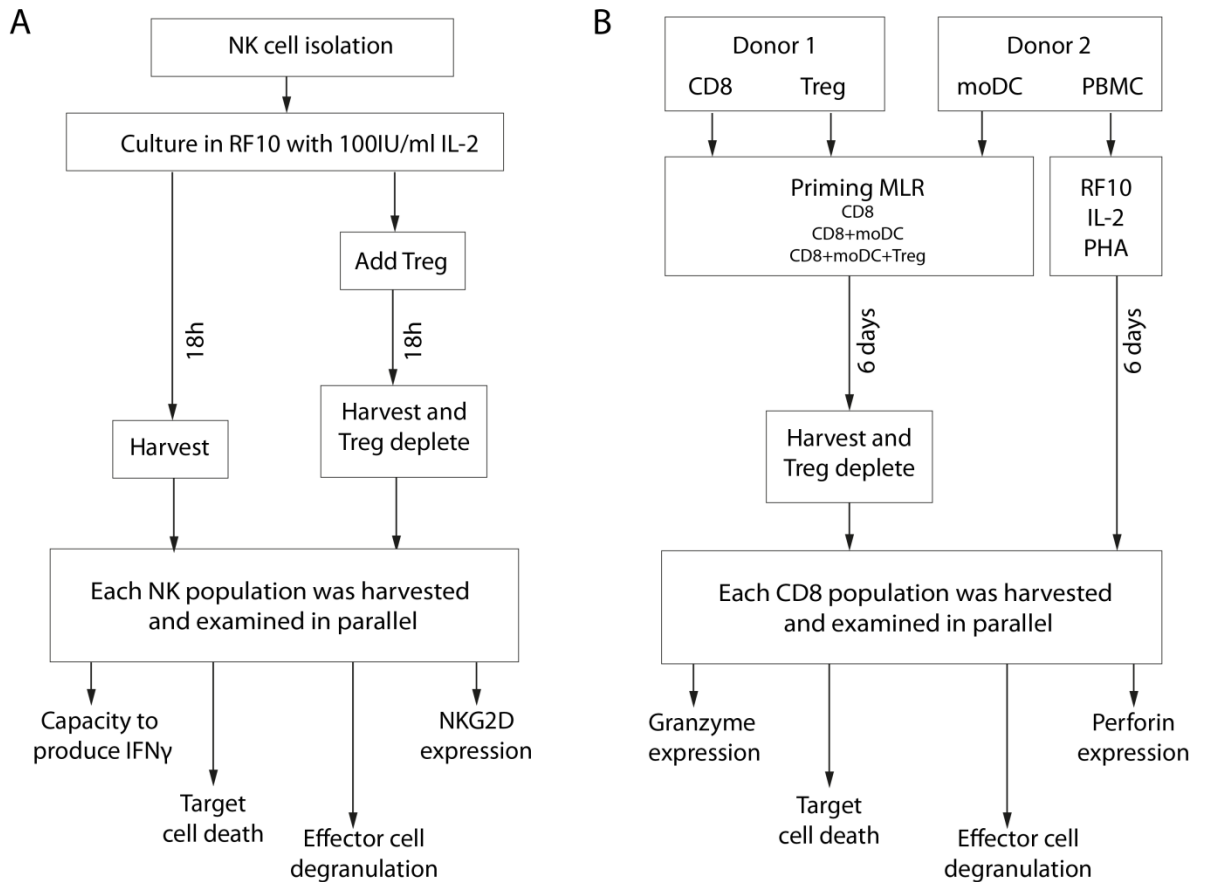
Figure 6.1 Experimental designs to test the effect of Treg on effector cell functions.
A. NK cells were incubated overnight in presence/absence of Treg. Treg were the removed prior to functional testing either by IC staining, cell surface staining of co-culture with K562 target cells.
B. CD8^{+} T cells were primed in MLRs and assessed for their functional capacity. PHA blasts generated form the same donor as the moDC were used as target cells to measure degranulation and target cell death

6.2.4 Acknowledgements

Acknowledgement to

1) Dr L Nicholson for generation of K562 growth curves

6.3 Results

Results presented in this chapter showed the impact of Treg on the GvL effects of NK cells and cytotoxic T lymphocytes at a cellular level. Also considered is the potential association between the numbers of Treg in serial patient samples post transplantation and the incidence of leukaemia relapse.

6.3.1 Methodology development

Results in this section have shown that the optimization of methods used to measure the effect Treg have had on the cytotoxicity of NK cells and CD8$^+$ T cells *in vitro*. The methods developed were adapted from two previous studies (Kim et al., 2007, Jardine et al., 2012).

Isolation of NK cells

NK cells were successfully isolated from normal healthy donor PBMC by MACS depletion of non-NK cells using an antibody cocktail of biotin conjugated antibodies against T cells, B cells, Stem cells, DCs, monocytes, granulocytes and erythroid cells (Miltenyi). The average NK cell purity post isolation was 90.3%±1.21SEM based on double staining for CD3 and CD56 (Figure 6.2)
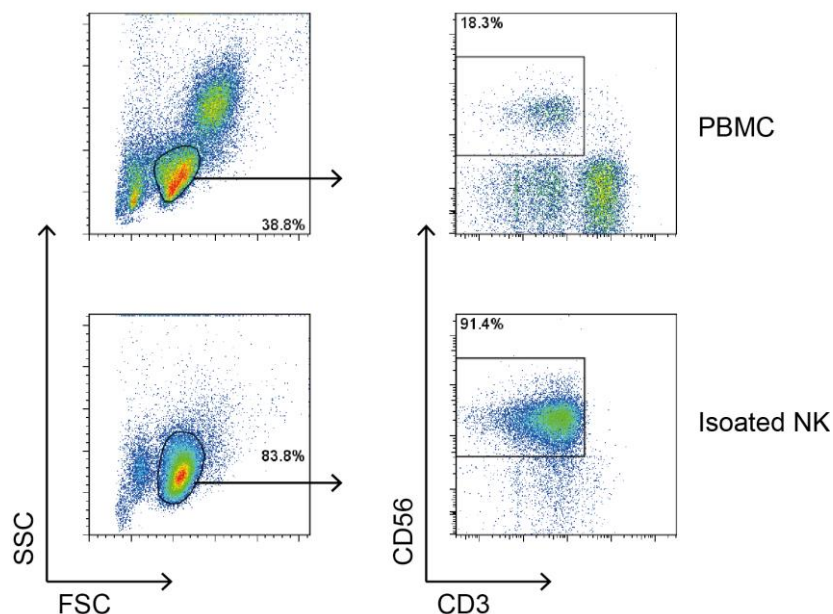
Figure 6.2 Isolation of NK cells from PBMC.
Data collected in BD Canto II and analysed with FlowJo version 7.6 (TreeStar). Data representative of PBMC before isolation and NK cell fraction after isolation in 10 independent experiments.

Maintenance of K562 cell line in culture

The kinetics of K562 growth was monitored with 3 starting cell concentrations of 0.1x10^6 cells/ml, 0.25x10^6 cells/ml and 0.5x10^6 cells/ml. The results showed that the log phase expansion of K562 cells appeared to be between 48 hours and 96 hours of culture (Figure 6.3). To ensure the consistency of the functional tests, particular attention was given to maintain the target cells in log phase growth prior to being used in functional assays.
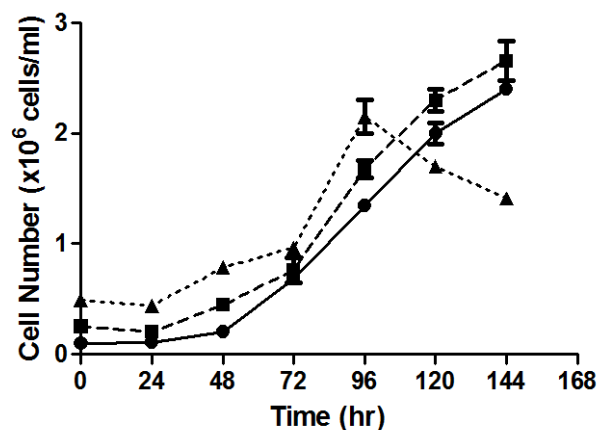
Figure 6.3 Representative K562 growth curve.
K562 were seeded at 0.1x10^6 cells/ml (●), 0.25x10^6 cells/ml (■) and 0.5x10^6 cells/ml (▲). Cell number was assessed at regular time points. Error bars represent duplicate cell counts.

Gating strategies to assess target cell death and effector cell degranulation

Traditionally radioactive ^{51}Chromiun release assays were used to quantify target cell death, measuring ^{51}Chromium released from lysed cells (Morales and Ottenhof, 1983). Despite this becoming the 'gold standard' for measuring cytotoxicity its use is often undesirable due to the hazardous nature of radioactive materials. Additionally the use of a flow based assay allows for target cell death to be quantified at a single cell level. The multi-parameter nature of flow cytometry allows for further sample analysis such as effector cell degranulation. Previous publications have reported that flow based assays do correlate well with the more traditional ^{51}Chromium release assays (Kim et al., 2007).

The K562 cell line is a human chronic myelogenous leukemia cell line, positive for the Philadelphia chromosome and commonly used as a target cell in NK assays (Lozzio and Lozzio, 1975). K562 cells do not express HLA-ABC

which makes them very susceptible to NK cytotoxicity due to their 'missing self' status. This provides a good marker to separate the K562 cells from the effector cells by staining for HLA-ABC (Class I). HLA-ABC staining resulted in a good separation between the K562 target cells and the NK effector cells. 7AAD staining was also used to accurately identify dead cells within the K562 population (Figure 6.4A). NK cells were identified as CD56$^+$CD3$^-$, NK cell degranulation was measured with CD107a staining and NKG2D expression was measured on the NK cells (Figure 6.4B). CD107a (lysosome-associated membrane protein-1) is a well reported functional marker of both NK and CTL cell cytotoxicity, as degranulation occurs CD107a is transported to the cell surface allowing antibody binding (Aktas et al., 2009, Alter et al., 2004).
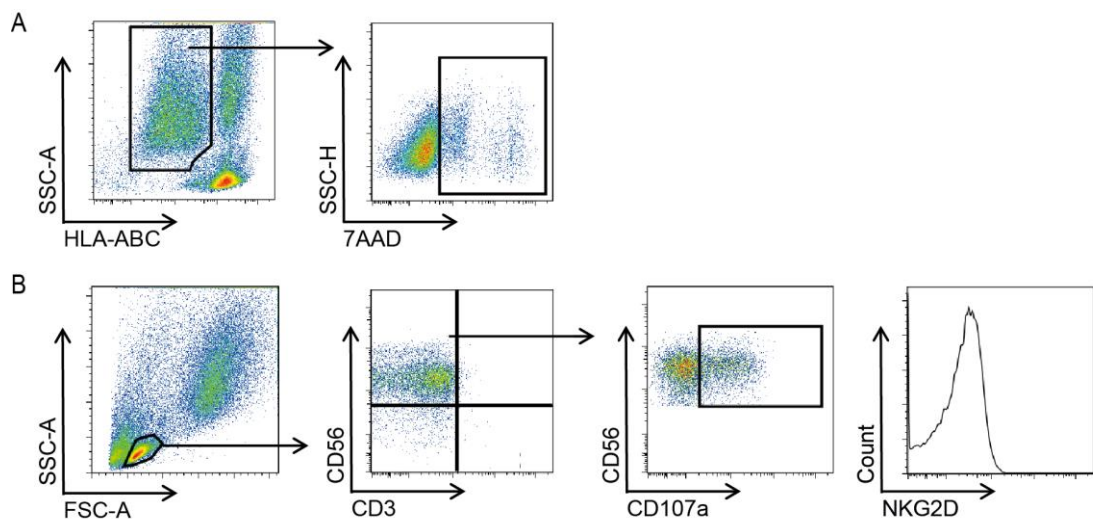
Figure 6.4 Gating strategies to assess NK cell function.
A) Target cells were selected based on negative expression of HLA-ABC then viability assessed by 7AAD staining. B) effector cells were selected based upon CD56$^+$CD3$^-$ and then assessed for CD107a expression and NKG2D. Appropriate isotype controls were used to determine gating strategies.

The target cells used to assess CTL killing ability were PHA blasts generated over 6 days from the same donor as the moDC used to stimulate the CD8$^+$ T cells. Target cells were subsequently separated from the effector cells by staining them with different dyes prior to co-culture (Figure 6.5). Target cells were stained with CD3-PerCPcy5.5 and effector cells with CD8-APC. Target cells could then be selected and assessed for viability with DAPI staining. Live effector cells were assessed for expression of CD107a.
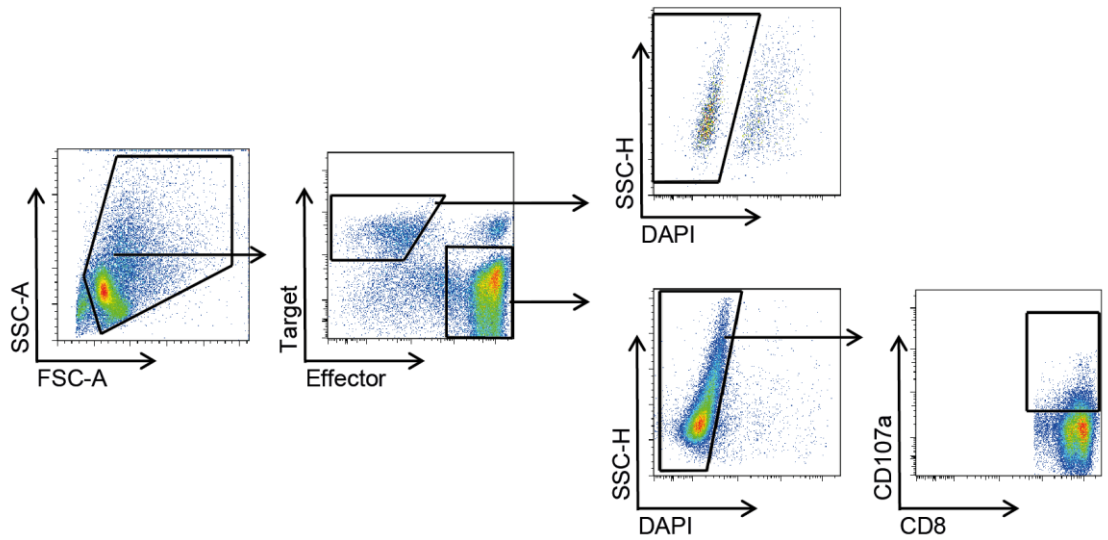
Figure 6.5 Gating strategies to assess CTL function.
Target cells were selected based on positive PerCPcy5.5 staining and effector cells were selected based on positive APC staining. Target and effector cells were then assessed for viability and degranulation (CD107a) respectively. Gate position was determined by appropriate isotype control.

6.3.2 Treg modulation of NK cell functions

The effect of Treg on NK cell mediated target cell death was assessed by treating NK cells with Treg cells at a 1:1 ratio overnight in RF10 with IL-2 to stimulate the NK cells. Treg were then removed prior to functional testing of NK cells with K562 target cells at a range of ratios. After 3 hours co-culture with the NK cells stimulated with or without Treg, target cell death was detected with 7AAD staining. Due to inter-experimental variation in the background viability of the K562 cells, the fold increase in cell death above background cell death of K562 only culture was used to standardise NK cell mediated cytotoxicity (Figure 6.6). A significant decrease in target cell death was observed at all NK:K562 ratios for the NK cells treated with Treg compared to untreated NK cells. The fold increases in target cell death were 3.07 ± 0.34SEM vs 1.82 ± 0.28SEM, 4.63 ± 0.49SEM vs 2.32 ± 0.35SEM and 10.32 ± 1.9SEM vs 3.76 ± 0.69SEM for untreated and Treg-treated NK cells at 1:1, 2:1 and 5:1 NK-K562 respectively ($p=0.041$, 0.015 and 0.004 for 1:1, 2:1 and 5:1 respectively).
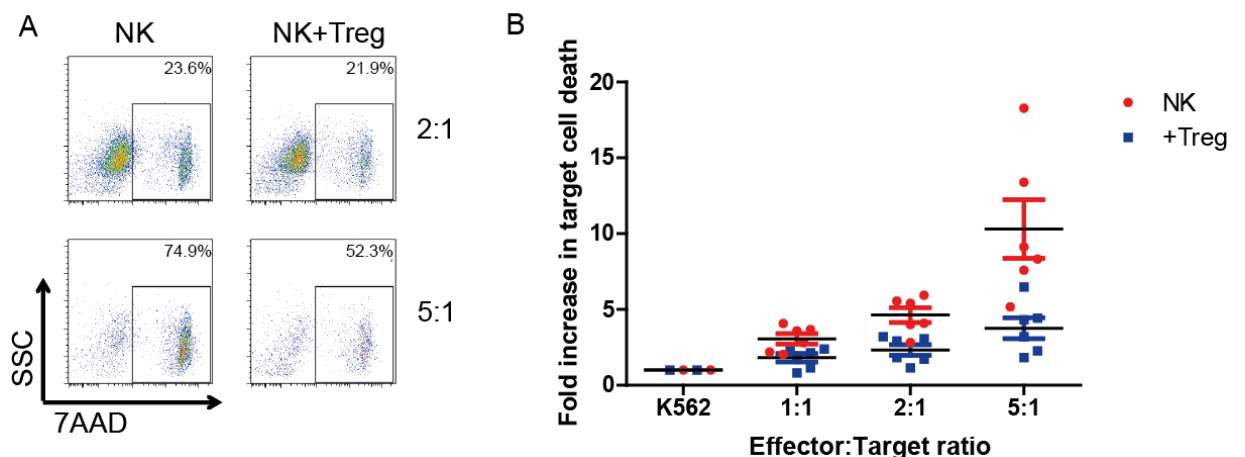
Figure 6.6 Treg treatment impaired NK cell mediated target cell death.
Untreated NK cells (●) and Treg treated NK cells (■) were co-cultured with a range of K562 cells for 3 hours. K562 viability was measured with 7AAD and calculated as fold increase over K562 only death. A) Representative plots, prior to plots shown gates were applied to target cells. B) Cumulative data from 6 normal donors.

In addition to target cell death, NK cell degranulation was analysed as a measurement of NK cell function. When CD107a is silenced it inhibits NK cell cytotoxicity therefore it is an integral part of NK cell functioning (Krzewski et al., 2013). In the absence of any target cells the NK cells did not degranulate

(0.60%±0.09SEM) and there was no difference between the Treg treated and untreated NK cells (p=0.83) (Figure 6.7). After 2 hours of co-culture with K562 target cells the percentage of degranulating NK cells was significantly decreased for NK cells that had been treated with Treg compared to untreated NK cells with 31.6%±2.71SEM vs 17.9%±1.24SEM, 26.1%±1.86SEM vs 17.3%±1.51SEM and 19.3%±1.87SEM vs 11.88%±0.94SEM at 1:1, 2:1 and 5:1 NK:K562 ratios respectively (p= 0.0002, 0.0005 and 0.0020 for 1:1, 2:2 and 5:1 respectively). Interestingly untreated NK cells exhibited a clear dose response, while Treg treated NK cells appeared to have less of a dose response to decreasing numbers of target cells.
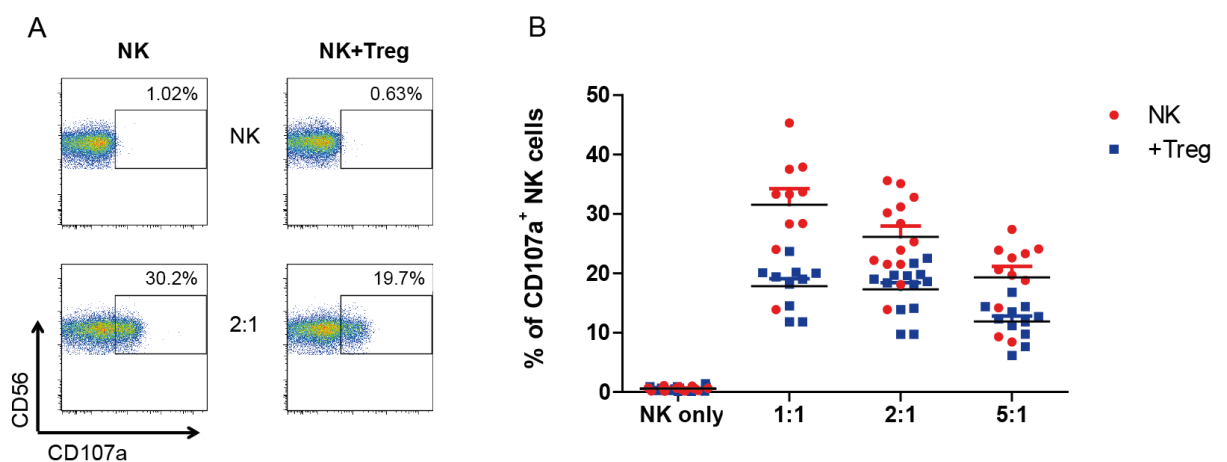
Figure 6.7 Treg treatment reduces NK cell degranulation.
Untreated NK cells (●) and Treg treated NK cells (■) were co-cultured with a range of K562 cells for 2 hours. CD107a expression was assessed by flow cytometry. A) Representative plots, prior to plots shown, gates were applied to live effector cells, upper row effector cells only, lower row effector:target ratio of 5:1. B) Cumulative data for 8 normal healthy donors.

NKG2D is an activating receptor expressed by NK cells, levels of NKG2D were measured on Treg treated and untreated NK cells prior to exposure to target cells. The MFI of NKG2D expression was significantly decreased for Treg treated NK cells (369.3±29.0SEM) compared to untreated (451.0±32.3SEM) (p<0.0001, paired t test) (Figure 6.8A&B). A significant decrease in NKG2D expression for Treg treated NK cells was also observed following exposure target cell ratios for untreated and Treg treated NK cells (411.7±46.4SEM vs 341.8±35.2SEM, 430.0±47.5SEM vs 331.7±37.7SEM and 401.7±47.1SEM vs 316.0±37.4SEM at 1:1, 2:1 and 5:1 ratios respectively. p=0.0412, 0.0040 and 0.0092 respectively, paired t test) (Figure 6.8C). NKG2D expression had no dose response to NK:K562 ratios for both Treg treated and untreated NK cells.
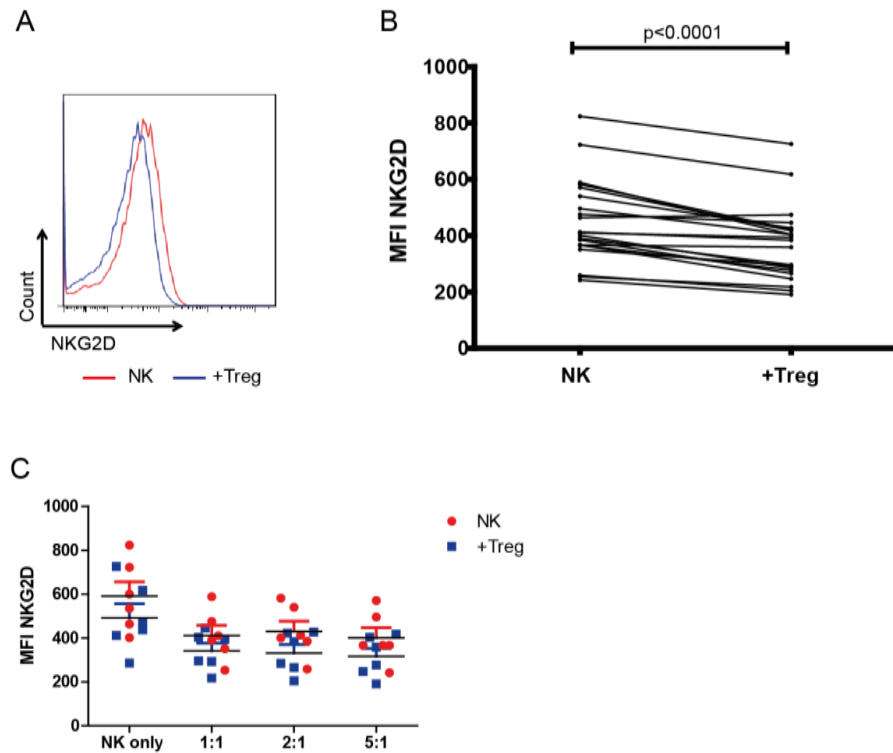
Figure 6.8 NK cell reductions in NKG2D expression following Treg treatment.
NKG2D expression was assessed by flow cytometry. A) Representative histogram, cells were previously gated on live NK cells B) Cumulative data, 4 measurements from 6 normal healthy donors. C) NKG2D MFI of NK cells with and without Treg treatment following exposure to K562 target cells n=6.

NK cells also produce IFNγ following stimulation which is able to influence the adaptive immune response (Vivier et al., 2011). The capacity of NK cells to produce IFNγ was assessed following Treg treatment and the results compared to untreated NK cells. Treg treated NK cells exhibited an impaired capacity to produce IFNγ following PMA and Ionomysin stimulation when compared to the untreated NK cells (15.1%±1.30SEM vs 22.9%±2.85SEM respectively p=0.0413, paired t test) (Figure 6.9).
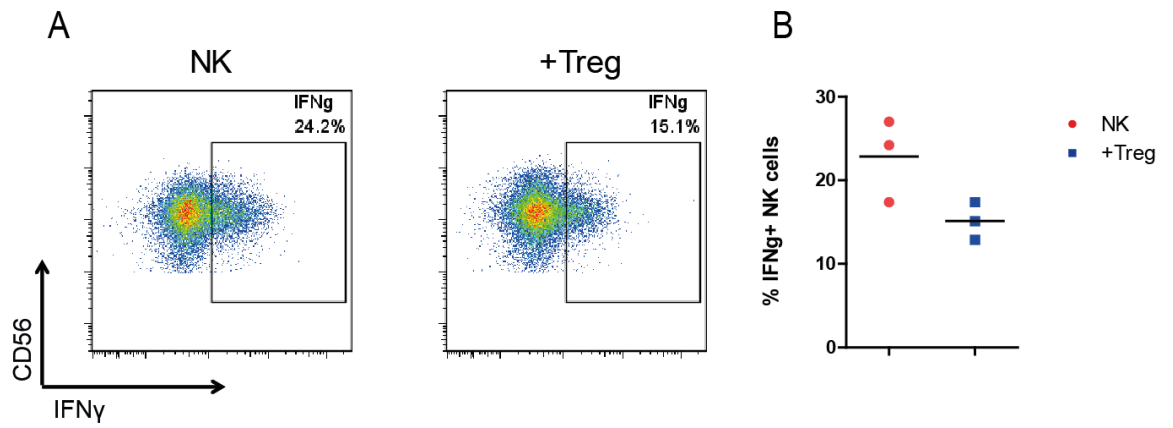
Figure 6.9 NK cells had reduced capacity to produce IFNγ following Treg treatment. Untreated NK cells (●) and Treg treated NK cells (■) stimulated with PMA/Iono/BFA and stained for intracellular IFNγ. A) Representative plots, prior to plots shown gates were applied on CD8⁺ cells. B) Cumulative data. n=3

6.3.3 Treg modulation of CTL functions

Cytotoxic T lymphocytes (CTL) are important mediators of the GvL effect and perforin and granzyme B are key components of CTL cytolysis machinery. To assess and compare CTL cytolytic function the levels of intra-cellular perforin and granzyme B were measured using flow cytometry. The results showed a significant decrease in granzyme B (6.6%±2.77SEM) in allo-reactive CD8$^+$ T cells generated in the presence of Treg compared to those generated in the absence of Treg (32.6%±3.86SEM) (p<0.0001, paired t test). Perforin levels were also significantly decreased to 5.8%±1.94SEM in the allo-reactive CD8$^+$ T cells generated in the presence of Treg, compared to those generated in the absence of Treg (33.8%±4.73SEM) (p=0.0003, paired t test) (Figure 6.10).
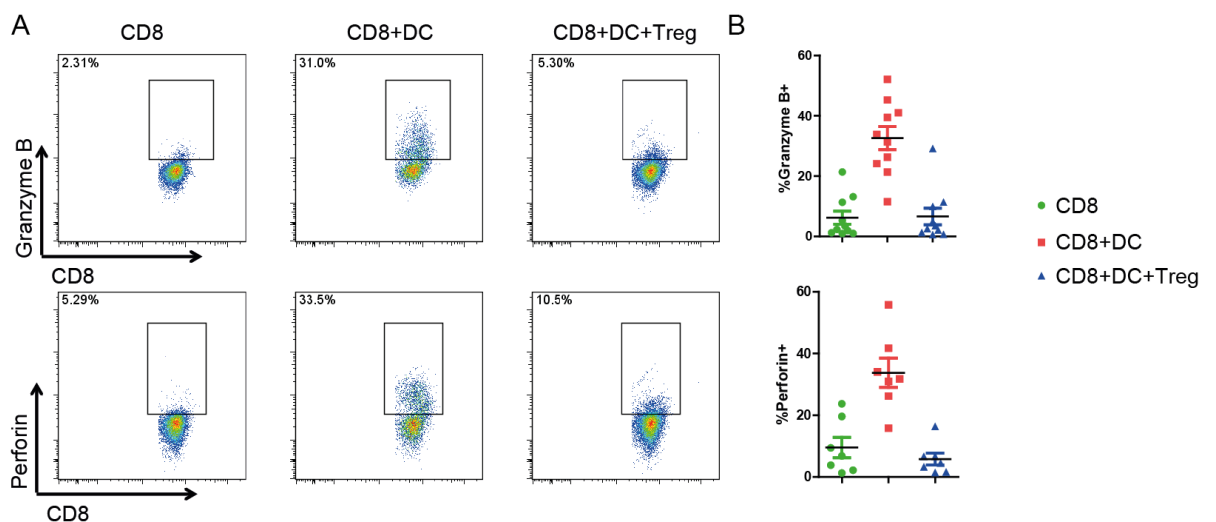
Figure 6.10 The presence of Treg during allo-stimulation resulted in lower perforin and granzyme levels in CTL.
CD8$^+$ T cells were allo-stimulated in the absence (■) or presence (▲) of Treg. Unstimulated CD8$^+$ T cells served as a control (●). A) Representative staining for granzyme and perforin, prior to plots shown gates were applied to CD8$^+$ T cells. B) Cumulative data from 7-10 independent experiments.

The ability of allo-reactive CD8$^+$ T cells to induce target cell death was assessed at a range of effector:target cell ratios ranging from 5:1 up to 100:1. At every ratio there was a significantly higher target cell death when allo-reactive CD8$^+$ T cells were generated in the absence of Treg compared to when Treg were present (p=0.013, 0.026, 0.030, 0.019 and 0.028 for 5:1, 10:1, 20:1, 50:1 and 100:1 ratios respectively, paired t test). Allo-reactive CD8$^+$ T cells generated in the presence of Treg resulted in a comparable target cell death to that of unstimulated CD8$^+$ T cell control (Figure 6.11).

Levels of CD8^{+} T cell degranulation were also assessed following co-culture with PHA blast target cells. The CTL degranulation at a range of effector:target ratios was compared between allo-reactive CD8^{+} T cells generated in the absence or presence of Treg. The CTL degranulation level from un-stimulated CD8^{+} T cells at the same effector:target ratio served as a background control and a culture of CD8^{+} T cells alone without target cells was also included as an additional background control. CD8^{+} T cells cultured alone showed very low levels of degranulation at only 0.90%±0.31SEM. Additionally co-culture of un-stimulated CD8^{+} T cells with the target PHA blasts exhibited less than 5% degranulation at all effector to target ratios.

High levels of effector cell degranulation was observed in allo-reactive CD8^{+} T cells generated in the absence of Treg demonstrating a significantly decreased degranulation at all effector:target ratios except at the 100:1 ratio where sample size was too small to reach statistical significance (p=0.0034, p=0.0089, p=0.0012, p=0.0453 and p=0.0629 for 5:1, 10:1, 20:1, 50:1, and 100:1 respectively). Indeed the allo-reactive CD8^{+} T cells generated in the presence of Treg showed degranulation levels as low as those of unstimulated CD8^{+} T cells.
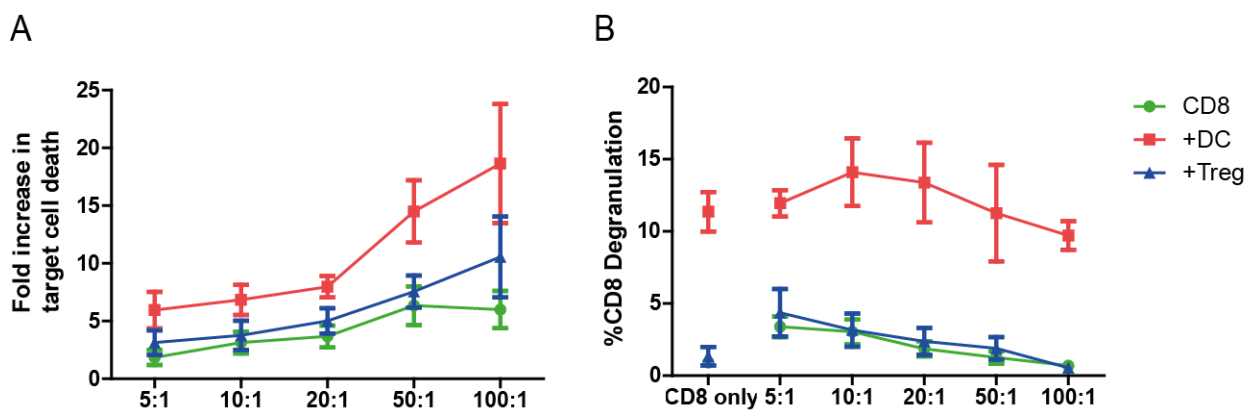
Figure 6.11 The presence of Treg during allo-stimulation resulted in decreased target cell death and effector cell degranulation.
CD8 cells were allo-stimulated in the absence (■) or presence (▲) of Treg. Unstimulated CD8 cells served as a control (●). Data acquired from 2-4 independent experiments. Error bars indicate mean±SEM. A) CD8 cells were incubated with PHA blasts for 2 hours and target cell death measured using flow cytometry. Fold increase in cell death was calculated as the fold increase over background levels in the absence of any effector cells. B) Levels of CD107a were measured by flow cytometry after co-culture with a range of ratios of target cells for 2 hours. Gates were applied to live CD8^{+} effector cells.

Next we assessed whether Treg mediated inhibition of CD8$^+$ T cell cytolytic function was reversible in the absence of Treg. Allo-reactive CD8$^+$ T cells were generated in the presence or absence of Treg, along with an unstimulated control as with previous experiments. After 6 days of priming culture, CD8$^+$ T cells primed in the presence of Treg were rested for 4 days in fresh medium following Treg depletion then tested for their ability to induce target cell death and degranulate. Preliminary data showed that allo-reactive CD8$^+$ T cells previously generated in the presence of Treg regained a certain level of cytotoxic function as indicated by markedly higher levels of target cell death and effector cell degranulation than those of CD8 alone control (Figure 6.12) whilst they were identically low before the reverse culture (Fig 6.11A & 6.11B). Due to technical difficulties it was not possible to assess the degranulation of rested allo-reactive CD8$^+$ T cells generated in the absence of Treg.
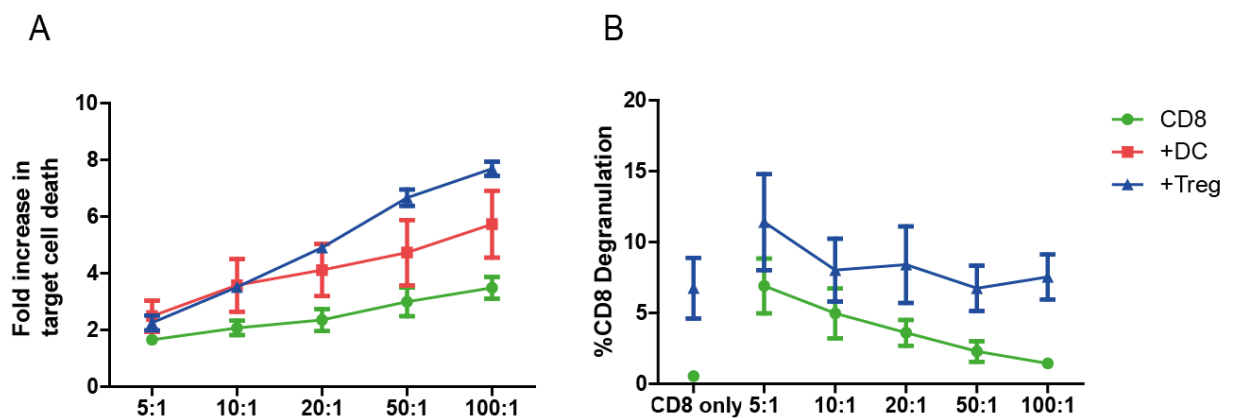
Figure 6.12 Resting CTL following allo-stimulation and Treg deletion allowed them to recover effector cell characteristics.
CD8$^+$ T cells were allo-stimulated for 6 days in the absence (■) or presence (▲) of Treg. Unstimulated CD8$^+$ T cells served as a control (●). Cells were then harvested and then Treg depleted. Cells were rested for 4 days then assessed for A) target cell killing and B) degranulation as previously described. N=3, error bars represent mean±SEM

6.3.4 Association between Treg content of PBMC and leukaemia relapse

A small cohort of transplant patients was examined to investigate if there was any association between Treg levels in patient PBMC post HSCT and the incidence of relapse (see Table 2.6 for patient characteristics). The *in vitro* data presented in this chapter, along with other published literature, suggested that Treg do have the potential to impair the GvL effects, and therefore could have an impact on relapse. Clinical data was obtained from 30 patients who received HSCT between 2008 and 2010. Within the patient cohort 8 suffered from leukaemia relapse and the remaining 22 did not. Patients who received DLI were excluded from the analysis.

The Treg content of serial PBMC samples from patients post HSCT were analysed by flow cytometry. The Treg content was measured as a percentage of CD4$^+$ T cells which were also either CD25^{HI}Foxp3$^+$ or CD25^{HI}CD127^{Low}. The normal range seen in healthy donors was between 3.7% and 9.0% (Figure 6.13), with a mean value of 6.10%±0.63SEM. When comparing the Treg content of samples taken from all the patients at all the time points no significant difference was seen between those who relapsed and those who didn't (p=0.9016). Patients who relapsed had an average Treg content of 13.5%±2.20SEM and those who did not relapse 12.1%±1.13SEM.
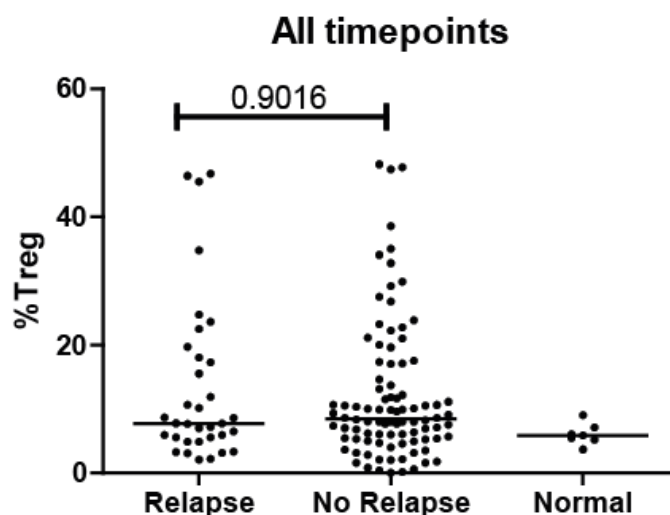
Figure 6.13 Treg content in the post HSCT patients.
Treg content calculated as a percentage of the CD4$^+$ T cells which were CD25^{HI}Foxp3$^+$ or CD25^{HI}CD127^{Low}. Samples from all time points post transplantation. Mann Whitney U values shown. Analysis of 30 patients and 7 normal donors.

Samples were then split into those taken early after transplant, at day 28 and 3 months, and those which were taken late after transplant, at 6 months, 9 months and 12 months. Early samples in the relapse group had a Treg content of 8.84%±2.20SEM while the no relapse group had a Treg content of 10.1%±1.65SEM (p=0.779). When the samples from late post-transplant were considered again there was no significant difference (p=0.423) in the Treg content between those who relapsed (17.7%±3.98SEM) and those who did not relapse (13.5%±1.47SEM) (Figure 6.14).
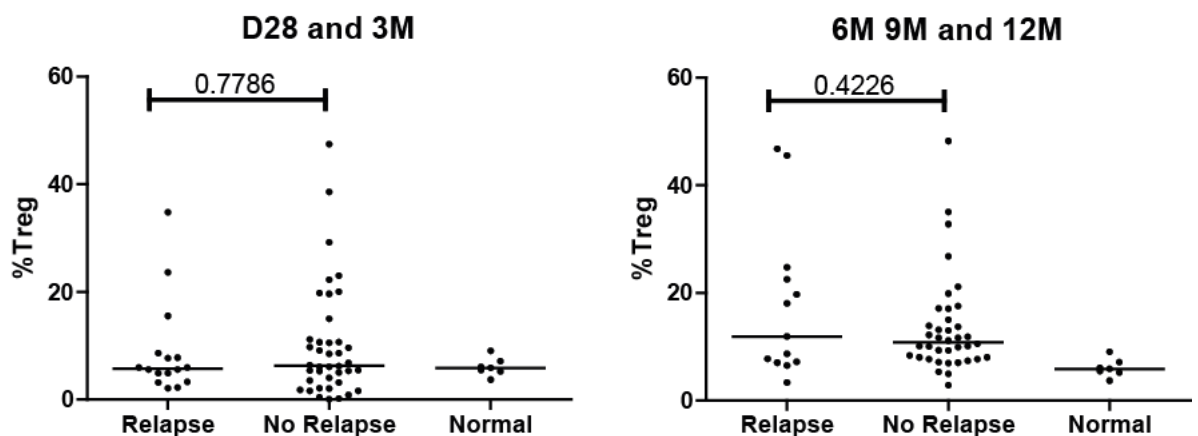
Figure 6.14 Treg content in early and late HSCT patient PBMC.
Treg content calculated as a percentage of the CD4$^+$ T cells which were CD25^{HI}Foxp3$^+$ or CD25^{HI}CD127^{Low}. Samples split into early (D28 and 3M) and late (6M, 9M and 12M) post-transplant. Mann Whitney U test values shown. Analysis of 30 patients and 7 normal donors.

Due to the importance of the GvL effect in AML patients, the Treg content of those patients who had an underlying AML diagnosis were separated out from the whole patient cohort. In total there were 14 AML patients, 3 who later relapsed and 11 who did not. When all the samples from all time points were analysed as one group there was a significant increase in the Treg content for the non-relapse group (12.3%±1.13SEM) than the relapse group (7.49%±1.51SEM) (p=0.46, Mann-Whitney U test) (Figure 6.15). Further analysis of the samples collected early (D28 and 3M) or later post-HSCT (6M, 9M and 12M) revealed a similar trend in early post-transplant samples (Figure 6.16). However, splitting samples into sub-groups reduced the sample size and hence reduced statistical power.
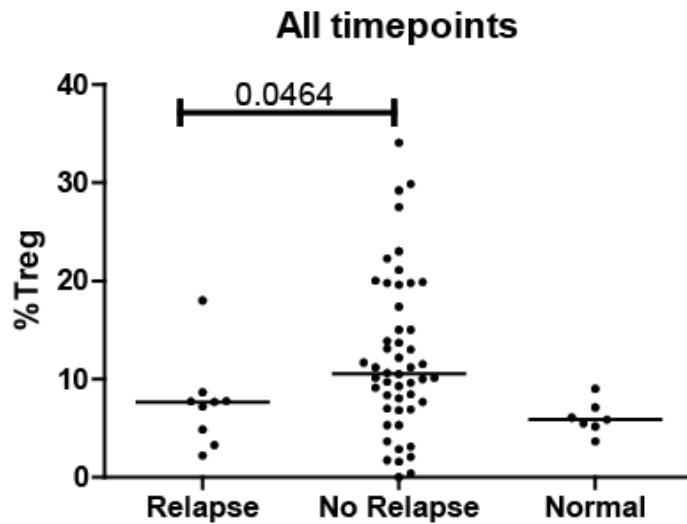
All timepoints

Figure 6.15 Treg content in AML patient cohort.
Treg content calculated as a percentage of the CD4$^+$ T cells which were CD25^{HI}Foxp3$^+$ or CD25^{HI}CD127^{Low}. Samples from all time points post transplantation Mann Whitney U test values shown. Analysis of 14 patients and 7 normal donors.

Although not statistically significant (p=0.099) there was a trend for increased Treg content in the non-relapse (11.7%±1.77SEM) cohort compared to the relapse (5.46%±1.09SEM) cohort early after transplantation. When the samples collected later after transplant were considered there was no difference in Treg content between the relapse and the non-relapse cohorts (p=0.423).
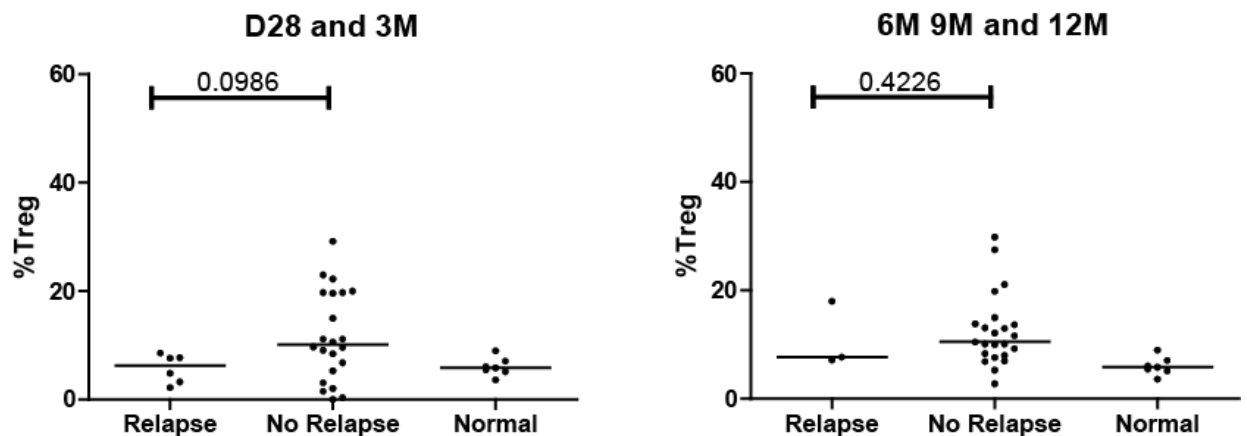
Figure 6.16 Early and late Treg content in AML patient cohort.
Treg content calculated as a percentage of the CD4$^+$ T cells which were CD25^{HI}Foxp3$^+$ or CD25^{HI}CD127^{Low}. Samples split into early (D28 and 3M) and late (6M, 9M and 12M) post-transplant. Mann Whitney U test values shown. Analysis of 14 patients and 7 normal donors.

Further investigation showed that it was the 3 month time point which was the most interesting. At 28 days post transplantation no difference was seen between relapse and non-relapse in terms of Treg content (p=0.788) whereas at 3 months there was a trend towards higher Treg content in the no-relapse (15.4%±2.69SEM) patients when compared to the relapse patients (5.3%±1.32SEM) (p=0.064) (Figure 6.17). Sample size was very low with only 3 samples available for AML patients who relapsed which may be the reason why statistical significance was not reached therefore further patient samples need to be tested before drawing any conclusions.
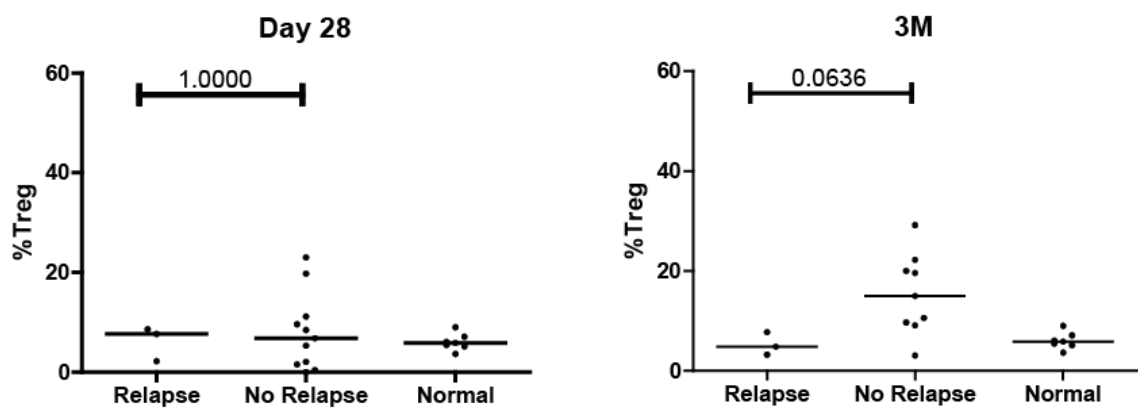
Figure 6.17 Treg content at 28 days and 3 months post HSCT in AML patients.
Treg content calculated as a percentage of the CD4$^+$ T cells which were CD25^{HI}Foxp3$^+$ or CD25^{HI}CD127^{Low}. Samples split into D28 and 3M post-transplant Mann Whitney U test values shown. Analysis of 14 patients and 7 normal donors.

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6.4 Discussion

Current research investigating the association between Treg and leukaemia relapse has been carried out in two distinct directions. One is assessing the frequency and function of autologous Treg in the peripheral blood or bone marrow of leukaemia patients against disease status or response to treatment (Shenghui et al., 2011, Yang and Xu, 2013). The other is dissecting the role of normal donor-derived allogeneic Treg infusion in GvHD and the GvL effect after HSCT. Research presented in this chapter is related to the later.

Data obtained from *in vitro* experiments described in this chapter have demonstrated a Treg mediated reduction in the cytolytic capacity of allo-reactive CD8^{+} CTL, which confirms the observation in a previous report (Wang et al., 2009). This finding was observed only when Treg were present during CD8^{+} T cell priming suggesting that ineffective activation and differentiation of CD8^{+} T cells could be one of the mechanisms leading to their reduced function (Andersen et al., 2006). Further study examining the effects of late Treg addition could strengthen this notion.

The results also indicated that the observed decrease in CD8^{+} T cell cytolitic function is at least partly due to the dysfunction of cytolytic machinery resulting from the inability of CD8^{+} T cells to produce granzyme B and perforin. These cytotoxic proteins are pre-synthesised and released upon encounter with target cells. The perforin/ganzyme pathway requires binding of perforin followed by penetration of the cell granule (granzyme A and B) which leads to activation of the caspase cascade and cell cytolysis takes place (Goker et al., 2001). The fact that intra-cellular levels of granzyme B and perforin were significantly decreased suggests that Treg modulation was targeted at inhibiting synthesis of these cytolytic proteins rather than preventing their release following exposure to target cells.

Preliminary data presented in this chapter has shown a possibility that Treg mediated loss of allo-reactive CD8^{+} T cell function may be partially reversible following removal of the Treg and subsequent resting of the CD8^{+} cells. This data requires further confirmation. Incorporating a re-stimulation step following Treg depletion and resting period may provide clearer evidence with regard to the reversibility of Treg mediated reduction in CD8^{+} T cell function. Nonetheless

this finding raised an interesting hypothesis that Treg infusion before or with HSCT may contribute more towards dampening down the very high magnitude early allo-reactive responses accountable for acute GvHD early post HSCT, but may have less influence on relatively lower magnitude and longer lasting allo-reactive responses required for delivering the beneficial GvL effect. It has been suggested that a lower number of allo-reactive donor T cells are needed to induce a GvL effect than to cause GvHD (Dazzi et al., 2000). Interestingly several results reported in this chapter and Chapter 5 have demonstrated that Treg modulation often resulted in a diminished rather than abolished immune response. It is worth noting that the long term GvL effect mainly depends on allo-reactive T cells derived from engrafted donor haematopoietic stem cells whilst infused Treg will only be present for a short period of time as they are already differentiated mature T cells.

The beneficial GvL effect is also mediated by NK cells. Treg mediated reduction in NK cell cytotoxic activity was detected in this study, along with reduced expression of the activating receptor NKG2D. However NK cell function was not completely negated. Therefore it is possible that *in vivo* Treg could impair but not completely abolish the NK cell mediated GvL effects. Other groups have also described Treg inhibition of NK functions through various mechanisms. Trzonkowski *et al* reported a contact dependent reduction in IFNγ production and cytotoxic activity by human NK cells in the presence of Treg *in vitro* but similarly Treg did not completely negate NK cell function (Trzonkowski et al., 2004). Treg have also been shown to inhibit murine NKG2D mediated cytolysis in a TGFβ dependent, IL-10 independent manner (Smyth et al., 2006). Data presented in this chapter has shown that Treg also induce a reduction in NKG2D expression on human NK cells in support of a previous report from Ghiringhelli *et al* (Ghiringhelli et al., 2005). Interactions between NKG2D and its ligands induces signalling through the adaptor molecule DAP10 inducing NK cell activation (Mincheva-Nilsson and Baranov, 2014). Therefore a Treg mediated reduction in NKG2D expression could result in less activating signals and therefore a decrease in NK cell activation. Although resting NK cells are cytotoxic, the strength of NK cell cytotoxicity greatly increases upon stimulation (Ralainirina et al., 2007). Consequently it is possible that the reason for the

decrease, but not negation, seen in NK cell function when cells were Treg treated was due to prevention of efficient NK cell activation.

In this study IL-2 was used to stimulate the NK cells and Treg treatment was seen to have a significant effect on their function. Recent mouse studies have shown that Treg are able to modulate murine NK cell function through local IL-2 deprivation (Sitrin et al., 2013, Gasteiger et al., 2013a, Gasteiger et al., 2013b). It is conceivable that during the Treg treatment the NK cells have access to lower levels of IL-2, compared to the untreated NK cells, and are stimulated to a lesser extent. Contradictory reports are available in the literature as to the role of Treg mediated IL-2 limitation on NK cell function. It has been reported in a previous human *in vitro* study that when NK cells were stimulated with either IL-2 or IL-15, Treg had no effect on their IFNγ secretory capacity (Ghiringhelli et al., 2005). A more recent report, supporting data presented in this chapter, has shown that human NK cells *in vitro* are suppressed by Treg limitation of available IL-2 (Bachanova et al., 2014). Interestingly during systemic viral infection IL-12 and IL-18 can drive CD25 expression on NK cells, possibly allowing them to compete with Treg for IL-2 (Lee et al., 2012) so although IL-2 can be important when investigating *in vitro* GvL effects it may be less so when assessing *in vivo* GvL effects of NK cells in a pro-inflammatory environment highlighting the difference in complexity between *in vitro* and *in vivo* settings.

Data presented in this chapter has also shown that following exposure to target cells the level of NKG2D expression is decreased on both the Treg treated and untreated NK cells. A similar down regulation in NKG2D expression following exposure to target cells has been reported in CD8^{+} effector cells. Upon stimulation of CD8^{+} CTL expression of NKG2D lessened due to interactions between NKG2D and its ligand inducing endocytosis and degradation of NKG2D expression (Groh et al., 2002) possibly due to up-regulation and subsequent shedding of NKG2D ligands by the CD8^{+} CTL (Maasho et al., 2005). It is possible that similar mechanisms regulating NKG2D expression on the NK cells following exposure to target cells are due to endocytosis and degradation following ligation.

The *in vitro* data which demonstrated a possible partial impairment of cellular GvL functions was obtained under isolated conditions which are unlikely

to fully reflect the complexity of the therapeutic setting after HSCT. Therefore an analysis of a small clinical cohort was carried out to investigate if there was any association between increased Treg levels and relapse. Examination of serial patient PBMC samples showed no association between a higher Treg content and incidence of leukaemia relapse. Apart from this observation there is only one such study available in the literature reporting that higher frequencies of Treg correlated with leukaemia relapse (Nadal et al., 2007). It is not surprising that the findings from the two studies contradict with each other. The conflicting outcome could be due to technical and clinical variations. Firstly, Nadal and colleagues identified the Treg population based on CD4$^+$CD25^{High} phenotype only whereas this study defined Treg using a combined phenotype of CD4$^+$CD25^{Hi} plus either CD127^{Low} or Foxp3$^+$. It is well established that CD4+CD25^{Hi} phenotype only is less reliable for identifying Treg as some activated conventional T cells may also express high levels of CD25 particularly in a pro-inflammatory environment, such as in the case of GvHD and infection (Liu et al., 2006). Secondly, the study presented in this chapter included a mixed cohort of patients whereas the observations from Nadal *et al* were based on an uniform cohort of patients with CML, which is particularly susceptible to the GvL effects of donor lymphocytes (Kolb, 2008) and therefore may be more sensitive to the effects of Treg impairment of GvL. Thirdly, Nadal *et al* correlated Treg content with molecular relapse which may be more sensitive than the clinical relapse which was used in this study. Fourthly, despite having an overall larger patient cohort, the Treg content in the Nadal *et al* study was examined at only one time point post HSCT with follow-up times ranging from just weeks to years post transplantation whereas this study was able to follow the Treg content of individual patients at regular time points for up to one year post HSCT. In addition other clinical variables such as GvHD status, conditioning regimens and DLI could also contribute to the discrepancy between the two studies. Both studies have their shortcomings; however they are the only such observations available in the current literature. Therefore a well-designed prospective study with large longitudinal series may provide more informative conclusion.

Taken together, based on the data and discussion presented in this chapter, along with the work of others, it is possible to hypothesise that the use of Treg

infusion as a clinical procedure to mitigate GvHD may not result in a substantial loss of beneficial GvL effects post HSCT. However, the final verdict lies in the long-term clinical outcome of patients with leukaemia who have received Treg infusion as part of their HSCT procedure. Two clinical trials, included allogeneic Treg infusion as part of their HSCT procedure, for either a heterogeneous or a mainly AML patient cohort, have reported no increased incidence of relapse when compared to a historical cohort based on a two year follow-up (Brunstein et al., 2011, Di Ianni et al., 2011). One of these clinical trials has recently published a follow-up study, including an additional second cohort of patients, reporting that adaptive therapy with Treg and conventional T cells prevents relapse in patients with acute leukaemia (Martelli et al., 2014). Although these are only small scale trials they have provided assurance that Treg can be used to supress GvHD without serious concerns in compromising the beneficial GvL effects. It is encouraging that no increased leukaemia relapse was observed in early clinical trials of Treg infusion. However longer term follow-up and further investigations are required before considering Treg as a routine immunotherapy for GvHD.

Furthermore the role of Treg in the GvL effect post HSCT remains to be fully elucidated. If indeed Treg do permit the GvL effect while preventing GvHD, it is important to understand how they are able to target GvHD but spare the GvL effect. Research in murine models has demonstrated that bone marrow stromal cells are able to neutralise Treg suppressive capacities, hence impede any potential adverse impact the Treg may have on a GvL effect (Martelli et al., 2014). Further investigations are required to establish if these could also be the case in human HSCT.

Chapter 7 - Concluding remarks

Despite substantial advances in HSCT, aGvHD still remains a significant risk post transplantation. The use of Treg for the prevention of GvHD is an appealing prospect and early stage clinical trials have shown promising results; reducing the incidence of aGvHD without adversely effecting transplant outcome in terms of relapse, transplant related mortality and engraftment (Brunstein et al., 2011). To date, the mechanisms by which Treg exert their function in protecting against GvHD are not fully elucidated. In addition, the important question of whether Treg mediated GvHD protection is at the cost of sacrificing the beneficial GvL effect remains largely unexplored. This project aimed to further investigate the mechanisms of action underlying Treg mediated protection against GvHD and to explore the potential impact of Treg therapy on the GvL effect following HSCT.

Due to the rarity of Treg, effective isolation and *ex vivo* expansion is a prerequisite for any Treg functional study. Data presented in Chapter 3 has demonstrated the robustness of the techniques used for Treg isolation/expansion and confirmed the potency of their immunosuppressive property. More importantly the data has provided further evidence demonstrating that *in vitro* expanded Treg can be cryopreserved without damaging their suppressive capacity (Mavin et al., 2013). This observation has not only allowed for more efficient use of resources with a reduced workload and increased consistency in functional research but also provided valuable information supporting the feasibility of using cryopreserved Treg in clinical trials especially where multiple infusions are required. If Treg were to be used routinely in a clinical setting the ability to store Treg prior to transfusion is critical, especially looking into the future considering the use of Treg as an 'off the shelf' cellular reagent. However, before Treg treatment becomes a routine clinical approach standard protocols need to be established to isolate, expand and store Treg under conditions that meet the requirements of regulatory bodies such as the Human Tissue Authority and the Medicines and Healthcare products Regulatory Agency. It would strengthen the quality control of expanded Treg to monitor the methylation status of the CNS2 element within the Foxp3 promoter region in the future as an indicator of Treg stability.

One of the highlights of this research is to have revealed a previously under-appreciated potential mechanism underlying Treg mediated protection against GvHD. Data presented in Chapter 4 has provided the first evidence in a human experimental setting that Treg are able to prevent cutaneous GvH tissue damage by blocking effector T cell invasion into target tissues (Mavin et al 2012). The data has further confirmed that Treg mediated impairment of effector cell migration into GvHD target tissue is associated with decreased expression of skin homing receptors on CD8$^+$ effector T cells paired with reduced expression of the counterpart chemo-attractant ligands in the target tissue. It is fully appreciated that the role of Tregs in GvHD is complex and multifactorial. However the findings of this research have provided a valuable piece of evidence linked into a very complicated mechanistic network by which Tregs exert their protection of GvHD. Further research into Treg modulation of effector T cell invasion into other GvHD target tissues could be of value. Additionally the impact of Treg on alternative, IFNγ independent, skin homing mechanisms could be considered, such as interactions between the chemokine receptor CCR10 and its ligand CCL27. Furthermore it is also worth considering the effects Treg may have on the CD8$^+$ effector T cells. Both murine and *in vitro* human studies have reported Treg modulation of CD8$^+$ effector T cells in an APC independent manner (Piccirillo and Shevach, 2001, Trzonkowski et al., 2004).

To date no human evidence is available directly demonstrating the impact of Treg treated moDC on modulating GvHD. Data presented in Chapter 5 has shown, for the first time in an *in vitro* human GvHD skin explant model, that allo-reactive CD8$^+$ T cells stimulated with Treg treated moDC are less able to induce severe GvH target tissue damage. This could result from the overall outcome of multi-factorial modulation by Treg, including weakening of DC priming, blocking effector T cell migration and IFNγ secretion as well as diminishing the ability of CD8$^+$ T cells to synthesise cytotoxic proteins such as granzyme B and perforin. The reduced ability of Treg treated moDC to prime allo-reactive CD8$^+$ effector T cells is closely related to Treg mediated multi-parameter impairment of moDC both phenotypically and functionally. Furthermore the data also provides the first evidence in humans suggesting that Treg driven development of a tolerogenic status in moDC involves a MARCH1 dependent mechanism (Mavin

et al. manuscript in preparation). Further investigation into the requirement for Treg derived IL-10 in MARCH1 regulation could provide further insight into regulation of co-stimulatory molecules on Treg-DC. Interestingly the presence of Treg throughout the allo-stimulation process resulted in the magnitude of suppression being markedly greater than that when only the DC were treated with Treg, suggesting that the most potent Treg modulation is likely to rely on multiple regulatory mechanisms, influencing DCs and effector cells simultaneously. Further investigations are required to establish the molecular mechanisms by which Treg are able to drive DCs into a tolerogenic status and therefore induce a less severe cutaneous GvH reaction. This could be further investigated with gene expression profiling using PCR arrays. The findings in this research could also be strengthened by exploring molecular pathways, such as the NF-κB signalling pathway underlying Treg mediated resistance to LPS maturation.

Despite the large amount of literature available relating to the beneficial effects of Treg protection against GvHD following HSCT, the effects of Treg on GvL are relatively unexplored. The findings from both *in vitro* experiments and clinical sample analysis presented in Chapter 6 are not able to provide a definitive conclusion with regards to whether Treg infusion would have a negative impact on the beneficial GvL effects. Considering the Treg mediated functional inhibition of cellular components that are critical for delivering beneficial GvL effects it is tempting to conclude that Treg treatment may lead to a compromised GvL response. However, based on the preliminary data showing reversibility of Treg supressed allo-reactive CTL function, the observation that the cytotoxic functions of CTL and NK cells were not completely negated by Treg treatment and the lack of association between higher Treg content in PBMC and leukaemia relapse, it is reasonable to suggest a 'threshold' concept. The idea being that Treg infused before or at the time of HSCT may predominantly target the overwhelming allo-immune responses and extensive inflammation that contribute to the development of aGvHD rather than GvL effect, where the consistent lower magnitude cytotoxic responses are required. In addition, infused Treg may only function early after HSCT and are therefore less likely to influence the long-term GvL effect following full engraftment and immune reconstitution. The research presented in this thesis,

although preliminary, supports the view that the use of Treg infusion as a clinical procedure to modulate GvHD may not result in a substantial loss in beneficial GvL effects post HSCT. Further investigation is required to confirm the preliminary results showing the reversibility of Treg mediated suppression of CD8$^+$ T cell cytolytic functions. The *in vitro* studies are capable of providing invaluable insight into the cellular mechanisms of GvL; however, it is only partially representative of what occurs *in vivo*. Early clinical trials have reported no adverse effects of Treg infusion on the GvL effects. However, the final conclusion lies in the long-term follow-up of multi-centre prospective clinical trials, including a large patient cohort and careful control of all relevant clinical parameters. In the meantime the powerful effect of Treg on modulating allo-reactivity may be utilised to aid other clinical needs where the GvL effect is not a concern, for example to ameliorate GvHD in HSCT for patients with non-malignant disorders or to reduce rejection in solid organ transplantation.

In summary, this project has been conducted to examine the role of Treg modulation in the context of both GvHD and the GvL effect in HSCT. This thesis has provided new evidence and new insight to address the highly complex mechanisms by which Treg modulate GvHD, which has advanced the knowledge and understanding related to the role of Treg in HSCT. The research outcome may also shed light on future clinical trials using Treg as an immunotherapy for GvHD and beyond.

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Appendix – Publications, presentations and ethics

Poster presentations

Mavin E., Dickinson A.M., Wang X.N. *The use of third party Treg to ameliorate graft-versus-host reactions*. In: Bone Marrow Transplantation: 39th European Group for Bone and Marrow Transplantation. 2013, London, UK: Nature Publishing Group.

Mavin E.R., Dickinson A.M., Wang X.N. *Feasibility of using regulatory T cells as an "off the shelf" cellular reagent*. In: Immunology: Special Issue: Volume 137 Issue Supplement. Abstracts of the European Congress of Immunology, 5-8 September 2012. Glasgow, Scotland: Wiley-Blackwell Publishing Ltd

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Madhra M.M., Mavin E., Grainger A., Barge D., Wang X.N., Gennery A.R. *Regulatory T cell subsets and T cell receptor repertoire in Omenn Syndrome.* Poster presentation UKPIN 2011.

Madhra M., Mavin E., Grainger A., Barge D., Wang X.N., Walter J., Matther D., Notarangelo L.D., Gennery A. *Regulatory T cell subsets, T cell receptor repertoire and autoantibodies in RAG1-deficient Omenn Syndrome.* In: Journal of Clinical Immunology. 2012, 32(suppl 1):S1-S379 pages 309-310.

Published abstracts (oral presentation)

Wang X, Turner B, O'Boyle G, Douglass S, Ahmed S, Mavin E, Collin M, Ali S, Dickinson AM. *Blocking effector T-cell trafficking contributes to regulatory T-cell mediated suppression of GvHD reactions*. In: Bone Marrow Transplantation: 37th European Group for Bone and Marrow Transplantation. 2011, Paris, France: Nature Publishing Group.

Publications

Mavin E., Ahmed S.S., O'Boyle G., Turner B., Douglass S., Norden J., Collin M., Ali S., Dickinson A., Wang X.N. 2012. *Regulatory T Cells Inhibit CD8+ T-Cell Tissue Invasion in Human Skin Graft-Versus-Host Reactions*. Transplantation 15:456-464.

Mavin E., Dickinson A., Wang X.N. 2013. *Do cryopreserved regulatory T cells retain their suppressive potency?* Transplantation 95:e68-70.

O'Boyle G., Fox C.R., Walden H.R., Willet J.D., Mavin E.R., Hine D.W., Palmer J.M., Barker C.E., Lamb C.A., Ali S., Kirby J.A.. 2012. *Chemokine receptor CXCR3 agonist prevents human T-cell migration in a humanized model of arthritic inflammation*. Proc Natl Acad Sci U S A 109:4598-4603.

Manuscripts in preparation

O'Boyle G., Barker C E, Mavin E.R., Wang X.N., Newton P.J., Fox C.R., Walden H.R., Willet J.D., Hine D.W., Palmer J.M., Lamb C.A., Douglass S., Ali S., Kirby J.A. 2014. *A small molecule agonist of the chemokine receptor CXCR3 prevents experimental graft-versus-host disease.*

Mavin E., Nicholson L., Ahmed S.A., Dickinson A.M., Wang X.N. 2014. *Treg modulation of dendritic cell function contributes to Treg mediated suppression of GvH reactions.*

Travel awards

Faculty of Medical Sciences Postgrad Travel award. 2014

British Society for Immunology Travel Award. 2014

Internal/Post Graduate conference presentations

North East Post-Graduate Conference, 21st October 2011. Poster presentation. *Regulatory T cell suppressive functions are not affected by cryo-preservation.*

Manchester University Post Graduate Research Day. 11th May 2012. Oral presentation. *Regulatory T cells in HSCT*

North East Post-Graduate Conference, 26th October 2012. Oral presentation. *Regulatory T cells in haematopoietic stem cell transplantation*.

Institute of Cellular Medicine Seminar Series. April 2013 and Feb 2014 '*Regulatory T cells in HSCT'*

NHS

National Research Ethics Service

Newcastle & North Tyneside 1 Research Ethics Committee

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07 November 2007

Professor Anne Dickinson
Professor of Marrow Tranplant Biology
Newcastle University
Clinical & Laboratory Sciences
Medical School
Newcastle upon Tyne
NE2 4HH

Dear Professor Dickinson

Full title of study:	**The development of new diagnostic tests, new tools and non-invasive methods for the prevention , early diagnosis and monitoring for haematopoietic stem cell transplantation (HSCT)**
REC reference number:	**07/H0906/131**

Thank you for your letter of 16 October 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Application	A+B+SSIF	28 August 2007

This Research Ethics Committee is an advisory committee to North East Strategic Health Authority

07-H0906-131 071107 The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Investigator CV	A M Dickinson	28 August 2007
Protocol	Version 1	28 August 2007
Covering Letter	A M Dickinson	28 August 2007
Peer Review	037703	
Participant Information Sheet: BMT Donor	Version 2.3	16 October 2007
Participant Information Sheet: Healthy volunteers	Version 2	16 October 2007
Participant Information Sheet: BMT patient	Version 2	16 October 2007
Participant Information Sheet: Autologous transplant	Version 2	16 October 2007
Participant Consent Form: Healthy volunteers	Version 2	16 October 2007
Participant Consent Form: Autologous transplant	Version 1	13 August 2007
Participant Consent Form: BMT Donor	Version 1	13 August 2007
Participant Consent Form: BMT Patient	Version 1	13 August 2007
Response to Request for Further Information	A M Dickinson	16 October 2007
Letter from funder	037703	09 October 2006

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from
http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following
   a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
   b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
   c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
   d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
   e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

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| 07/H0906/131 | **Please quote this number on all correspondence** |

With the Committee's best wishes for the success of this project

Yours sincerely

P.P.

Professor Peter A Heasman
Chair

Enclosures:

Standard approval conditions

Copy to:

Newcastle upon Tyne Hospitals NHS Foundation Trust
Research & Development
Clinical Research Facility
4th Floor - Leazes Wing
Royal Victoria Infirmary
Queen Victoria Road
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NE1 4LP

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