Role of human interleukin-17 producing T cells (Th17) and regulatory (Foxp3^{+}) T cells in graft versus host disease (GvHD)

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for the degree of Doctor of Philosophy

By

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Declaration of Originality

I herein, Abdullah Saleh Alsuliman, declare that the work in this thesis is my own and has not been submitted for the award of any degree at any other university. Where colleagues have made valuable contributions they have been acknowledged.

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DEDICATION

This thesis is dedicated to my father “Saleh” and mother “Norah” to whom I owe my life and always been an excellent example for me. Your blessings and encouragement were a real inspiration for me to follow my dreams.

I would also like to dedicate this work to my lovely wife “Tamadur” and son “Saleh”. Tamadur, there are numerous reasons I want to personally thank you for. Your love and endless support has been a cornerstone for the achievement of this work. Saleh, the smile and joy you shower me with every day are my daily rewards.

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ABSTRACT

Allogeneic stem cell transplantation (AHSCT) remains the only curative option for a number of patients with haematological malignancies. The interplay between a number of T cell subsets including Th1, Th2, Th17 and regulatory-T cells (Treg) has been linked to the establishment of graft-versus-leukaemia (GvL) as well as its deleterious counterpart graft-versus-host-disease (GvHD). A recent study defined a subset of memory antigen-specific $CD8^+$T cells, with the ability to efflux cytotoxic drugs through the (ABC)-superfamily multidrug efflux complex. I hypothesized that a subset of $CD4^+$T cells, capable of drug efflux through the MDR pump also exists. I confirmed the existence of $CD4^+$T cells with 'stem-like' properties, capable of rapidly effluxing Rhodamine123 as well as cytotoxic drugs such as daunorubicin. I confirmed that the effluxing properties of these cells are through ABCB1 and ABCC1 transporters. Similar to their CD8 counterpart, the rhodamine-effluxing subset of $CD4^+$T cells had a memory phenotype and was enriched within the $CD161^+$T cell compartment. Lastly, using CMV and Flu as model antigens, I showed that viral-specific memory $CD4^+$T cells are enriched within the rhodamine-effluxing $CD4^+CD161^+$T cell population. Once I characterized these cells, I hypothesized that, through effluxing corticosteroids and other immunosuppressant drugs,$CD161^+CD4^+$ T cells may also play a role in acute GvHD. $CD161^+CD4^+$ T cells are precursors of Th17 cells. I demonstrated that, whereas Tregs are prone to apoptosis when exposed to corticosteroids, $CD4^+CD161^+$Th17 cells rapidly efflux and survive exposure to corticosteroids and other immunosuppressive drugs *in vitro*. Inhibition of the ABCB1 and ABCC1 with inhibitors such as vinblastine induced apoptosis of this $CD4^+CD161^+$Th17 steroid-refractory T cell subset. I observed that patients with steroid-refractory GvHD had significantly higher frequencies of $CD161^+CD4^+$ T cells compared to patients with no or steroid-responsive GvHD. I also enumerated $CD161^+CD4^+$ T cells and $Foxp3^+$T cells in the stem cell graft and donor lymphocytes and found an association between higher $CD4^+CD161^+$ and lower $Foxp3^+$T cells frequencies and the risk of GvHD post-transplant. These data advance our understanding of potential mechanisms of steroid-refractory GvHD and have important implications for the development of novel therapeutic approaches for the treatment of this challenging group of patients.

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List of Abbreviations

ABCB1	-	ATP-binding cassette B1
AHSCT	-	Allogeneic Haematopoietic Stem Cell Transplantation
aGvHD	-	acute graft versus host disease
APC	-	antigen presenting cells
rATG	-	rabbit anti-thymocyte globulin
BD	-	Becton Dickinson
BFA	-	brefeldine A
BSA	-	bovine serum albumin
CFSE	-	carboxyfluoresceindiacetatesuccinimidyl ester
CMV	-	cytomegalovirus
CTL	-	cytotoxic T lymphocytes
CTLA-4	-	cytotoxic T lymphocytes antigen-4
CsA	-	cyclosporine A
DC	-	dendritic cells
DLI	-	donor lymphocyte infusion
DMSO	-	dimethylsufoxide
EM	-	effluxing media
FCS	-	Foetal calf serum
FSC	-	forward side scatter
Foxp3	-	forkhead box P3 transcription factor
GATA3	-	Trans-acting T cell-specific transcription factor
G-CSF	-	granulocyte-colony stimulating factor
GITR	-	glucocorticoid induced tumour necrosis factor receptor
GvHD	-	Graft versus host disease
GVL	-	Graft versus leukaemia
HLA	-	human leukocyte antigens
HSC	-	haematopoietic stem cells

Iono	-	ionomycin
IPEX	-	immunodysregulation polyendocrinopathy enteropathy X-linked
LTT1	-	Lectin-like transcript1
mAb	-	monoclonal antibodies
MAIT	-	mucosal-associated invariant T cells
MFI	-	median florescence intensity
MHC	-	major histocompatibility antigens
mHA	-	minor histocompatibility antigens
mTOR	-	mammalian target of rapamycin
NK	-	natural killer
NKT	-	natural killer T cells
PBMC	-	peripheral blood mononuclear cells
PBSC	-	peripheral blood stem cells
PBS	-	phosphate buffered saline
PMA	-	phornol 12-myristate 13-acetate
RORγt	-	retinoic-related orphan receptor γ transcription factor
RPMI	-	Rosewell Park Memorial Institute medium
RT	-	room temperature
SNP	-	single nucleotide polymorphism
SSC	-	side scatter
SRf	-	steroid-refractory
SRs	-	steroid-responsive
STAT	-	signal transducer and activator of transcription
Teb-1	-	T box transcription factor
Teff	-	T effector
TCR	-	T cell receptor
Th	-	T helper
Treg	-	regulatory T cell

CHAPTER 1. INTRODUCTION

1.1 Allogeneic Stem Cell Transplantation

Allogeneic Haematopoietic Stem Cell Transplantation (AHSCT) remains the only curative option for many malignant haematological conditions. The development of reduced intensity conditioning regimens and improved supportive care have broadened the indications for the procedure and in Europe alone, over 24,000 stem cell transplants are currently performed each year(ESH-EBMT 2008). In addition to the treatment of haematological malignancies, AHSCT is increasingly employed in a number of non-malignant conditions including inherited haemoglobinopathies, metabolic disorders and congenital immunodeficiency syndromes. However, treatment of malignant haematological conditions remains the most common indication for AHSCT, where harnessing the powerful anti-tumour capacity of the donor immune system is the cornerstone of long term disease eradication and prevention of relapse.

The success of AHSCT is influenced by a number of variables including the underlying condition, disease status, patient / donor age and importantly, the degree of HLA matching between donor and recipient(ESH-EBMT 2008;Ringden et al. 2003).A number of complications hamper the beneficial effect of AHSCT and increase procedure related morbidity and mortality including infection, conditioning regimen toxicity and graft versus host disease (GvHD)(Gratwohl et al. 2005). The control of these complications and other procedure-related complications, coupled with the ability to maximise immune mediated, graft-derived anti-tumour effect, are key to the further improvement of transplant outcome.

1.2 Graft versus Leukaemia and Graft versus Host Disease

1.2.1 Historical overview

The beneficial graft versus tumour / leukaemia effect (GVL) was observed in early murine experiments in the 1950s where it was first demonstrated that the eradication of leukaemia was possible(Barnes and Loutit 1957). However this success was accompanied by a fatal, wasting diarrheal syndrome that would now be recognised as GvHD. In contrast to the setting of solid organ transplantation, where the major concern is rejection of the donor organ by the recipient immune system, AHSCT reconstitutes an active, donor-derived immune system which, on the one hand, is able to recognise the recipient's residual leukaemia cells as foreign and eradicate them, and on the other, has the capacity to recognise the host tissues as foreign, resulting in immune attack of the recipient– GvHD. Some of the most compelling evidence for the power of anti-leukaemia alloresponses comes from the efficacy of donor lymphocyte infusion (DLI), introduced into clinical practice in the early 1990s to enhance anti-leukaemia immune responses and treat post-transplant disease relapse(Deol and Lum 2010;Helg et al. 1998). In addition, the ability of reduced intensity conditioned (non-myeloablative) transplants to eradicate haematological malignancy relies largely on immunologic GVL reactions.

Both GvL and GvHD involve the recognition of minor histocompatibility antigens presented on the recipient's antigen presenting cells (APC), which accounts for the clinical overlap in GvL and GvHD.

The biological overlap in the immunological mechanisms at play in GvL and GvHD has been clearly demonstrated. Haematological disease relapse is more common in patients without any evidence of GvHD; conversely allograft T cell depletion results in reduced GvHD but increased relapse rate(Alyea 2008). Nevertheless there is experimental evidence that GvL and GvHD could be separated and a number of research groups are actively pursuing this goal.

1.2.2 Clinical manifestation of acute GvHD

The clinical syndrome of GvHD manifests as tissue injury mediated both by inflammatory cytokines and cytotoxic effector T cells. The syndrome has been divided into two phases: acute, occurring earlier than 100 days post-allograft and chronic, occurring after 100 days. Acute GvHD primarily affects the skin, liver and gastrointestinal tract, whereas chronic GvHD typically has a multisystem, (classically) autoimmune-syndrome pattern and may have a wider distribution of affected tissues **(Table 1.1)**. Temporal division of the two manifestations is however challenging. Acute GvHD is not necessarily followed by the chronic form and conversely chronic GvHD can arise de novo without prior acute GvHD. In addition late-onset GvHD with the clinical pattern of acute GvHD is recognised(Mielcarek et al. 2003). These problems led to a working group consensus statement in 2005 from the National Institutes of Health that proposed new criteria for diagnosis and classification of chronic GvHD for clinical trials. According to the consensus criteria, clinical manifestations, rather than time after transplantation, should be used in clinical trials to distinguish chronic GvHD from late acute GvHD, which includes persistent, recurrent, or late-onset acute GvHD(Filipovich et al. 2005).

Organ involved	Acute GvHD	Chronic GvHD
Skin	Maculopapular rash, ulceration	Abnormal pigmentation, alopecia, sclerosis
Upper gastrointestinal tract	Nausea and vomiting	Xerostomia, ulceration, oesophageal strictures
Lower gastrointestinal tract	Watery diarrhea (>500ml), abdominal pain, ileus	Weight loss
Liver	Cholestatichyperbilirubinaemia	Jaundice, transaminitis
Eyes	-	Sicca syndrome, conjunctivitis
Lungs	-	Obliterative bronchiolitis, restrictive defects, pleural effusions
Bone marrow	-	Cytopaenias
Musculoskeletal	-	Myositis, fasciitis, contractures

Table 1.1: Clinical Patterns of Acute and Chronic GvHD

1.2.3 Target antigens for GvHD

The most important antigens in the pathogenesis of GvHD are the human leukocyte antigens (HLA)(Ferrara et al. 2009). These proteins are highly polymorphic and expressed on almost all nucleated cells in the body. The degree of mismatch between donor and recipient HLA proteins is directly related to the risk of GvHD(ESH-EBMT 2008;Ferrara, Levine, Reddy, & Holler 2009).However GvHD still occurs in fully HLA-matched transplants, indicating the importance of other proteins, termed minor histocompatibility antigens (mHA) in driving the allo-immune T cell response. In contrast to HLA molecules, mHAs may be expressed ubiquitously, such as HY and HA-3 or may exhibit tissue specificity such as HA-1 and HA-2, which are restricted to haematopoietic tissues(Loiseau et al. 2007). The potential exploitation of this tissue separation to extract useful GvL effect from unwanted GvHD is one avenue of current active research.

1.2.4 Pathophysiology of acute GvHD

Acute GvHD is initiated by massive cytokine storm, induced by the conditioning regimen itself. Damaged host tissues produce pro-inflammatory cytokines and chemokines, resulting in APC activation and increased cross-presentation of polypeptide antigens to donor immune cells. This may be further enhanced by the presence of infection, the underlying haematological malignancy itself and the systemic translocation of additional inflammatory stimuli across damaged mucosa(Pidala and Anasetti 2009;Xun et al. 1994). These activated APCs then stimulate donor T cells to proliferate and produce large amounts of cytokines, particularly interferon-gamma (IFNγ), interleukin-2 (IL2) and tumour necrosis factor-alpha (TNFα), which in turn promote the stimulation of cellular mediators including cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.

These mediators destroy target tissues via cell lysis pathways such as Fas-Fas Ligand, perforin and granzyme, resulting in clinical GvHD(Hill et al. 1997;Jacobsohn et al. 2003;Mielcarek et al. 2003)**(Figure 1.1).**

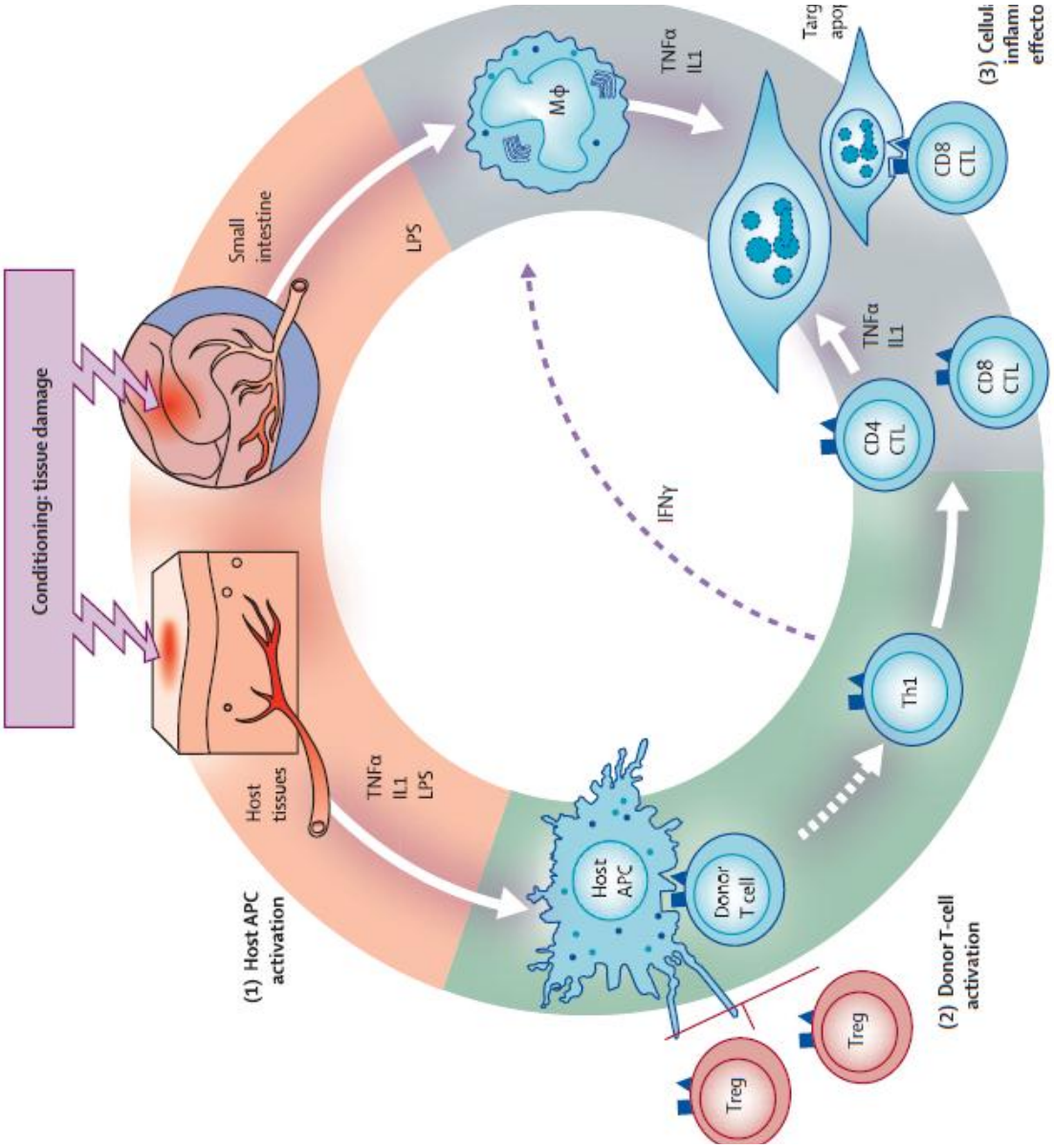


Figure 1.1: Pathophysiology of acute GvHD. Diagram shows the sequential development of acute GvHD and the involvement of cellular and soluble mediators. Adopted from Ferrara et al, Graft versus Host Disease, Lancet 2009.

1.2.5 Acute GvHD grading

Grading criteria for acute GvHD have evolved over time. The first system was published by Glucksberg in mid-seventies (Glucksberg et al. 1974). These criteria measure the involvement of each organ and give an overall grading from I-IV. Nevertheless, a newer version of this system came in 1994, so-called modified Seattle Glucksberg criteria (Przepiorka et al. 1995)and it included the percentage of skin involvement and using a system known as the “rule of nines” the body surface area involvedcan be calculated (Hettiaratchy & Papini, 2004). The international bone marrow transplant registry (IBMTR) has also introduced another grading systemclassifying GvHD severity from A-D according the outcome of the transplant (Rowlings et al, 1997). While IBMTR grading criteria minimize bias in scoring by physicians, modified Seattle Glucksberg system was better in predicting survival. Nevertheless, there are no advantages for the one system over the other(Cahn et al, 2005).

1.2.6 GvHD prophylaxis and treatment

Prophylaxis and treatment of acute GvHD consist of pharmacological agents to interrupt the cascade of inflammation .Prior to AHSCT, patients undergo preparative regimens aimed at eradicating residual cancer cells as well as suppressing recipients’ immune system to allow the donor cells engraftment. The calcineurin inhibitors (e.g. cyclosporine and tacrolimus) and methotrexate constitute the usual standard of care for prophylaxis. However, failure of prophylaxis regimens to suppress the undesired allo-reaction i.e. GvHD, necessitate prompt intervention. High dose corticosteroids are usually the front-line in the management of active GvHD. A high proportion of patients however fail to respond to corticosteroids. Steroid-refractory GvHD and complications associated with immunosuppressive regimens continue to thwart the

success of AHSCT(Jacobsohn et al. 2003).Several interventions, such as mTOR inhibitors and mycophenolatemofetil (MMF), were employed in an effort to reverse steroid resistance(Dignan et al. 2012). Severe GvHD has a poor prognosis, with <25% 5 year survival for disease grade III or above despite maximum intervention(ESH-EBMT 2008;Jacobsohn et al. 2003).Harnessing the power of regulatory T cells for the improved control of GvHD and consequent reduction in transplant related morbidity and mortality is an enticing strategy, although to be a successful approach it will be essential to ensure that donor engraftment, anti-tumour allo-responses (GvL)and protection against infection are not adversely affected.

1.3 T cell Biology and subsets

T cells are fundamental in the adaptive immune response, mostly as a consequence of their huge diversity, in addition to their ability to produce a wide spectrum of cytokines. They belong to a group of cells known as lymphocytes. Generally, T cells originate in the bone marrow from a haematopoietic stem cell precursor (HSC). They then emigrate to the thymus where they undergo maturation, negative and positive selection and tolerance induction to self-antigens, which help in identification and destruction of the self-reactive clones, thus maintaining immune tolerance(Abbas A.K. et al. 2007).

Historically, these cells have been identified by the surface expression of CD3 in addition to the mutual expression of either TCR αβ or γδ. T cells with TCR αβ subunits are further characterized by the surface expression of CD4 and CD8, which differentiate the two major subsets of T cells, namely helper (Th) and cytotoxic (Tc) T cells, respectively. Additionally, according to their ability to produce specific cytokines, these two subsets can be categorized into Th1/Tc1, Th2/Tc2

and Th17/Tc17(Abbas A.K., Lichtman A.H., & Pillai 2007). However, other T cell subsets, albeit in much smaller numbers, exist including γδ T cells, double negative ($CD4^-$ $CD8^-$) and double positive ($CD4^+CD8^+$) T cells.

1.3.1 Helper T cell subsets

When naïve $CD4^+$ helper T cells (Th) encounter their cognate antigens through TCR complex- MHC-II interaction, they tend to differentiate into effector phenotype. T-helper cells were classically subdivided into Th1 and Th2(Boyton and Altmann 2002); however, the recent identification of IL-17-producing $CD4^+$cells (Th17), and TGF-β-induced regulatory T cells (iTreg), has changed the dogma of Th classifications(Wan and Flavell 2009). The differentiation fate of these Th subsets is largely governed by a number of factors including the cytokine milieu during the differentiation process as well as their transcriptional factor network. Accordingly, these subsets can be distinguished by their cytokine production and their master transcriptional factor. Also, the affinity of their cognate antigen could have an influence on their lineage commitment(Boyton & Altmann 2002;Zhu and Paul 2010). Interestingly, although the commitment of each Th cell to one phenotype is absolute, a great degree of plasticity has been observed within these subsets as discussed later(Zhou et al. 2009).

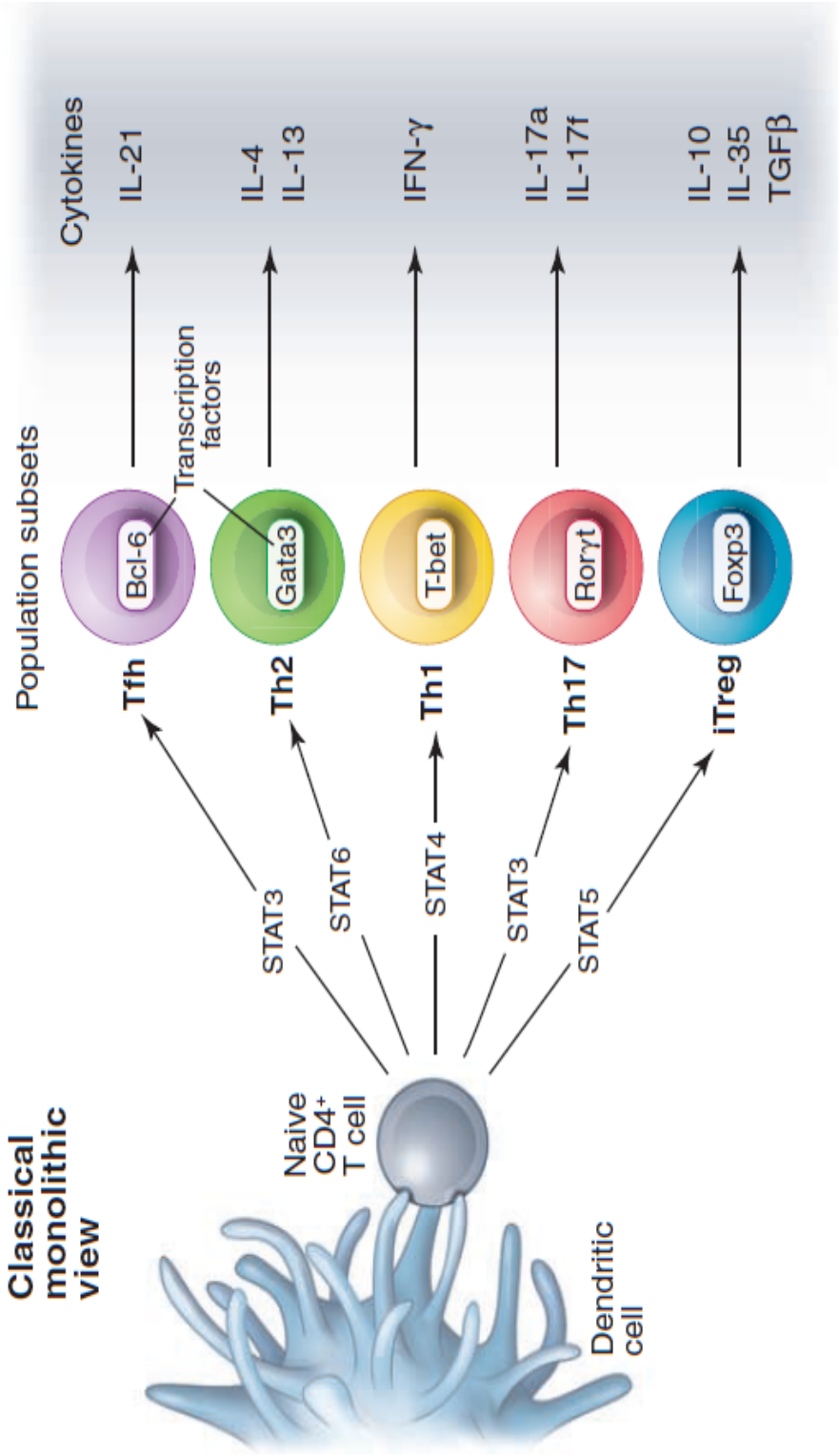


Figure 1.2: CD4$^+$ helper T cell subsets. This illustration shows the different T cell subsets, their lineage-specific transcriptional factors and cytokines.Adopted from O'Shea et al, Science, 2010.

1.3.1.1 T helper type 1(Th1)

The cytokine milieu in addition to the master transcriptional network can drive the differentiation and fate of T cells.

Th1 cells are key players in the clearance of intracellular microorganisms (viruses and some bacteria), mostly through their ability to produce large amounts of IFNγ, which is considered as a major signature of this Th subset. Interleukin-12, produced by cells of the innate immune system, in addition to IFNγ, produced by T cells and NK cells, act as a positive feedback loop to induce polarization toward a Th1 phenotype. The transcriptional network controlling Th1 differentiation includes signalling transducer and activator transcription 1 and 4 (STAT1 and 4), as well as T box transcription factor (Tbet) (Zhou, Chong, & Littman 2009;Zhu & Paul 2010).

1.3.1.2 T helper type 2(Th2)

In contrast to Th1 cells, Th2 cells produce a wider range of immunomodulatory cytokines including interleukin-4 (IL-4), IL-5, IL-9, IL-13, and IL-25. During antigen recognition, the presence of IL-4 polarizes naïve $CD4^+$ T cell differentiation towards Th2 cell fate. Additionally, this process results in the activation of STAT6. The action of both IL-4 and STAT6 consequently up-regulates GATA3, the master regulator for this subset's differentiation fate. The main role of this subset of T helper cells is protecting the body against parasitic infections such as helminths. It also plays a key role in allergic reactions(Zhu & Paul 2010).

1.3.1.3 T helper type 17(Th17)

A role of interleukin 17 in the immune response has been known for some time(Kolls and Linden 2004); however it is only in the last few years, that IL-17-producing $CD4^+$ T cells were defined as a new Th subset. This notion was supported by the fact that these cells could not produce cytokines of other Th lineages (IFNγ and IL-4), in addition to the requirement for a distinct set of cytokines to drive their differentiation including TGF-β, IL-6, IL-21 and IL-23 and most importantly the expression of their unique master transcription factor, retinoic acid-related orphan receptor (ROR)γt(Ivanov et al. 2006;Miossec et al. 2009). Polarization of Th17 cells with the aforementioned cytokines (IL-6, IL-21 and IL-23) activates STAT3,which in turn induces RORγt expression. Additionally, other transcriptional factors including the AP-1 family members, BFAT and Runx1, have been shown to be involved in the induction of the Th17 master regulator(Zhu & Paul 2010). Th17 cells mainly produce IL-17A, in addition to IL-17F, IL-21 and IL-22(Miossec, Korn, & Kuchroo 2009). They play an important role in expelling extracellular microorganisms e.g. fungi and bacteria(Weaver et al. 2006).Although phenotypically Th17 cells do not have specific characteristics; the origin of this subset has been proposed to be $CD4^+$ $CD161^+$ T cells (unlike Th1 and Th2 cells) (Cosmi et al. 2008).

1.3.2 CD161-expressing T cells

Human CD161, also known as NKR-PA1, is made of disulfide-linked homodimer subunits and was originally identified on murine NK cells. In humans, the vast majority of NK cells express CD161, in contrast to only approximately one quarter of all T cells (TCR-αβ, -γδ and double negative)(Lanier et al. 1994a;Maggi et al. 2010). Two related ligands for CD161 have been recognized:Lectin-liketranscript1 (LTT1), expressed on activated APC and T cells, and proliferation-induced lymphocyte-associated receptor (PILAR),expressed on T cells post-TCR engagement(Aldemir et al. 2005;Huarte et al. 2008).

Generally, CD161-expressing CD4 and CD8 T cells reside within the memory T cell repertoire(Takahashi et al. 2006). However, a very small percentage of naïve $CD161^{+}CD4^{+}$ T cells were also identified in cord blood(Cosmi et al. 2008). Unlike $CD4^{+}$ T cells which have intermediate expression of CD161, $CD8^{+}$ T cells express CD161 at two levels: intermediate and high(Takahashi, Dejbakhsh-Jones, & Strober 2006). Several reports have examined the origin and function of the $CD8^{+}$ $CD161^{hi}$ population. $CD8^{+}$ $CD161^{hi}$ T cells co-expressing IL-18Rα, were shown to have a chemo-effluxing, memory-like stem cell phenotype(Turtle et al. 2009). Likewise, Billerbeck et al identified a similar population with tissue-homing properties, but with Tc17 phenotype(Billerbeck et al. 2010). More recently, $CD161^{hi}$ IL-18Rα^{+} $CD8^{+}$ T cells were shown to be Vα7.2^{+} MAIT cells (mucosal associated invariant T cells)(Dusseaux et al. 2011).

In contrast, CD161-expressing $CD4^+$ T cells have been less well characterized. Takahashi and colleagues showed that this population has a pro-inflammatory profile(Takahashi, Dejbakhsh-Jones, & Strober 2006). $CD4^+$ T cells with intermediate expression of CD161 were also found with migratory capability across the endothelial monolayer (Poggi et al. 1997); nevertheless, this ability could be revoked by blocking CD161 using monoclonal antibodies(Bezouska et al. 1994).Moreover, the naïve populations of $CD4^+CD161^+$ T cells, identified in umbilical cord blood, were shown to be the origin of IL-17-producing T cells(Cosmi et al. 2008).These data suggest that CD161-expressing T cells are likely to represent a mixture of different T cell subsets, some of which may have stem cell-like memory properties.

1.3.3 Regulatory T cells

Maintaining immune tolerance to self antigens is crucial for the immune system to prevent autoimmunity. A variety of cells are involved in this process, including naturally occurring regulatory T cells (Tregs)(Albert et al. 2005;Cohen et al. 2002;Hoffmann et al. 2002;Sakaguchi 2005),IFNγ/IL-10-secreting T regulatory type 1 (Tr1)(Groux et al. 1997;Levings et al. 2001a), transforming growth factor-β-secreting Th3 regulatory cells(Weiner 2001), natural killer T cells (NKT)(Seino et al. 2001), and double negative cells ($CD4^-CD8^-$)(Zhang et al. 2000).Of these, naturally occurring regulatory T cells have been the principal focus of transplantation tolerance studies.

1.3.3.1 Naturally occurring regulatory T cells

Although studies have suggested that naturally occurring regulatory T cells are generated as a distinct population in the thymus, $CD4^+CD25^{hi}Foxp3^+$ Tregs that are identical to the thymus-derived population, can also be generated after antigen-induced proliferation of $CD4^+$ T cells in peripheral tissues in both mice and humans(Apostolou and von 2004;Cobbold et al. 2004;Vukmanovic-Stejic et al. 2008). $CD4^+CD25^{hi}Foxp3^+$ Tregs constitute 5-10% of total $CD4^+$ T cells and play a pivotal role in immune tolerance by inhibiting auto-reactive T cell populations(Sakaguchi et al. 2001;Takahashi et al. 1998). As such they are critical in the maintenance of normal immune homeostasis(Dudda et al. 2008). In addition to their ability to suppress autoimmune reactivity, they can suppress reactions directed against non-self antigens(Sakaguchi 2005). Naturally, Tregs are anergic when stimulated *in vitro*. However, co-stimulation with anti-CD28 and high concentrations of IL-2 has been shown to break this hypo-responsiveness.

By contrast, *in vivo* studies have shown the ability of these cells to accumulate and proliferate at the site of inflammation upon second antigen recall(Vukmanovic-Stejic et al. 2008).

1.3.3.2 Characterization of naturally occurring regulatory T cells

Regulatory T cells can be identified phenotypically by the expression of CD4 and the IL-2 receptor α chain, CD25(Sakaguchi et al. 1995). In addition, they are characterised by negative or low expression of CD127, the IL-7 receptor α chain(Liu et al. 2006). More recently, the forkhead/winged helix transcription factor protein (FoxP3), which is constitutively expressed in Tregs and involved in their development and suppressive functions, has been employed as an intracellular

marker for Tregs(Fontenot et al. 2003;Hori et al. 2003). Mutation or disruption in the FoxP3 gene leads to a fatal multi-organ autoimmune disease in both human and mice, namely immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and the scurfy mouse phenotype, respectively(Bennett et al. 2001;Brunkow et al. 2001), demonstrating the critical role of this transcription factor in Treg function.

T cells are divided into naïve and memory according to the expression of different isoforms of CD45. Whereas naïve T cells express CD45RA (Valmori et al. 2005) the memory counterpart possess the CD45RO isoform. This classification of T cell subsets has been shown to correlate with Tregs functions and propagation (Vukmanovic-Stejic et al. 2006;Hoffmann et al. 2006).More detailed characterization of Treg cell subpopulations, naïve and memory, based on the deferential expression of CD45RA and FoxP3 has also been reported (Miyara et al. 2009). Further characterization was also introduced on the basis of the expression of homing receptors, namely CD62L and CCR7 (Sallusto et al. 1999; Tosello et al. 2008). In transplant setting, regulatory T cells expressing CD62L have exhibited higher suppressive potential and were able to control GvHD than their $CD62L^-$ counterparts (Ermann et al. 2005;Taylor et al. 2004).

Other markers that are commonly used for Treg identification, include cytotoxic T lymphocytes antigen-4 (CTLA-4), glucocorticoid induced tumour necrosis factor receptor (GITR) and more recently tumour necrosis factor receptor 2 (TNFR2)(Chen et al. 2010;Shimizu et al. 2002;Takahashi et al. 2000). Although FoxP3 and CTLA-4 are among the most reliable markers for the identification of regulatory T cells, they cannot be employed for the selection of functional Tregs due to their intracellular

expression. To overcome this problem, several groups have focussed on finding a reliable surface marker. Recently, CD39 (the ectoenzyme which degrades ATP to AMP) and CD49d (the α-chain of the integrin VLA-4 (α4β1)), have been used along with $CD4^+$, $CD25^{hi}$ and $CD127^{low}$ as markers to facilitate the isolation of functionally active untouched $Foxp3^+$ Tregs(Borsellino et al. 2007;Kleinewietfeld et al. 2009;Mandapathil et al. 2009). Other markers include the tumour-necrosis factor receptor family member CD27(Ruprecht et al. 2005)and HLA-DR(Baecher-Allan et al. 2006), although both are limited in their selectivity.

1.3.3.3 Regulatory T cells-mechanisms of action

The exact mechanisms by which $CD4^+CD25^+Foxp3^+$ regulatory T cells exert their anti-inflammatory and immunoregulatory functions are not fully understood. To date, efforts to identify these mechanisms have cast some light on the topic but progress has been hampered by conflicting data. The majority of *in vitro* studies have shown that Tregs require cell contact to exert their suppressive effect(Nakamura et al. 2001). However, a key role of immunoregulatory cytokines such asIL-10, TGF-β and IL-4 in T cell-mediated suppressive activities has also been demonstrated in both *in vitro* and *in-vivo* neutralization studies(Asseman et al. 1999;Powrie et al. 1996;Seddon and Mason 1999).

In addition to these two main mechanisms, Edinger et al have shown perforin dependency of these cells in the control of allo-reactions in GvHD(Edinger et al. 2003). In a recent study by Cai and colleagues, granzyme-B was reported to be an important player in the suppressive action of Tregs against anti-tumour effector T cells (mediating GVL), but not important in suppressing allo-reactive effector T cells

in GvHD; this suggests that the manipulation of granzyme-B could allow separation of the two phenomena(Cai et al. 2010;Cao et al. 2007).

1.3.3.4 Targets of regulatory T cells

A variety of effector cells, most notably those of the adaptive immune system such as $CD4^+$and $CD8^+$T cells, as well as other immune subsets such as NK and B cells have been shown to be the main targets for Treg suppressive activity(Iikuni et al. 2009;Kim et al. 2006;Levings et al. 2001b;Pandiyan et al. 2007;Piccirillo and Shevach 2004;Simon et al. 2007). Interestingly, it has been shown that once activated, T regulatory suppressor function are non-specific(Thornton and Shevach 2000) and therefore, it may also be possible for these cells to control innate immunity. Conversely, recent work has shown that there may in fact be counter-regulation of regulatory T cells by innate immune mechanisms(Kim et al. 2009).

The manipulation of regulatory T cells in the allogeneic stem cell transplant setting has the potential to achieve two opposing, although not necessarily mutually exclusive effects: their depletion to enhance anti-tumour immunity (Heier et al. 2008;Imai et al. 2007;Kline et al. 2008) or harnessing their regulatory capacity to achieve protection from autoimmunity, graft rejection and critically, GvHD. The segregation and optimisation of these two effects is at the forefront of current allogeneic stem cell transplant research.

1.3.4 Plasticity between T cell subsets

Different Th cell subsets have been historically considered as terminally differentiated, recognised mainly by their ability to produce specific cytokines and express a unique transcriptional profile. However, recent compelling data suggest more flexibility in the phenotype of differentiated T cell subsets, hence challenging this paradigm. This flexibility has been noted to include the shared production of cytokines that were previously assigned for a specific phenotype such as IL-10 (considered to be of Th2 origin) or IL-21(Saraiva and O'Garra 2010;Suto et al. 2008). Additionally, lack of fundamental factors for differentiation could drive this plasticity. For instance, Th17 cells, in the absence of TGF-β which is required for their development, can acquire a Th1 phenotype(Hirota et al. 2011;Lee et al. 2009b). Likewise, regulatory T cells under certain circumstances upregulate the Th1 master regulator T-bet and produce IFNγ(Dominguez-Villar et al. 2011;Oldenhove et al. 2009). Furthermore, in addition to their own transcription factors and cytokines, T cell subsets may express transcription factors and/or produce cytokines from T cells of a different phenotype. For example, although regulatory T cells were defined by the expression of Foxp3, under certain conditions they were shown to express the Th17 master transcriptional factor, RORγt, and produce IL-17(Voo et al. 2009). Similarly, $Foxp3^+$ regulatory T cells were shown to have the ability to express the master regulators of Th1 (T-bet) and Th2 (GATA3)(Koch et al. 2009;Wang et al. 2011;Wohlfert et al. 2011). Finally, a subset of Th17 cells with high pathogenic potential in murine models was also found to co-express other transcription factors such as T-bet(Ghoreschi et al. 2010;Yeh et al. 2011).

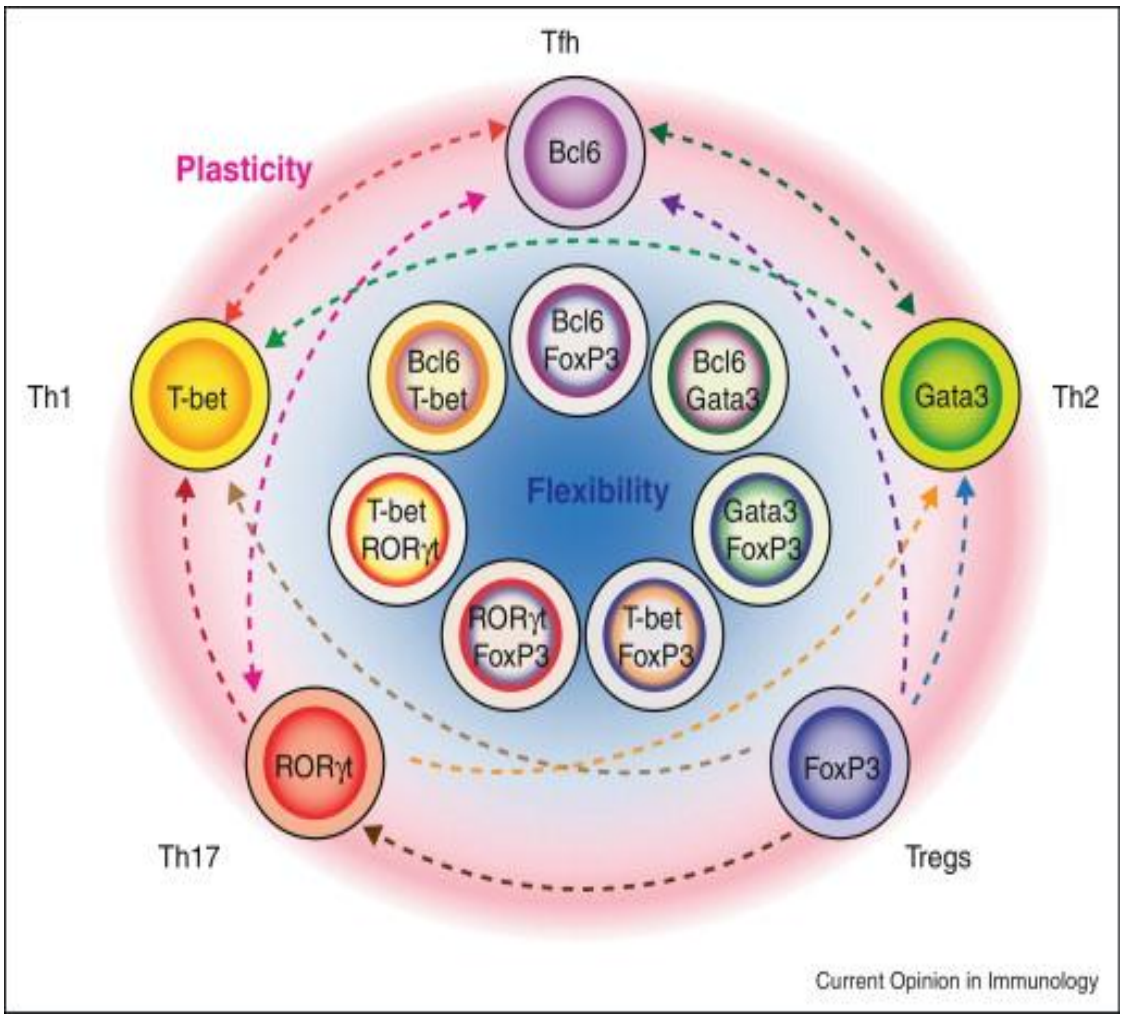


Figure 1.3: Plasticity between helper T cell subsets. The Figure shows the potential to acquire different phenotype through mutual expression of the master regulators. Adopted from Nakayamada et al, 2012.

1.4 $CD4^+$ T cell subsets and GvHD/GvL

1.4.1 Animal studies of Th17, $CD4^+CD161^+$T cells, Tregs and GvHD

1.4.1.1 Helper T cell type 17 and $CD161^+CD4^+$ T cells

Historically, acute GvHD was thought to be mediated exclusively by IFNγ-producing T cells (Th1)(Allen et al. 1993). Administration of T helper 1 inhibitors was found to protect mice from GvHD (Lu et al. 2001) and selective deletion of the Th1 transcription factor T-bet prevented GvHD while allowing GvL. However, other studies in murine models reported that IFNγ-deficient mice developed much more severe GvHD compared to the wild-type(Murphy et al. 1998;Yang et al. 1998). These conflicting findings shifted the focus of GvHD researchers to study the role of other T cell subsets in the pathogenesis of GvHD.

Recently, the role of Th17 cells in GvHD pathogenesis has been addressed. Carlson et al demonstrated that infusing IL-17-producing T cells generated *in vitro* into lethally-irradiated mice was sufficient to induce acute GvHD in skin and lung. Interestingly, neutralizing IL-17 by a monoclonal antibody abrogated this effect(Carlson et al. 2009). In another study, although no difference was observed in GvHD related deaths in recipients of wild type (WT) and IL-17 knock-out (KO) T cells, transfer of IL-17 KO $CD4^+$ T cells into allogeneic recipients delayed the onset of GvHD when compared to WT $CD4^+$ T cells(Kappel et al. 2009). Furthermore, suppression of IL-17 production from G-CSF-treated $CD4^+$ T cells by SOCS3 induction was shown to ameliorate acute GvHD(Joo et al. 2012). On the other hand, IL-17-deficient donor $CD4^+$T cells accelerated GvHD in the recipients through enhancing Th1 differentiation(Yi et al. 2008), suggesting an immunomodulatory function of Th17 cells on Th1 cells.

In a different approach to assess the importance of Th17 cells in GvHD pathogenesis, two different groups have evaluated the consequence of interfering with the Th17 specific transcription factor RORγt. Yu and colleagues have shown that deleting both RORγt and T-bet, but not RORγt alone, ameliorates GvHD while sparing its beneficial counterpart, GvL(Yu et al. 2011). In contrast, a more recent report determined that deleting both isoforms of RORc (RORt and RORγt) in CD4 and CD8 was sufficient to mitigate the severity of GvHD in allo-recipients(Fulton et al. 2012).

Other reports have also supported the involvement of Th17 cells in GvHD mouse models. For instance, several studies have shown that blockade of cytokines involved in Th17 differentiation such as IL-6, IL-21 and IL-23 can reduce the incidence of GvHD(Bucher et al. 2009;Chen et al. 2009b;Das et al. 2010;Tawara et al. 2011). Additionally, the mechanism of action halofuginone, an inhibitor of skin collagen deposition in GvHD, was shown to be mediated through inhibition of Th17 differentiation and function(Cheng et al. 2012).

1.4.1.2 Regulatory T cells

Several studies using animal models have demonstrated the prominent role of regulatory T cells in the prevention of GvHD and more interestingly in the separation of GvHD from favourable GvL. Depletion of $CD4^+CD25^+$T cells has been shown to accelerate and exacerbate GvHD whereas the adoptive transfer of these cells can rescue mice from such harmful effects(Taylor et al. 2002b). Furthermore, $CD4^+CD25^+$T cells can prevent GvHD-related damage of the thymus and peripheral lymphoid tissues, which in turn facilitates the development and expansion of an enhanced T cell repertoire, resulting in improved reconstitution of functional immune responses following AHSCT(Nguyen et al. 2008). Regulatory T cells can also prevent the production of pro-inflammatory cytokines, such as IFNγ, IL-17, IL-1β and G-CSF that are mediators of chronic GvHD(Chen et al. 2007).

Different sub-populations of Tregs have different regulatory potency. $CD4^+CD25^+CD62L^+$ were shown to be more effective at preventing GvHD, whereas their counterpart $CD62L^-$ fraction did not demonstrate the same anti-GvHD capacity. This differential effect may be related to the ability of the $CD62L^+$ Treg sub-population to home to secondary lymphoid organs more efficiently and therefore more effectively exert their protective function against acute GvHD(Ermann et al. 2005;Taylor et al. 2004).It is possible to break the anergic status of Tregs *in vitro* using TCR ligation, co-simulation and cytokines. *Ex-vivo* expanded Tregs are even more suppressive than their fresh counterparts(Jones, Murphy, & Korngold 2003;Taylor, Lees, & Blazar 2002b).

Recently, Beyersdorf et al showed that Tregs isolated from animals that were pre-treated with super-agonistic anti-CD28 displayed enhanced suppressive activity and, following adoptive transfer, protected recipients from acute GvHD without abrogating the beneficial GVL effect of CD28 pre-activated effector T cells(Beyersdorf et al. 2009). The suppressive ability of regulatory T cells has been shown by some groups to be antigen-specific, as third party recipients are not protected from GvHD(Joffre et al. 2004; Trenado et al. 2003). Conversely, others have shown that once activated, regulatory T cells act in a non-specific manner(Thornton & Shevach 2000). $CD4^+CD25^+$T cells can also suppress CD8-mediated GvHD across minor histocompatibility barriers (Jones, Murphy, & Korngold 2003) and were shown to be suppressive in xenogenic antigen-mediated GvHD(Cao et al. 2009b).

The paucity of regulatory T cells in the peripheral blood and their unknown specificity has been a challenge to employing these cells in transplantation tolerance. However, several groups have successfully expanded polyclonal, as well as antigen-specific regulatory T cells using several different expansion strategies. Taylor and colleagues have evaluated different methods for polyclonal expansion, including immobilized anti-CD3 with low dose IL-2 in the presence of TGF-β (yielding the highest frequency and most suppressive population), and soluble anti-CD3 with APC or high concentration IL-2(Taylor, Lees, & Blazar 2002b). Stimulating Tregs with anti-CD3/CD28 microbeads with IL-2 (Taylor et al. 2004;Trenado et al. 2006) or with IL-15(Karakhanova et al. 2006)has also been shown to result in successful expansion of polyclonal regulatory T cells.

An alternative approach is the use of lentivirus to ectopically express Foxp3 in $CD4^+CD25^-$ T cells. These engineered regulatory T cells were shown to be

phenotypically and functionally similar to natural T regulatory cells and able to suppress GvHD while preserving GVL in a murine EL4/DsRed leukaemia model(Cao et al. 2009a).

Although polyclonal regulatory T cells have been shown to possess sustainable phenotypic and functional characteristics of regulatory T cells(Godfrey et al. 2004) and have the ability to delay or protect from GvHD, their effect on beneficial immune responses is not fully understood. Therefore, the idea of expanding regulatory T cells with a particular specificity, so-called recipient allo-antigen specific T cells, has emerged in transplantation models. The advantage of using antigen-specific over polyclonal regulatory T cells has been proven in different settings such as diabetes (Tang et al. 2004) and solid organ transplantation. Trenado et al have shown that recipient-specific regulatory T cells, generated by expanding donor regulatory T cells with recipient APCs, were able to prevent GvHD while preserving GVL; with some variation according to tumour cells used(Trenado et al. 2003). The specificity of this protection was further assessed using a graft rejection model whereby mice were transplanted with syngeneic bone marrow (BM) and two different allogeneic BMs along with syngeneic splenocytes (stimulator), in the presence of allo-activated $CD4^{+}CD25^{+}$ Tregs. Only BM, from which APCs were used to activate the Tregs, was protected from rejection, whilst the third party BM was not(Joffre et al. 2004). As would be expected, expanded antigen-specific regulatory T cells were even more suppressive than polyclonal ones. As is the case with adoptive immunity, specificity is preferable. Many protocols have been and continue to be developed aiming to generate antigen-specific regulatory T cells. However, further research is needed to achieve the required specificity to enable these powerful cells to be translated to human applications.

1.4.2 Clinical evaluation of Th17 and regulatory T cells in GvHD

1.4.2.1 Helper T cells type 17 and $CD4^+CD161^+$ T cells

Th17 involvement in the pathogenesis of acute GvHD in humans has also been investigated. In a report measuring the frequencies of Th17 cells using ELISPOT and IL-17 plasma levels by ELISA, patients with acute GvHD were shown to have higher Th17 frequencies and increased IL-17 plasma levels when compared to healthy controls and patients with no sign of aGvHD(Dander et al. 2009). Likewise, Zhao and colleagues showed that patients who received more Th17 cells in the graft were more likely to develop acute GvHD. The authors demonstrated that the frequency of $IL\text{-}17^+$ T cells was higher at GvHD onset and response to treatment for GvHD was associated with a reduction in Th17 cells. In keeping with murine studies, exposure to G-CSF was found to decrease the number of Th17 cells(Zhao et al. 2011). Similarly, in a recent report by Bossard et al, the absolute number of Th17 cells in the intestinal mucosa was higher in patients with aGvHD compared to healthy controls and patients with no GvHD. Th17 infiltration was assessed indirectly using immunohistochemistry staining for$CD161^+$ $CCR6^+$ and the transcription factor RORγt. Moreover, they found that the intestinal mucosa of patients with aGvHD had more plasmacytoid dendritic cells, which have been shown to play a role in Th17 differentiation(Bossard et al. 2012).

In contrast, findings from two other groups do not support a role for Th17 cells in acute GvHD pathogenesis. One group showed that T cells isolated from the skin of patients with aGvHD produce more IFNγ, but not IL-17 or IL-22. In addition, they found no significant difference in the frequencies of Th17 cells in the peripheral

blood of patients with or without aGvHD patients(Broady et al. 2010). Using immunohistochemistry, Ratajczak et al found no difference in Th17 infiltration of aGvHD targets such as skin and gastrointestinal tract in aGvHD and no GvHD patients(Ratajczak et al. 2010).

Analysis of single nucleotide polymorphism (SNP) is another approach used to evaluate the role of Th17 in aGvHD pathogenesis. A Japanese group showed that recipients with 197A genotype at rs2275913 in the promoter region of the IL-17A gene were more likely to develop aGvHD(Espinoza et al. 2011b). Furthermore, in a separate report, the same group demonstrated that T cells from healthy controls with the 197A allele produced more IL-17 than T cells from donors with the 197G allele. They concluded that a graft from a donor with the 197A allele is more likely to contribute to the risk of aGvHD(Espinoza et al. 2011a). Similarly, a comprehensive genetic analysis of IL-23/Th17 variants in patients undergoing T-depleted AHSCT has shown that TC and CC genotype of the patients at rs8193036 in the IL-17A gene were associated with higher risk of aGvHD(Carvalho et al. 2010).

1.4.2.2 Regulatory T cells

The translation from murine Treg models into the human clinical setting remains in its infancy. Early work in the field yielded conflicting data, partly because of difficulties in the use of markers to accurately identify true regulatory T cells and discriminate them from activated T cell effectors. Indeed some early studies reported a correlation between GvHD occurrence and *high* Treg frequency, defined as $CD4^{+}CD25^{+}$T cells, both in the graft (Stanzani et al. 2004)and in the recipient(Clark et al. 2004). Others failed to show a correlation between $CD4^{+}CD25^{+}$content and outcome in the setting of donor lymphocyte infusion (DLI); although the addition of a further discriminatory marker defining Treg as $CD25^{hi}$ $CD45RB^{low}$, did show a relationship between higher DLI $CD4^{+}CD25^{hi}CD45RB^{low}$ cells and lower severity of GvHD(Mutis et al. 2005). However, these early works used $CD25^{+}$ as the only marker for identifying Treg which is also expressed on activated T cells, rendering these data difficult to interpret due to the probable overlap between regulatory and activated T cell populations in these studies.

The use of Foxp3 as a marker for the identification of regulatory T cells served to aid progress in the field and human observational studies demonstrated that decreased Foxp3 expression, measured by flow cytometry and PCR techniques, correlates with a heightened incidence of both acute and chronic GvHD post allogeneic stem cell transplant(Miura et al. 2004;Zorn et al. 2005). Rezvani et al have shown an association between the Treg content of the graft and GvHD(Rezvani et al. 2006). The number of transfused Treg within the graft has been shown to also affect overall survival post-transplant(Vela-Ojeda et al. 2010). Furthermore, high percentage and

absolute number of $Foxp3^{+}CD4^{+}$T cells in both donor graft and the recipient blood early after AHSCT was found to correlate with a low incidence of GvHD(Rezvani et al. 2006).However, when $CD25^{+}$T cell content, which had previously been employed as a marker for regulatory T cells, was analysed independently, there was a direct correlation between $CD4^{+}CD25^{+}$ and increased incidence of GvHD - further confirming the inadequacy of $CD25^{+}$ as a stand-alone marker for Tregs. Even following the selective depletion of $CD25^{+}$T cells in the graft using anti-CD25 immunotoxin, the $CD4^{+}Foxp3^{+}$ regulatory T cell compartment reconstituted promptly(Mielke et al. 2007).These data were further supported by a recent study, reporting that the balance between Tregs and T-effector cells is important in determining outcome from GvHD(Matthews et al. 2009).In contrast, others failed to show a correlation between Foxp3 expressing Treg and the incidence of GvHD(Meignin et al. 2005).The discrepancy in these results is probably related to the heterogeneity of the patient groups and the treatment regimen in the different studies.

Recent published data of the comparative examination of pathological specimen have shown an association between the Treg frequencies in the affected organs and severity of GvHD. Rieger et al demonstrated a post-transplant shift in the T cell repertoire during GvHD with reduced numbers of peripheral blood $CD4^{+}CD25^{hi}$ regulatory T cells, accompanied by a reduction in gastrointestinal (GI) mucosal Foxp3 expression(Rieger et al. 2006).

Others have reported higher number of Tregs in the skin of patients with aGvHD that responded to therapy compared to those with resistant aGvHD, illustrating their important role, even in established disease(Fondi et al. 2009). These data have led to a proposal to consider Treg frequencies as diagnostic and prognostic biomarkers for aGvHD(Magenau et al. 2010).

The potential suppressive effect of Treg on the useful anti-tumour responses in humans remains largely unknown. Whereas in some murine studies, the protection from aGvHD has been reported without the loss of anti-tumour immune responses, clinical data reported in the literature are conflicting. Nadal et al examined a cohort of patients transplanted for chronic myeloid leukaemia (CML) and found that, although an increment in Tregs may have an advantageous effect on graft survival in the early post-transplant phase, this was associated with a loss of GvL effect and a consequent increase in relapse rate. Furthermore, their data failed to support a role for Tregs against aGvHD(Nadal et al. 2007). The weight of published data however favours regulatory T cells as potentially useful therapy in GVHD without reducing GvL.

1.4.3 Treg and Th17 imbalance in GvHD

The disturbance in the balance between regulatory T cells and Th17 cells have been proposed to play a role in autoimmune diseases as well as AIDS pathogenesis(Alunno et al. 2012;Kanwar et al. 2010;Niu et al. 2012;Prendergast et al. 2010). However, the importance of Treg and Th17 ratio in the stem cell transplantation setting is poorly understood. In mice, G-CSF treatment has been shown to attenuate acute GvHD. In this study, although the percentage of $CD4^+$ $Foxp3^+$ Treg did not change significantly, exposure to G-CSF reduced the number of Th17 cells, resulting in a skewing in the Treg/Th17 ratio in favour of protection against aGvHD(Joo et al. 2012). In addition, monoclonal antibodies used to inactivate IL-6 have been shown to shift the balance between Treg and Th17 cells(Chen et al. 2009b;Noguchi et al. 2011). Likewise, neutralizing IL-21, another Th17 differentiation-related cytokine, has been shown to control GvHD through manipulating the Treg/Th17 ratio(Bucher et al. 2009).

In human studies, the observed contribution of IL-17-producing $CD4^+$T cells to the onset of acute GvHD has also been associated with a drop in the frequencies of $CD4^+CD25^+Foxp3^+$ T cells(Dander et al. 2009). Additionally, despite demonstrating the indispensable role of Th17 in aGvHD, Ratajczak and colleagues concluded that the Th17/Treg ratio is a more useful predictive marker for skin and gastrointestinal GvHD(Ratajczak et al. 2010).

1.5 Immunosuppressive drugs used for prophylaxis and treatment of GvHD and their impact on Th17 and Regulatory T cells.

Various strategies are employed to prevent or attenuate GvHD through dampening pathogenic alloresponses and suppression of effector T cells subsets e.g. Th17 cells.

1.5.1 Corticosteroids

The use of corticosteroids as a first-line intervention for acute GvHD is routine in the management of this disease. Irrespective of the variable outcome of this treatment modality, it is favourably used due to its anti-inflammatory as well as cytotoxic activity. However, the effect of steroids on Th17 and regulatory T cells has not been well addressed. The effect of steroids treatment on IL-17 production is controversial(Liu et al. 2009;McKinley et al. 2008;Prado et al. 2011a;Yang et al. 2009).In addition, previous data have suggested that $Foxp3^+$ Treg cells are resistant to the effect of steroids; however the suppressive ability of Tregs was impaired in this study(Karagiannidis et al. 2004;Prado et al. 2011b).

1.5.2 Immunosuppressive drugs used for GvHD prophylaxis

1.5.2.1 Anti-thymocyte globulin (ATG)

Anti-thymocyte globulin has been used for the prophylaxis and treatment of GvHD as well as wider clinical applications that harness its potent immunosuppressive action e.g. in the management of aplastic anaemia. More recently it has been suggested that ATG may preferentially deplete conventional T cells, while sparing regulatory T cells(Feng et al. 2008).

However, work seeking to evaluate the notion that rabbit anti-thymocyte globulin (rATG) may provide protection from GvHD through the enhancement of regulatory T cells, has shown that although Foxp3 expression increases in human T cells exposed to rATG, the increase in $Foxp3^+$ T cells is only transient. Furthermore, Foxp3 positive T cells induced following exposure to rATG do not appear to have immunosuppressive capacity, even at the time of maximal Foxp3 expression(Broady et al. 2009).

1.5.2.2 mTOR inhibitor (Rapamycin)

Rapamycin, an mTOR inhibitor, has been shown to effectively induce Tregs both *in vitro* and *in vivo*(Battaglia et al. 2005;Thornton et al. 2004).Furthermore, rapamycin was found to prevent the differentiation of Th17 *in vitro*(Kopf et al. 2007).This agent has been used successfully in the prophylaxis and treatment of GvHD post AHSCT(Ghez et al. 2009). However, in contrast to other classical prophylactic agents such as cyclosporine and tacrolimus, rapamycin exerts a differential effect on regulatory and conventional effector T cell populations, leading to an increase in the Treg:T effector ratio(Coenen et al. 2007). Recent data from the murine setting have shown that post allograft, rapamycin results in an increase in the frequencies of Tregs in GvHD target organs, such as the skin, although circulating numbers of Tregs are unaffected(Palmer et al. 2010).Similarly, a recent report by Shin and colleagues has shown that administering rapamycin and IL-2 together expanded Treg more efficiently and provided protection against aGvHD(Shin et al. 2011). These studies have not investigated the effect of rapamycin on the Th17 subset. Such pathological data in the human allograft setting is as yet unavailable, principally due to the difficulties in obtaining such samples from patients.

1.5.2.3 Calcineurin inhibitors (CNI)

Calcineurin inhibitors are widely used in GvHD prophylaxis prior to AHSCT. By interfering with calcineurin activity, these agents inhibit IL-2 dependent T cell activation. However, their exact influence on the Th17 and Treg axis has not been well studied. Previous studies have generated conflicting data on the effect of calcineurin inhibitors on Tregs; on the one hand they support a role for low doses of calcineurin inhibitors in inducing regulatory T cells(Brandt et al. 2009;Wang et al. 2009b) whereas other data suggest that they may reduce Tregs in favour of pro-inflammatory T cell expansion(Lopez-Flores et al. 2011;Miroux et al. 2009;Miroux et al. 2012;Wang et al. 2006). Unexpectedly, although they interfere with IL-2, previously shown to prevent Th17 differentiation, CNI generally have a suppressive effects on IL-17 production(Cho et al. 2007;Chung et al. 2012;Fabrega et al. 2009;Tsuda et al. 2012;Yago et al. 2012;Zhang et al. 2008). However, further studies are needed to understand the influence of CNI on the Treg/Th17 balance and their role in GvHD.

1.5.3 Cytokine therapy

IL-2 is an essential cytokine in the maintenance of the pool of Treg cells *in vivo* and is also critical to their function. Furthermore, in the lymphopenic setting such as might be encountered immediately following allogeneic stem cell transplantation, IL-2 may preferentially promote the expansion of the Treg compartment(Zhang et al. 2005a). IL-2 directly up-regulates Foxp3 gene expression through the phosphorylation of STAT proteins that subsequently translocate to the nucleus and bind to regulatory motifs in the Foxp3 gene(Wuest et al. 2008).Conversely, manipulation of STAT proteins by IL-2 was found to suppress

Th17 differentiation (Laurence et al. 2007) and the silencing of IL-2 gene expression was shown to reverse this effect(Quintana et al. 2012).Exogenous use of this cytokine had therefore been proposed as a potential immunomodulatory therapy to boost the number of Tregs post AHSCT.

In support of this, Rouse et al, have shown the reciprocal effect of IL-2 on Treg and Th17 cells and their impact on controlling experimental autoimmune encephalomyelitis(Rouse et al. 2012).For Treg to preferentially expand *in vivo*, adequate numbers of Tregs is necessary at baseline (Thornton, Donovan, Piccirillo, & Shevach 2004). Recently Zorn et al reported the feasibility of a clinical study of low-dose IL-2 combined with infusions of donor $CD4^+$ lymphocytes to expand Tregs *in vivo*(Zorn et al. 2009). Concerns remain that this approach may diminish the GVL action of effector T cells. Therefore, carefully monitored, prospective clinical trials are required to explore the efficacy of this strategy further.

1.5.4 Extracorporeal photopheresis (ECP)

ECP is used for the treatment of chronic graft versus host disease, although the exact mechanism for the efficacy of this therapy remains unclear. Leukapheresed peripheral blood mononuclear cells are treated with UVA radiation in the presence of a DNA alkylating agent that induces apoptosis. It is proposed that upon phagocytosis by antigen presenting cells, tolerogenic signals are induced resulting in reduced graft versus host disease. The precise nature of these tolerogenic signals and the cell phenotypes involved have been an area of active investigation. A number of groups have shown that induction or differentiation of regulatory T cells is

an important component of this tolerogenic response(Lamioni et al. 2005;Maeda et al. 2005). In addition to an increase in Treg frequencies, each successive cycle of treatment with ECP leads to an increase in the suppressive capacity of these cells(Rao et al. 2009;Schmitt et al. 2009). Di Biaso et al recently demonstrated that only patients responding to ECP have a rise in their fraction of circulating Tregs whereas patients who fail to respond to the therapy have no such rise in Treg numbers. In addition they identified a concurrent decrease in the $CD4^+$ T cells producing interleukin-17 in patients responding to ECP(Di, I et al. 2009).

Recently, Pappa and colleagues have shown a similar reciprocal association between Treg and Th17 cells in systemic sclerosis patients treated with ECP(Papp et al. 2012). These studies further support the importance of the recently described Th17 axis in the pathology of GvHD and its frequent reciprocal relationship with the regulatory T cell axis(Carvalho et al. 2010;Dander et al. 2009).

1.6 Regulatory T cells as cellular therapy for acute GvHD

The most established model of cellular therapy is in the setting of DLI. DLI is employed to augment the graft versus leukaemia response post AHSCT, either to treat relapsed disease or pre-emptively to prevent relapse, particularly in the context of reduced intensity conditioning transplant regimens. Increasingly other cell-based therapies are being used in the clinic – mesenchymal stem cells have been employed as successful GvHD therapy(Dazzi and Marelli-Berg 2008) and illustrate the feasibility of expanding the necessary GMP (good manufacturing practices) standards to a range of cellular products including regulatory T cells for therapeutic adoptive transfer.

Tackling the source of regulatory T cells, given their low frequencies in peripheral blood, has been a major block in their development as a viable cellular therapy. Several methodologies have been under development to overcome this obstacle, either through polyclonal or antigen-specific expansion of these cells. The *in vitro* conversion of conventional T cells ($CD4^+CD25^-$) into polyclonal cells with a Treg phenotype has been achieved using specific cytokines, such as retinoic acid(Wang et al. 2009a) and/or TGF-B(Chen et al. 2003;Park et al. 2004) in addition to IL-2, which plays a critical role in the expansion of Tregs. The use of agonistic antibodies against stimulatory and co-stimulatory molecules, mainly CD3 and CD28, has also been shown to be effective in expanding these cells(Godfrey et al. 2004;Hoffmann et al. 2004;Taylor et al. 2002a;Taylor, Lees, & Blazar 2002b). Anti-CD28 super-agonist were shown to preferentially expand the Treg compartment(Kitazawa et al. 2009), however translation of this approach in a recent clinical trial failed to yield significant preferential Treg generation(Suntharalingam et al. 2006). Several studies have also

focused on the expansion of antigen-specific regulatory T cells, employing cellular mediators to promote Treg expansion.

Yamazaki and colleagues have directly expanded Tregs using dendritic cell stimulation(Yamazaki et al. 2006). Allogeneic B cells(Chen et al. 2009a) or CD40-activated B cells(Tu et al. 2008) have also been suggested as stimulators to generate and expand alloantigen-specific Tregs. CD40-activated B cells were shown to be more efficient at generating Tregs with more suppressive activity, compared to immature dendritic cells(Zheng et al. 2010). An alternative methodology has been *ex-vivo* selection of $CD4^+CD25^+$ natural regulatory T cells and their *in vitro* expansion. Regulatory T cells are selected according to $CD4^+CD25^+$ phenotype for either direct adoptive transfer or *in vitro* expansion; this raises the issue of activated conventional T cell contamination of the collected product, which might in turn skew the benefits expected. It is therefore important to further characterize these cells phenotypically to achieve accurate selection and expansion of a pure regulatory T cell product.

The technology is now ripe for transfer into the human clinical setting, in carefully conducted and closely monitored trials – only then will it become possible to glean answers to questions on optimal dose, timing, compatibility with other immunosuppressive drugs and critically, safety in terms of relapse of underlying disease and susceptibility to infection.

1.7 Adoptive Treg Transfer – Clinical Trials

The first human study of adoptive transfer of *ex vivo* expanded $CD4^+CD25^+CD127^-$ T regulatory cells in the treatment of GvHD was reported in October 2009 in two patients, using cells expanded from buffy coats taken from family members(Trzonkowski et al. 2009). The first patient, who was being treated for chronic GvHD, achieved significant alleviation of symptoms and reduction of pharmacologic immunosuppression. However, the second patient, who was being treated for grade IV acute GvHD, experienced only transient improvement. A number of phase I clinical studies, currently ongoing, have employed both freshly isolated donor Tregs administered alongside DLI, and Tregs expanded from umbilical cord blood administered in the double cord blood transplant setting(Claudio Brunstein 2009;Riley et al. 2009). Recently, Di Ianni and colleagues conducted a phase I/II clinical trial involving twenty-two patients with haematological malignancies, receiving a haploidentical AHSCT (Mauro Di Ianni and Mauro Di Ianni* 2009). Patients received freshly isolated $CD4^+CD25^{hi}$ regulatory T cells after transplant conditioning therapy followed by infusion of mobilized $CD34^+$ stem cells and conventional T cells from their haploidentical donor. The infused Tregs:Tcons ratio was established at 1:1.5 and no post-transplant prophylaxis against GvHD was used. Strikingly, only 2 of 26 evaluable patients developed GvHD. The authors concluded that in the setting of haploidentical transplantation, the success of which has been thwarted by infection-related deaths due to aggressive immunosuppression needed to prevent GvHD, infusion of freshly purified Tregs may allow safe administration of high doses of T cells, providing protection from GvHD and facilitating robust immune reconstitution.

Hypothesis and Aims

I hypothesize that the balance of regulatory T cells and IL-17-producing $CD4^+$ T cells may play a significant role in the pathogenesis of aGvHD.

The aims of this thesis are two-fold: I will define stem cell-like memory $CD4^+$ T cells with drug-effluxing capacity and will evaluate if they play a role in long-term memory against pathogens. I will examine if stem-like memory $CD161^+CD4^+$ T cells have a Th17 cytokine profile and will evaluate if they resist the lymphocytotoxic effect of corticosteroids, a substrate of the ABCB1 transporter complex, through their MDR pump, thereby contributing to steroid-refractory GvHD.

The specific aims of this thesis are:

1. To evaluate the existence of stem cell-like memory T cells with drug-effluxing ability in the peripheral blood of healthy controls and patients with AML.
2. To examine the functional phenotype of drug effluxing $CD4^+$ and $CD8^+$ T cells including their Th1 and Th17 profile.
3. To examine mechanisms of steroid-refractory GvHD by assessing the *in vitro* and *in vivo* resistance of T cell subsets (Th1, Th17 and Tregs) to corticosteroids as well as calcineurin and mTOR inhibitors.
4. To evaluate the impact of donor T cell subsets (Th1, Th2, Th17 and Tregs) infused with the graft on risk of GvHD.

CHAPTER 2. MATERIAL AND METHODS

2.1 Patients and Samples

Informed consent for the use of patient material was obtained from all patients at Imperial NHS Trust, Hammersmith Hospital. The study was approved by both the National Research Ethics Committee (NREC) and the local ethics review board. Peripheral blood mononuclear cells (PBMC) were collected from patients undergoing AHSCT prospectively before and at regular time points following AHSCT: days 30, 60, 90, 120 and 180 days post-transplant.

Informed consent for the use of archived patient material was obtained from all patients at initial presentation to Imperial NHS Trust, Hammersmith Hospital.

2.2 Sample processing

2.2.1 Isolation of Peripheral Blood Mononuclear Cell (PBMC)

A maximum of 20-30ml of whole blood was collected from patients and healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient separation technique (Lymphoprep). Briefly, 15 ml heparinised whole blood was diluted 1:1 with RPMI 1640 media (GIBCO / Invitrogen) in a 50ml falcon tube and 10ml of lymphoprep (Axis Shield, UK) was gently layered under the diluted blood.

After centrifugation at 1800 rpm for 30 min, the interface layer between plasma and lymphoprep, which contains mononuclear cells, was collected and re-suspended in fresh RPMI 1640. Cells were washed twice with RPMI 1640 media at 1200 rpm for 10 min. PBMC were resuspended in 10% fetal calf serum (FCS)/ RPMI, counted using Trypan Blue (TB) for cell viability assessment.

2.2.2 Cell freezing

PBMCs were suspended in freezing media [RPMI-CM with 20% foetal calf serum (FCS) and 20% DimethylSufoxide (DMSO) (Sigma-Aldrich, UK)] on ice and aliquoted into cryovials and stored in -80 ^{o}C (max 1 week) before transfer to liquid nitrogen.

2.2.3 Cell thawing

Cryovials of PBMCs were transferred from liquid nitrogen on dry ice. Each vial was thawed in a water bath at 37 ºC. Once thawed PBMCs were transferred immediately to a 15 ml falcon tube containing 10 ml thawing media (RPMI 20% FCS containing 50,000 unit DNase). PBMCs were centrifuged at 1200 rpm for 10 min. PBMCs were then re-suspended in 1 ml media and cell count and viability were assessed.

2.3 *In vitro* culture

PBMC were isolated from healthy subjects and suspended in RPMI 1640 medium (GIBCO, UK) supplemented with 10% FCS, gentamycin, and L-glutamine. Next, cells were distributed in flat-bottom 96 well-plate at 4 X 10^5 cells/well. Cells were then cultured at 37℃/5% Co2 for 5 consecutive days in the presence or absence of escalating dose of selected ABCB1 substrates **(discussed later in sections 4.2.1 and 4.2.2)**. Cells were either left un-stimulated or stimulated with anti-CD3/CD8 beads (Dynabeads, Invitrogen, UK) according to the manufacturer's protocol (2µl beads / 8X10^4 cells). Finally, cells were harvested on a daily bases and then prepared according to the required assay. In some experiments, in addition to abovementioned drugs, inhibitors of ABCB1 transport such as cyclosporine, PK11195 and vinblastine were added to the culture.

For daunorubicin experiments, PBMC from healthy subjects chronically infected with CMV (CMV-seropositive) were used. Cells were then cultured in the presence or absence of 0.1 mM daunorubicin for 44–48 hr. Cells were then harvested, washed and prepared for further experiments.

2.4 Intracellular staining (Th1/Th2/Th17 and Foxp3)

PBMCs were thawed and rested overnight at 37 ºC in 5% Co_2, to minimize background cytokine production secondary to lymphocyte manipulation. PBMC were counted and plated at 5 $x10^5$ cells/ 200 µL RPMI supplemented with 10% FCS per well of a 96-well plate (BD Falcon™, Oxford, UK). Cells were stimulated using 4 different conditions:

1- No stimulation (negative control),

2- PMA (50ng/ml)/Ionomycin (2µg/ml) (Sigma Aldrich),

3- Anti-CD3/CD28 Dynabeads(Invitrogen) and

4- CMV lysate (5µg/ml) or CMV pepmix (0.25 µg/ml)

Brefeldine A (12.5 µg/ml) or monensin (Golgi-plug, Sigma Aldrich) was added for the last 4-6h of the culture. Next, cells were harvested, washed and stained with surface markers at RT for 15 min. Cells were then washed, fixed and permeabilized with eBioscience Foxp3 Staining Buffer Set (eBioscience, UK) and stained with FITC, PE and APC conjugated antibodies for intracellular markers **(Table 2.1)**. Cells were acquired using BD FACSCalibur or BD LSRFortessa (BDbiosystem, USA) (Figure II-2)**.** Data analysis was performed on FlowJo software (Treestar, San Carlos, CA).**Figure 2.1** shows an example of intracellular staining for cytokines and Foxp3 compared with their correspondent isotypes

Antibody	Volume	Manufacturer (Clone)
IFNγ	5 µl	BD (25723.11)
IL-2	5 µl	BD (5344.111)
IL-17	5 µl	eBiosciences (eBio64DEC17)
TNFα	1 µl	BD (MAb11)
Foxp3	5 µl	eBiosciences (PCH101)

Table 2.1: Clones of Monoclonal antibodies used for intracellular staining

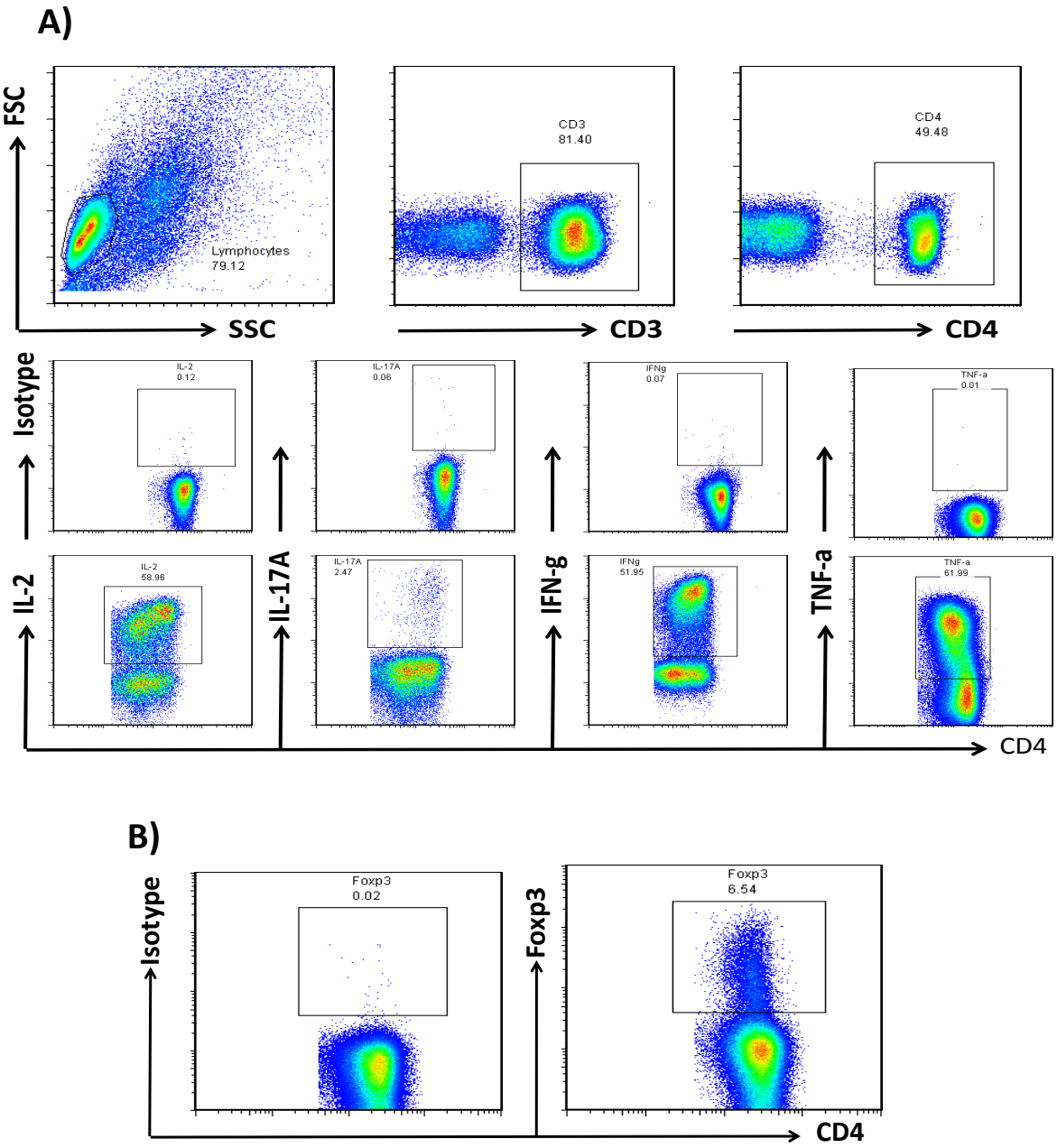


Figure 2.1: A representative example of intracellular staining. A, Gating strategy and cytokines staining; IL-2, TNFα, IFNγ and IL-17 were measured after stimulation with PMA and ionomycin. **B,** An example of Foxp3^{+} T cells staining. Positivity was determined using isotype controls.

2.5 Comprehensive phenotypic analysis of T cell subsets using multi-colour flow cytometry

I planned to comprehensively characterize T cell subsets in donor grafts, DLIs and PBMC collected from patients post-transplant, and relate the frequencies and functional phenotype of T cell subsets to the risk of GvHD post HSCT. The analyses were performed on BD LSRII and BD LSRFortessa, since both cytometers are equipped with 3-4 lasers, enabling detailed characterisation of T cell subsets using an extended (6-10 colours) antibody panel **(Table. 2.2).** Fluorochromes for the antibody panels were carefully selected to minimize fluorescence overlap. Briefly, freshly isolated or thawed PBMCs were stained with conjugated monoclonal antibodies (Mab) against a range of surface markers **(as defined in Table 2.2)** and incubated at room temperature for 10-20 minutes in the dark. Next, cells were washed with PBS or an equivalent staining buffer and spun for 5 minutes at 2000 rpm. If the panel included an intracellular marker, I proceeded to fixing and permeabilisation followed by intracellular staining as shown in **section 2.4.2**. Cells were refrigerated until acquisition on the BD LSRII or LSRFortessa (BD biosystem, USA).

Prior to acquisition, compensation was performed using BD compbeads plus (BD bioscience) to minimize overlap between fluorochromes as shown in **Figure 2.2A.** FlowJo (Treestar, San Carlos, CA) was used to analyse the data. **Figure 2.2B** shows an example of the gating strategy employed for the extended phenotypic T cell analysis. Following a lymphocyte gate, cell doublet and dead cell discrimination, I gated on live cells and the T cell subset of interest.

To precisely determine the cut-off to discriminate the positive from the negative population, I employed fluorescence minus one (FMO). **(Figure 2.2C)**.

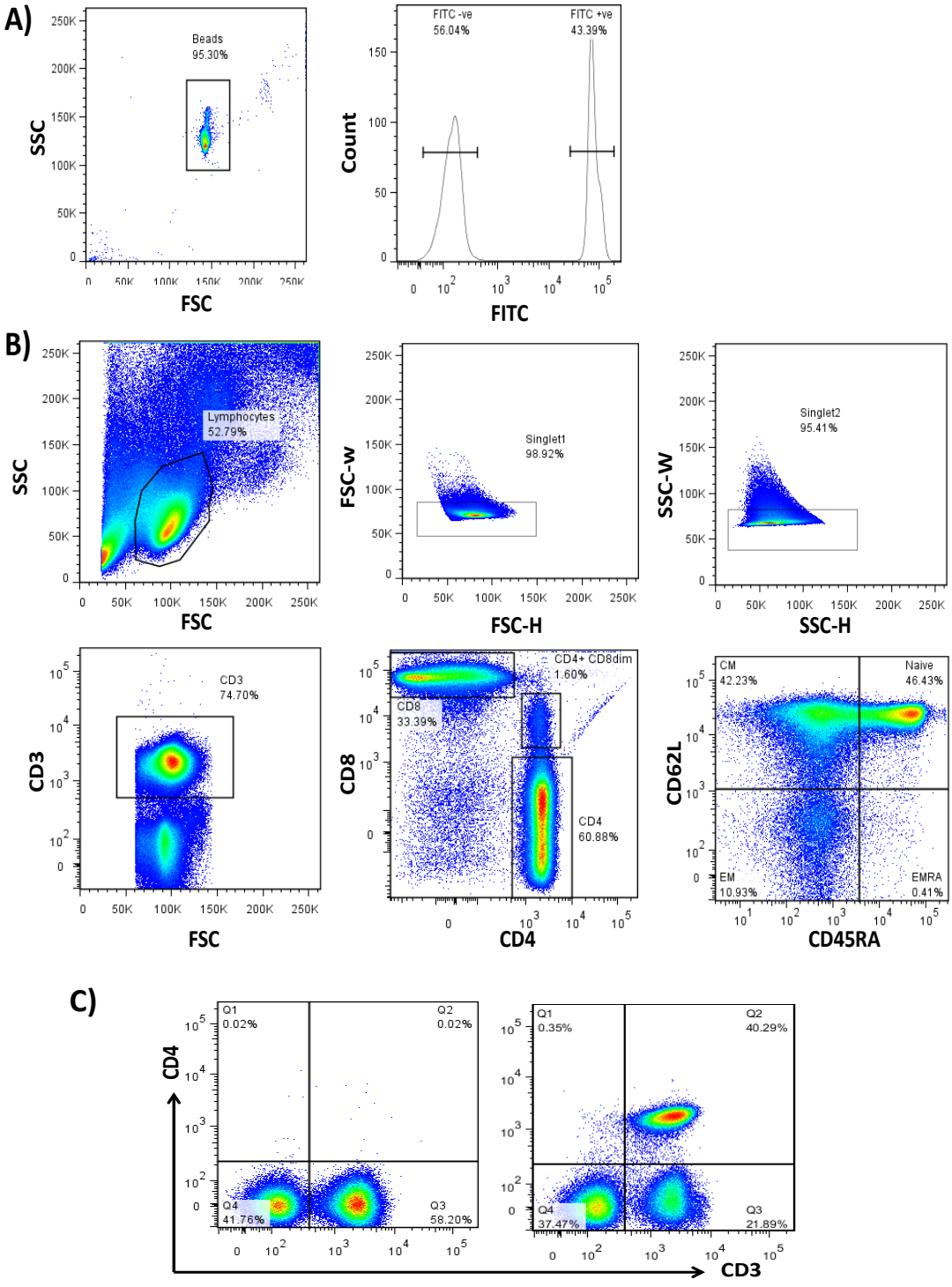


Figure 2.2: A representative example of extended flow cytometric panel analysis. **A**, Compbeads were used to aid in setting up the compensation on the flow cytometer by gating on negative and positive peaks.**B,** gating strategy for the extended panel including lymphocyte gating, doublet exclusion and subset selection e.g. $CD3^{+}$ $CD4^{+}$ $CD45RA^{+/-}$ $CD62L^{+/-}$ T cells. **C,** an example of fluorescence minus one for CD4 monoclonal antibody, staining without **(left)** and with **(right**) CD4 antibody (FMO).

Panel / FL (Clone)	Naïve / memory	T- helper subsets	Regulatory T cells	Rhodamine 123 efflux	Refractory GvHD
FITC	**CD31** **(WM59)**	**IFNγ** **(25723.11)**	**CD161** **(DX12)**	**Rh123** **-**	**TCR γδ $^{+}$ Vα24 TCR (11F2/C15)**
	5 µl	5 µl	10 µl	10µg/ml	10 µl / 5 µl
PE	**Foxp3 (PCH101)**	**IL-4 (MP4-25D2)**	**Foxp3 (PCH101)**	**CD25 (2A3)**	**CD25 (2A3)**
	5 µl	10 µl	5 µl	5 µl	5 µl
PerCP cy5.5	**CCR7 (150503)**	**IL-17 (eBio64DEC17)**	**CD127 (HIL-7R-M2)**	**CD127 (HIL-7R-M2)**	**CD127 (HIL-7R-M2)**
	3.5 µl	5 µl	3.5 µl	3.5 µl	3.5 µl
APC	**CD45RO (UCHL1)**	**IL-10 (JES3-19F1)**	**CD25 (M-A251)**	**CD161 (HP-3G10)**	**CD161 (HP-3G10)**
	5 µl	10 µl (1:14)	5 µl	5 µl	5 µl
APC-H7 (cy7)	**CD3 (SK7)**	**CD3 (SK7)**	**CD3 (SK7)**	**CD4 (RPA-T4)**	**CD4 (RPA-T4)**
	3 µl	3 µl	3 µl	3 µl	3 µl
PE-cy7	**CD4 (SK3)**	**CD4 (SK3)**	**CD4 (SK3)**	**CD62L (DREG-56)**	**CD62L (DREG-56)**
	2 µl	2 µl	2 µl	3 µl	3 µl
Pacific blue	**-**	**-**	**-**	**CD95 (DX2)**	**CD95 (DX2)**
				3 µl	3 µl
Qdot 605	**-**	**-**	**-**	**CD8 (3B5)**	**CD8 (3B5)**
				1 µl	1 µl
Qdot 655	**-**	**-**	**-**	**CD45RA (MEM-56)**	**CD45RA (MEM-56)**
				1 µl	1 µl
Horizon V500	**-**	**-**	**-**	**CD3 (UCHT1)**	**CD3 (UCHT1)**
				3 µl	3 µl
Aqua	**Live/ Dead marker**				
	1 µl				

Table 2.2: Antibodies, fluorochromes (FL), clones and volumes used in the extended phenotyping experiments.

2.6 *In vitro* efflux assays

2.6.1 Rhodamine 123 efflux experiment

Rhodamine 123 (Rh123) is a fluorescent dye, which easily crosses the plasma membrane. It can be visualized by a laser beam when excited at 505nm. This dye is the substrate of P-glycoprotein (P-gp), also known as ABCB1 transporter, a member of the ATP binding cassette (ABC) superfamily. Therefore, Rhodamine 123 can be used to study the efflux capacity of cells such as haematopoietic stem cells (Wagner-Souza et al. 2008).

To perform this assay, freshly isolated PBMC are suspended in effluxing media (containing RPMI and 1% bovine serum albumin (BSA)) and incubated with 10µg/ml Rh123 (Sigma) for 30 minutes on ice in the dark. Next, PBMC are washed with effluxing media at 1500 rpm for 10 minutes. To study the ability of the cells to pump the dye, the cells are divided into two tubes; one to be incubated for 30-60 minutes at 37C in 5% Co2,allowing the initiation of the efflux process and the other tube to be kept on ice to avoid spontaneous efflux of the dye. They are then washed with effluxing media and co-stained with surface antibodies to define the effluxing subset **(Table 2.2)**. Rhodamine 123 fluorescence can be detected at 530/30 nm. Cells are acquired on FACSCalibur or LSRFortessa (for extended panel) and data analysed using FlowJo software. **Figure 2.7a** shows an example of $CD4^+$ T cells, effluxing Rh123.

2.6.2 Daunorubicin efflux experiment

Daunorubicin is a member of anthracycline family of chemotherapeutic agents. It is commonly used for remission induction in acute myeloid leukaemia (AML) patients. Naturally this substance has a fluorescent capacity when exposed to laser beam (blue or green laser) and thus, can be detected by flowcytometery. Noteworthy, like rhodamine **(section 2.6.1),** daunorubicin is a substrate of the ABCB1 transporter.

PBMC freshly isolated from healthy controls were suspended in effluxing media (containing RPMI and 1% BSA). Cells were then loaded with 2.5µM (1.4µg/ml) daunorubicin (Sigma Aldrich, UK) and incubated for 20 minutes at 37 °C in 5% Co_2 in the dark. Next, PBMC are washed with effluxing media at 1500 rpm for 10 minutes to remove the excess dye. The cells are then transferred into two tubes; one incubated at 37 °C in 5% Co_2 in the dark for 30 minto allow the effluxing process to take place and the other was kept protected from light at RT as a negative control. Lastly, cells were washed with effluxing media and stained with surface antibodies **(Table 2.3).** Daunorubicin was excited using blue laser (488nm) and its fluorescence can be detected at 610/20 nm (PE-Texas Red). Cells were acquired on LSRFortessa (BD Biosystem, USA).

FL	PE-cy7	APC	PE-Texas Red	APC-H7	Pacific blue
MoAb (Clone)	**CD4 (SK7)**	**CD161 (Hp-3G10)**	**Daunorubicin**	**CD3 (SK3)**	**CD95 (DX2)**
volume	2ul	5ul		3ul	3ul

Table 2.3: Antibodies, fluorochromes (FL), clones and volumes used in daunorubicin effluxing experiment

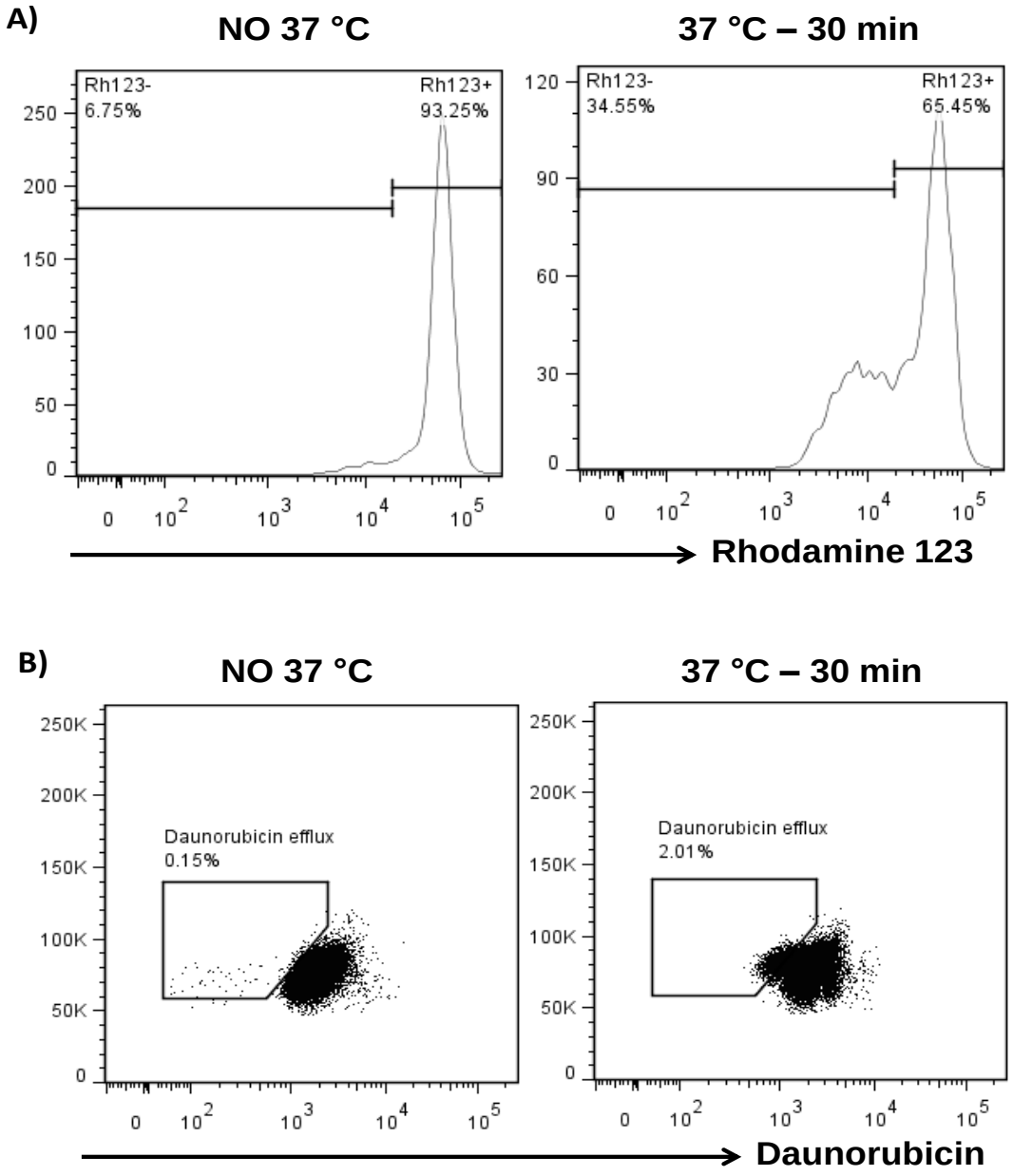


Figure 2.3: Representative experiment of *in vitro* effluxing assay. An example of effluxing experiment showing the ability of $CD4^+$ T cells to pump out **A,** rhodamin123 or **B,** daunorubicin

2.7 Staining with 5-(and-6) carboxyfluoresceindiacetatesuccinimidyl ester (CFSE) for the proliferation assay

5- (and -6)-carboxyfluoresceindiacetatesuccinimidyl ester (CFSE or CFDASE) dye has been widely used for the monitoring of *in vivo* lymphocyte migration as well as quantitative cell proliferation and divisions both *in vitro* and *in vivo.* Initially, this non-fluorescent dye can cross the intact cell membrane. Subsequently, the acetate groups are cleaved by esterases inside the cells, which in turn generate the fluorescent carboxyfluorescein molecules. During proliferation, the CSFE-labelled dividing cells halve the carboxyfluorescein molecules within their daughter cells, resulting in CFSE dilution with each division (Lyons and Parish 1994;Parish 1999;Parish et al. 2009;Weston and Parish 1990).

I used CFSE (eBioscience, UK) to monitor the proliferation of T cells in response to *in vitro* stimulation with anti-CD3 /CD28 Dynabeads (Invitrogen). Briefly, freshly isolated PBMC ($5X10^6$/ 1ml) are labelled with 2µM CFSE (eBioscience, UK) suspended in DMSO (eBioscience, UK) and serum-free PBS, and incubated for 10 minutes at room temperature (RT) in the dark. Equal volumes of neat FCS are then added for 5 minutes at RT/dark to stop the reaction. Next, PBMCs are centrifuged at 2000 rpm for 5 minutes and the supernatant discarded. CFSE labelled PBMC are washed twice in serum containing media (RPMI $^+$ 20% FCS), re-suspended in FCS (10%) containing RPMI (GIBCO, invitrogen) and plated in a 96 well plate for subsequent stimulation. An example of the temporal kinetic of CFSE dilution in T cells following anti-CD3/CD28 stimulation is depicted in **Figure 2.4.**

Proliferation assays were carried out over 5 days; cells were harvested and washed with PBS following centrifugation at 2000 rpm for 5 minutes. Cells were then stained with a range of surface markers according to experimental protocol and acquired on a FACSCalibur flow cytometer (BD biosystems).

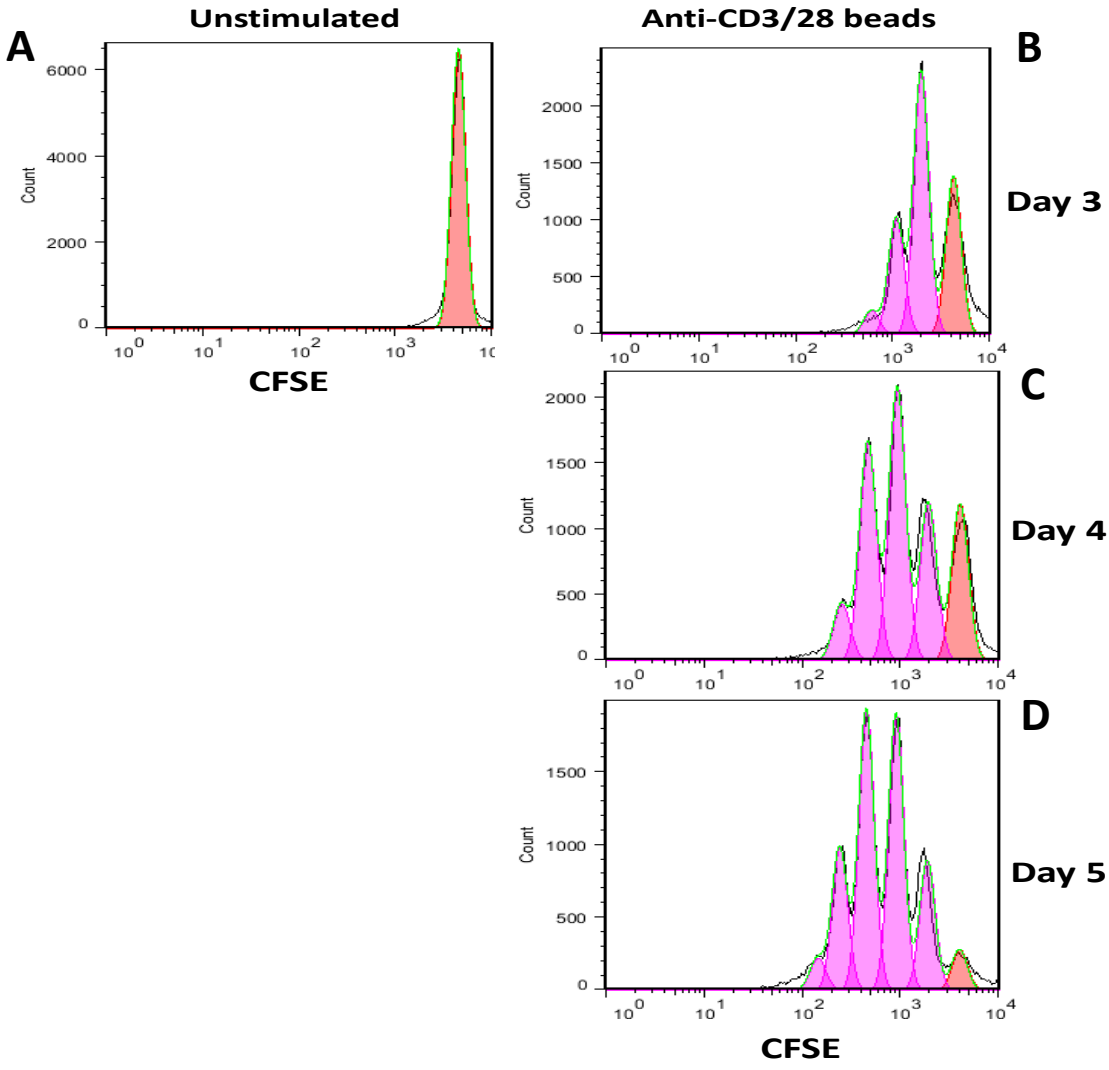


Figure 2.4: An example of FACS plots showing CFSE-labelled $CD4^+$ T cells before and after stimulation with anti-CD3/CD28 Dynabeads. The histograms demonstrate the kinetics of CFSE dilution within $CD4^+$ T cells following: **A,** no stimulation (negative control), **B,** three days of stimulation, **C.** four days of stimulation and **D**, five days of stimulation.

2.8 Apoptosis staining using Annexin V

Apoptosis is the process of physiological programmed cell death, recognised by a number of morphological and intracellular features including loss of plasma membrane asymmetry, cytoplasmic and nuclear condensation and DNA fragmentation. One of the earliest characteristics of this process is the disrupted asymmetry of the plasma membrane, which results in the flipping of the negatively charged phospholipids, namely phosphatidylserine (PS), from the inner to the outer layer of cell membrane, thereby exposing PS to the external surroundings. Annexin V is a protein that binds to PS in a Ca^{2+} dependent manner. Therefore, fluorochrome-conjugated Annexin V can be used to visualise apoptotic cells using flow cytometry and, used together with a Live/dead marker such as propidium iodide (PI) or an equivalent, can discriminate apoptosis from necrosis at a single cell level(Tait et al. 1989;Vermes et al. 1995).

To optimize the protocol for Annexin V staining, I treated freshly isolated PBMCs with escalating concentrations of test drugs for 5 days to induce apoptosis as shown in **section 4.2.2.**After culture, PBMC were washed, re-suspended in 500µl PBS, stained with 0.5 µL (1:1000) eflour660 live/dead dye (eBiosciences, UK). Cells were then incubated for 20 min at 4 °C, protected from light and then washed with PBS. PBMC were then washed again using 1X Annexin V washing buffer (BD bioscience, USA), prepared by diluting 10X Annexin V washing buffer with distilled water. This buffer is crucial for the assay as it contains Ca^{2+} required for the interaction between Annexin V and PS.

PBMC were then stained with 2µl of FITC-conjugated Annexin V (BD bioscience) along with surface antibodies including CD3, CD4 and CD161 **(section 2.4.1)** to identify apoptotic cells within each subset. PBMC were incubated for 20min at RT in the dark and washed in 2ml Annexin V washing buffer at 2000 rpm for 5 minutes. Lastly, cells were acquired on a FACSCalibur flow cytometry (BD biosystems). An example of Annexin V^+ (apoptotic) cells within $CD4^+$ T cells is shown in **Figure 2.5**

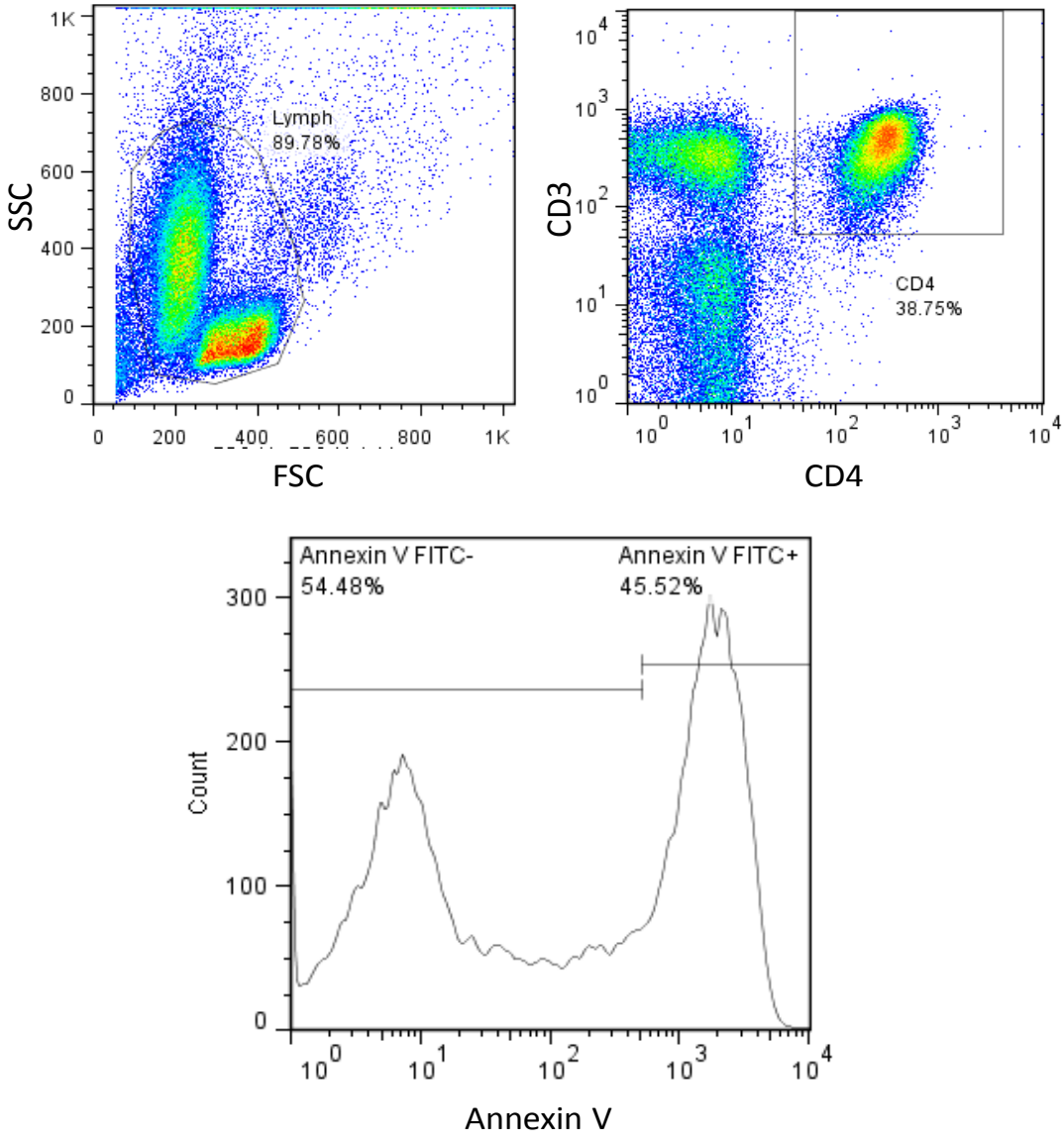


Figure 2.5: Representative experiment of Annexin V staining. Gating strategy with an example of Annexin V percentage within $CD4^+$ T cells are shown.

CHAPTER 3. STEM-LIKE CHARACTERISTICS IN A SUBSET OF CD161 EXPRESSING HUMAN MEMORY CD4$^+$ T CELLS FACILITATES THEIR SURVIVAL AFTER CHEMOTHERAPY

3.1 Introduction

CD4$^+$T cells play an indispensable role in shaping the adaptive immune system through their interaction with a variety of cells including B cells and CD8$^+$T cells(Shedlock and Shen 2003;Sun et al. 2004). CD4$^+$T cells are required for control of viral infection and play a significant role in the control of chronic viral infections such as protection against cytomegalovirus (CMV) disease(Salmon-Ceron et al. 2000);(Pourgheysari et al. 2009). Several studies in animal models and humans have shown that the absence of CD4$^+$T cell help results in severe dysfunction and failure of persistence of virus-specific CD8 T cells(Cardin et al. 1996;Kemball et al. 2007;Lilleri et al. 2006;Lilleri et al. 2008;Matloubian et al. 1994;Walter et al. 1995;Zajac et al. 1998). Furthermore, the success of adoptively transferred CMV specific CD8$^+$T cells in controlling infection post-transplant is largely dependent on the presence of anti-CMV CD4$^+$T cells(Lilleri et al. 2006;Walter et al. 1995).

The establishment of a wide range of long-lived pathogen-specific T cells, which are ready-to-act upon second encounter with the specific pathogen, is a fundamental property of the adaptive immune response. Following encounter with antigen, T cells proliferate vigorously in an antigen-specific manner. A subset of antigen-specific

memory T cells, with slow proliferative potential under normal homeostatic conditions, will persist for life (Hammarlund et al. 2003);(Surh and Sprent 2008). The mechanisms employed by long-lived antigen-specific memory T cells for long-term persistence in humans are not clearly understood.

Patients with acute myeloid leukaemia (AML) undergoing repetitive cycles of cytotoxic chemotherapy for remission induction experience severe, albeit short-lived lymphocytopaenia(Sung et al. 2009). Yet they rarely suffer serious viral reactivations such as CMV disease, supporting the existence of chemo-resistant populations of viral-specific memory $CD8^+$ and $CD4^+$T cells with the ability to self-renew and reconstitute immunity against infectious agents.

The notion that a stem-like subset exists within the T cell repertoire has been clearly addressed in $CD8^+$T cells. In mice, a postmitotic $CD8^+$T cell subset with naïve phenotype ($CD44^{lo}CD62L^{hi}$) and self-renewal ability was found to differentiate into memory subsets and cause graft-versus-host disease (GvHD) upon transfer in a secondary host.(Zhang et al. 2005b) Similarly, Gattinoni and colleagues have recently described the homolog of this subpopulation in humans. These cells were defined as $CD45RA^+CD62L^+$ $CD95^+$(Gattinoni et al. 2011), were enriched within the viral specific T cell population and exerted cytotoxic activity in a tumour model. Despite expressing naïve T cells markers, both subsets in mice and humans, were shown to exhibit stem cell signatures, including the expression of Sac-1 (only in mice), Bcl-2, IL-2Rβ and CXCR3(Gattinoni et al. 2005b). A recent study also reported the

existence of a memory subset of $CD8^+$T cells ($CD45RA^-$ $CD95^+$) with stem cell like properties, defined phenotypically on the basis of high expression of CD161 (NKR-P1A), a marker primarily associated with natural killer (NK) cells(Lanier et al. 1994b;Turtle et al. 2009). $CD8^+CD161^{hi}$T cells have the ability to rapidly efflux cytotoxic drugs through the ATP-binding cassette (ABC)–superfamily multidrug efflux protein ABCB1, shared expression of molecules with haematopoietic stem cells and were quiescent in non-lymphopenic individuals. A more recent study however, suggested that $ABCB1^+CD161^{hi}$ $CD8^+$T cells may in fact represent a subset of mucosal associated invariant T (MAIT) cells(Dusseaux et al. 2011) . Whereas much of our understanding of T cell memory has been attained through studies of $CD8^+$T cells, recent studies have also suggested the existence of IL-17-producing $CD4^+$ (Th17) stem cell like cells(Kryczek et al. 2011;Muranski et al. 2011).

Here I show the existence of a stem-like memory cells with high efflux capacity within the $CD4^+$T cell compartment. I report that these $CD4^+$T cells are enriched within the long-lived viral-specific Th1 memory T cell repertoire and share a similar phenotype to stem cell like memory $CD8^+CD161^{hi}$T cells.

3.2 Results

3.2.1 CMV-specific $CD4^+$ T cells survive induction chemotherapy in patient with AML

$CD8^+$ T cells specific for CMV, EBV, and influenza are detectable in patients with AML after recovery from chemotherapy(Turtle et al. 2009).I sought to investigate whether $CD4^+$ T cells specific for CMV are also present in the PB of patients with AML following treatment with chemotherapeutic agents. PBMC from five AML patients collected at diagnosis and following recovery from induction chemotherapy with daunorubicin, a lymphocytotoxic ABCB1 substrate, were stimulated with a pool of overlapping MHC class II CMV pp65 peptides for 6 hr, and using IC cytokines analysis to look for IFNγ and TNFα production, the frequencies of CMV-specific $CD4^+$ T cells were assessed directly *ex-vivo*. I found no significant difference in the frequencies of CMV-specific $CD4^+$ T cells in AML patients at diagnosis compared to healthy controls. Following recovery from the first cycle of remission induction chemotherapy, there was a nearly fivefold increase (median 4.98, range 1.6-11.7) in the frequencies of CMV-specific $CD4^+$ T cells in the PB of AML patients compared to diagnosis, p= 0.036. **(Figures 1A and B),** suggesting that a subset of CMV-specific memory $CD4^+$ T cells might preferentially resist and therefore survive the lymphocytotoxic effect of daunorubicin.

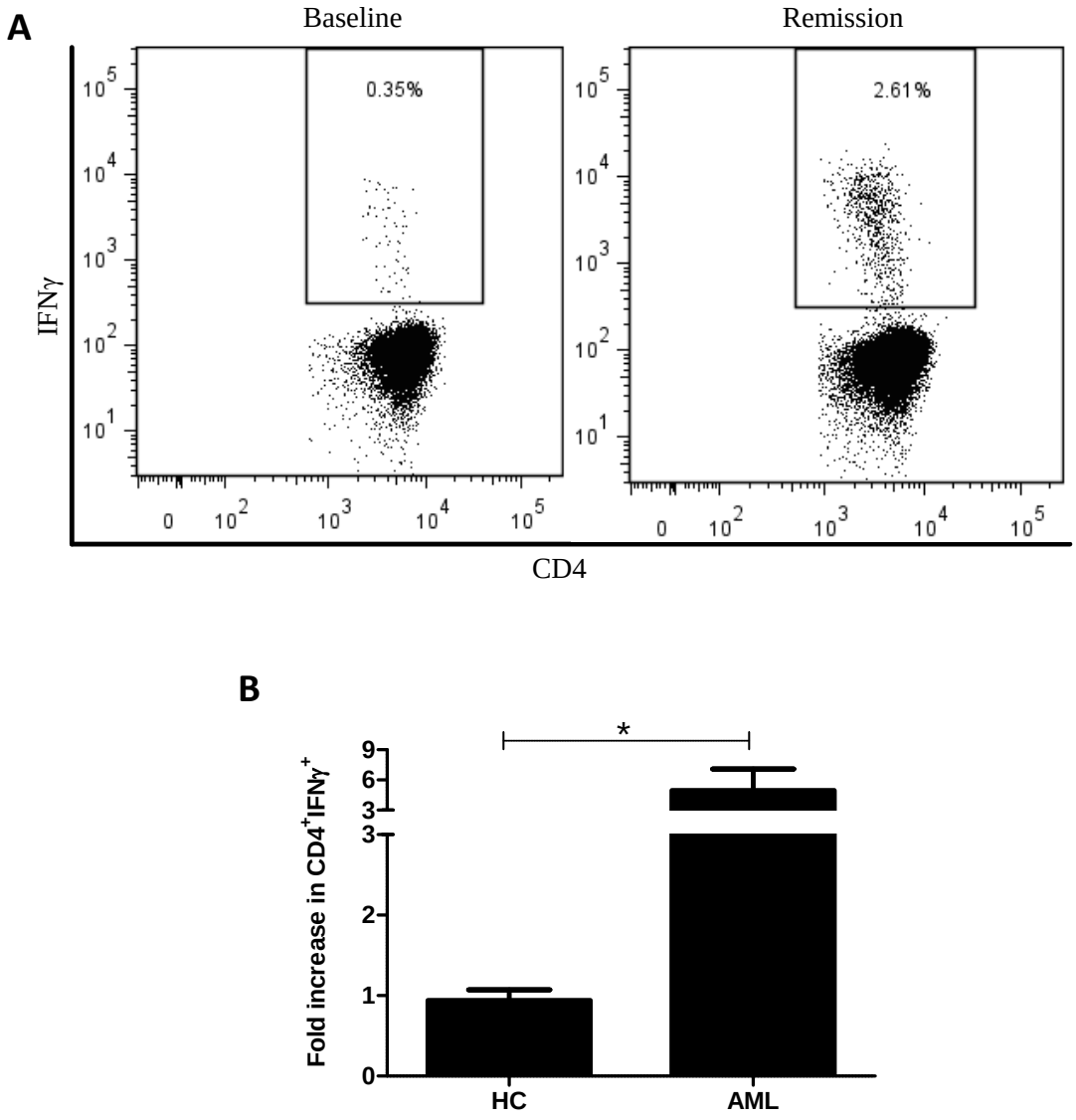


Figure 3.1: CMV-specific $CD4^+$ T cells in AML patients. A, a representative FACS plot depicting IFNγ-producing CMV-specific $CD4^+$ T cells in an AML patient before (baseline) and after recovery from chemotherapy (remission). **B,** Significant fold increase in CMV specific $CD4^+$ T cells in AML patients post-daunorubicin treatment (***p***=<0.036).

3.2.2 CMV specific $CD4^+$ T cells have an effector memory phenotype and express CD161

Multi-parameter flow cytometry was used to further characterize the phenotype of CMV-specific $CD4^+$ T cells **(Figure 2A)**. As expected, CMV-specific $CD4^+$T cells fell within the memory T cell compartment, with high expression of CD95 and low expression of CD45RA and the adhesion molecule L-selectin (CD62L). CMV-specific $CD4^+$ memory T cells from HD and AML patients (n=11) displayed significantly higher expression of CD161 (84.98 ± 2.7%), compared to only 33.83 ± 3.2% of the total $CD4^+$ T population ($p < 0.001$) **(Figures 2B and C)**. Such a profile is similar to that of viral-specific stem-like memory $CD161^+CD8^+$ T cells, reported to preferentially survive chemotherapy(Turtle et al. 2009).CD161 was also recently identified as a hallmark of Th17 cells(Cosmi et al. 2008). However, stimulation of PBMC from CMV seropositive donors with a pool of overlapping MHC class II CMV pp65 peptides failed to induce detectable IL-17 or IL-10 production, indicating that CMV-specific $CD161^+CD4^+$ T cells are in fact a unique subset of Th1,cells and are distinct from Th17 and Th2 cells.

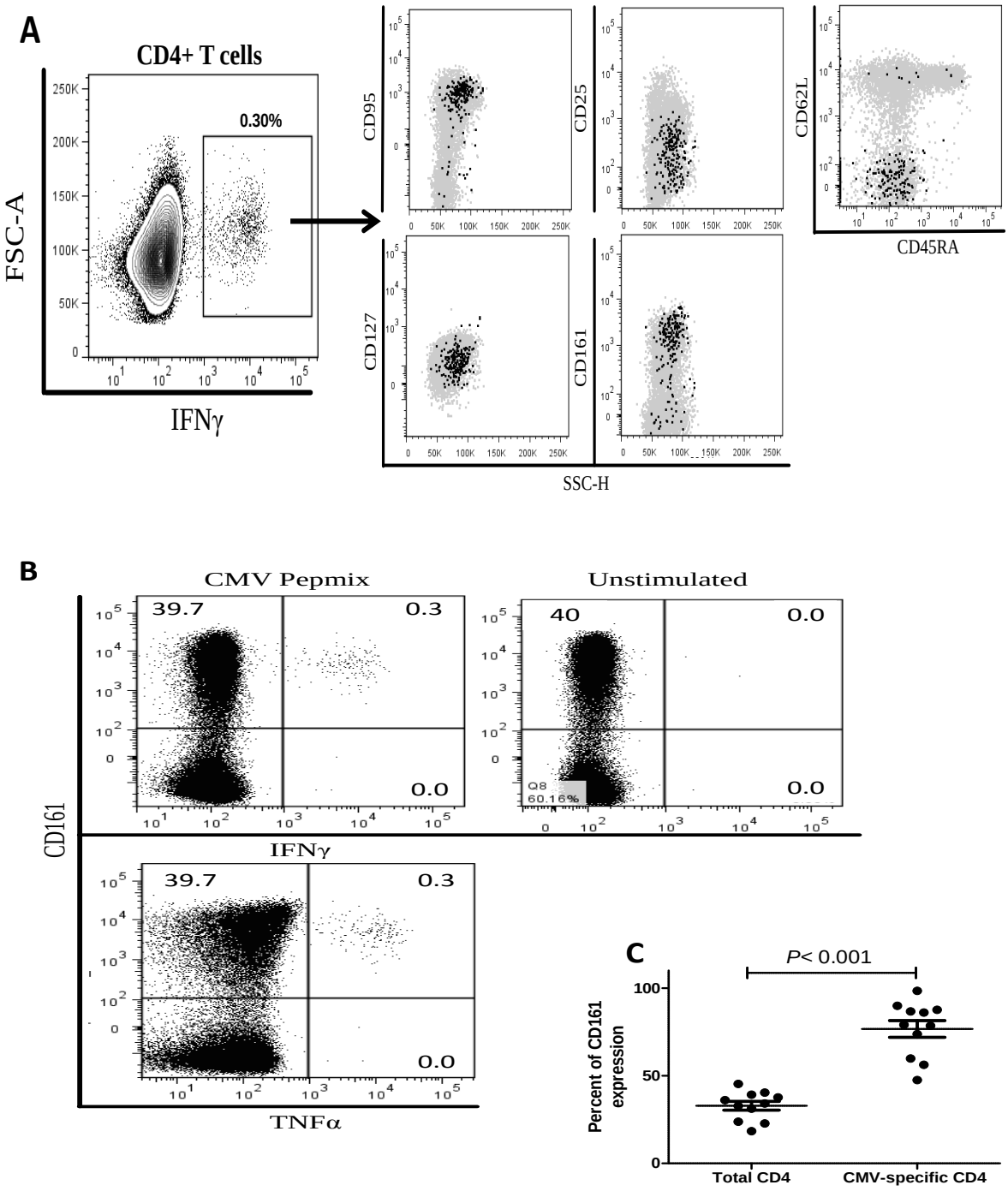
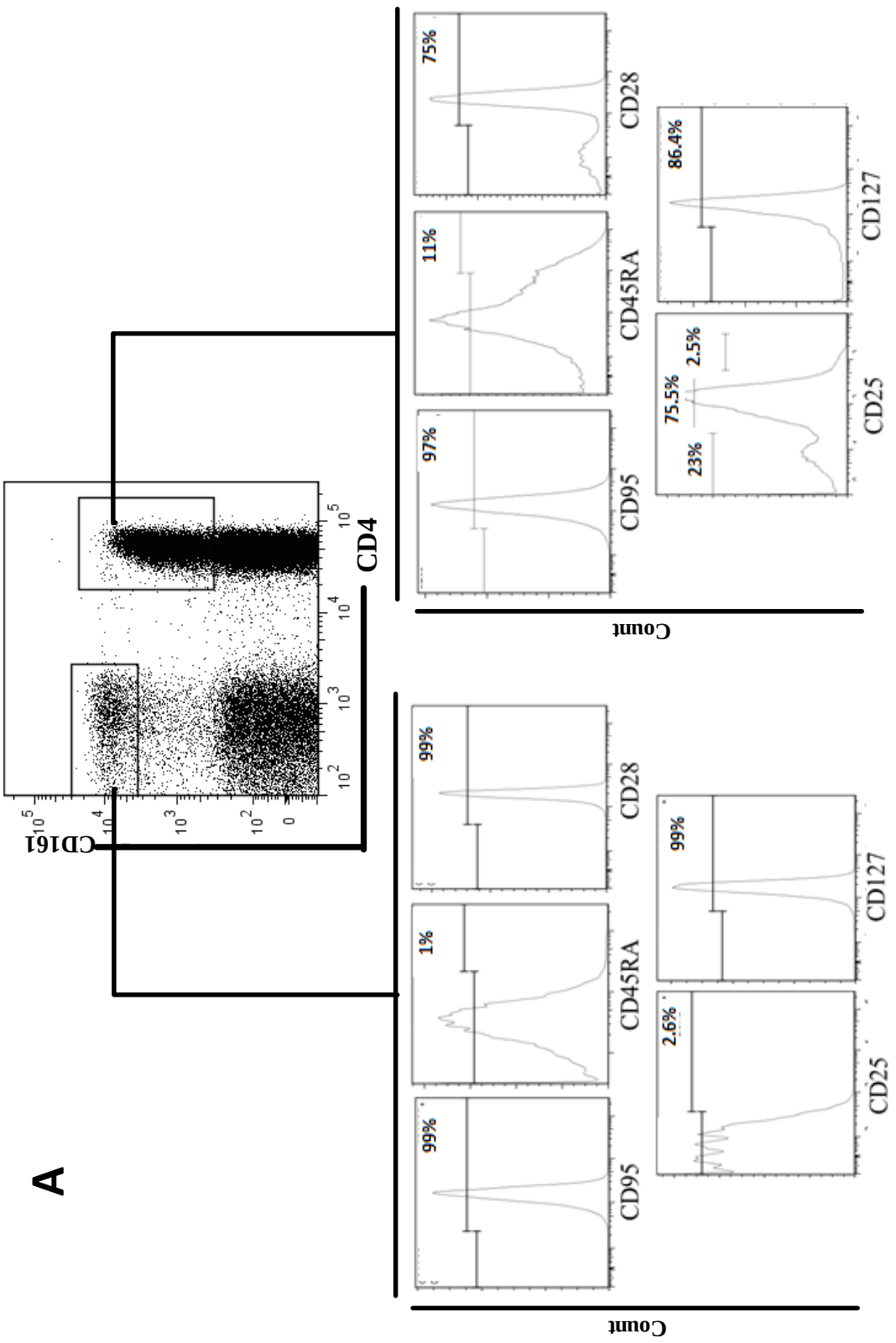


Figure 3.2: Phenotypic characterization of CMV specific CD4$^+$ T cells. PBMC were stimulated with an overlapping pool of MHC class II CMV pp65 peptides. Cells were gated on IFNγ-producing CD4$^+$ T cells and examined for the co-expression of a panel of surface markers as outlined in Materials and Methods. **A**, A representative FACS plot showing the phenotype of IFNγ-producing CMV-specific CD4$^+$ T cells (**black**) overlaid on total CD4$^+$ T cells (**grey**) is presented. CMV-specific T cells are predominantly CD95$^+$, CD45RA$^-$, CD62L$^-$ and CD127$^+$. **B,** A representative FACS plot showing that the majority of IFNγ$^+$ and TNFα$^+$ CMV-specific CD4$^+$ T cells also express CD161. **C,** CD161 expression on CMV specific CD4$^+$ and total CD4$^+$ T cells (***p*** <0.001). The majority of CMV specific CD4$^+$ T cells co-express CD161.

3.2.3 $CD161^{+}CD4^{+}$ T cellsshare a similar phenotype with stem cell-like memory $CD8^{+}CD161^{hi}$ T cells

To characterize $CD161^{+}CD4^{+}$ T cellsfurther, freshly isolated PBMC from healthy controls (N=7) were stained with antibodies to CD16, TCR Vα24, CD8β, and γδ TCR to exclude NK, NKT, $CD8^{dim}$ and γδ T cells respectively, thereby allowing selective analysis of $CD4^{+}$ and $CD8^{+}$ T cells **(Figure 3A)**. Whereas in $CD8^{+}$ T cells, two populations of CD161 expressing T cells could be defined, a $CD8^{+}CD161^{hi}$ and a $CD8^{+}CD161^{int}$ subset, $CD4^{+}$T cells displayed intermediate levels of CD161 expression. $CD4^{+}CD161^{+(int)}$ and $CD8^{+}CD161^{hi}$ T cells shared remarkable phenotypic similarities, including high expression of CD127, CD95 and CD28 (**Figure 3A**). Using CD95 and CD45RA to distinguish memory cells from naïve T cells, and CD62L, CCR7 and CD28 to divide memory cells into central memory and effector memory cell subsets, $CD4^{+}CD161^{+}$ and $CD8^{+}CD161^{hi}$ T cells were defined as memory T cells and were divided almost equally into effector and central memory subsets (**Figure 3B**). $CD4^{+}CD161^{+}$ memory T cells exhibited a significantly higher expression of CD127 compared to the total $CD4^{+}$ T cell population (MFI 986.6 ± 120 vs. 595.8 ± 61.7, p= 0.02) **(Figure 3C)**. These cells also expressed CD25 (IL-2Rα), with the vast majority enriched within the $CD25^{int}$ population. Analysis of Foxp3 expression as a marker for $CD4^{+}CD25^{+}$ regulatory T cells (Tregs) revealed that Tregs are in fact mainly confined to the $CD4^{+}CD161^{-}$ T cell fraction and not the $CD161^{+}CD4^{+}$ T cell subset**(Figure 3D)**. Overall, these data demonstrate that $CD4^{+}CD161^{+}$ are phenotypically very similar to the recently described $CD8^{+}CD161^{hi}$ stem cell-like memory T cells **(Figure 3A-C)**(Turtle et al. 2009).


A
CD161
CD4
Count
CD95
CD45RA
CD28
CD25
CD127
99%
1%
99%
2.6%
99%
97%
11%
75%
75.5%
23%
2.5%
86.4%

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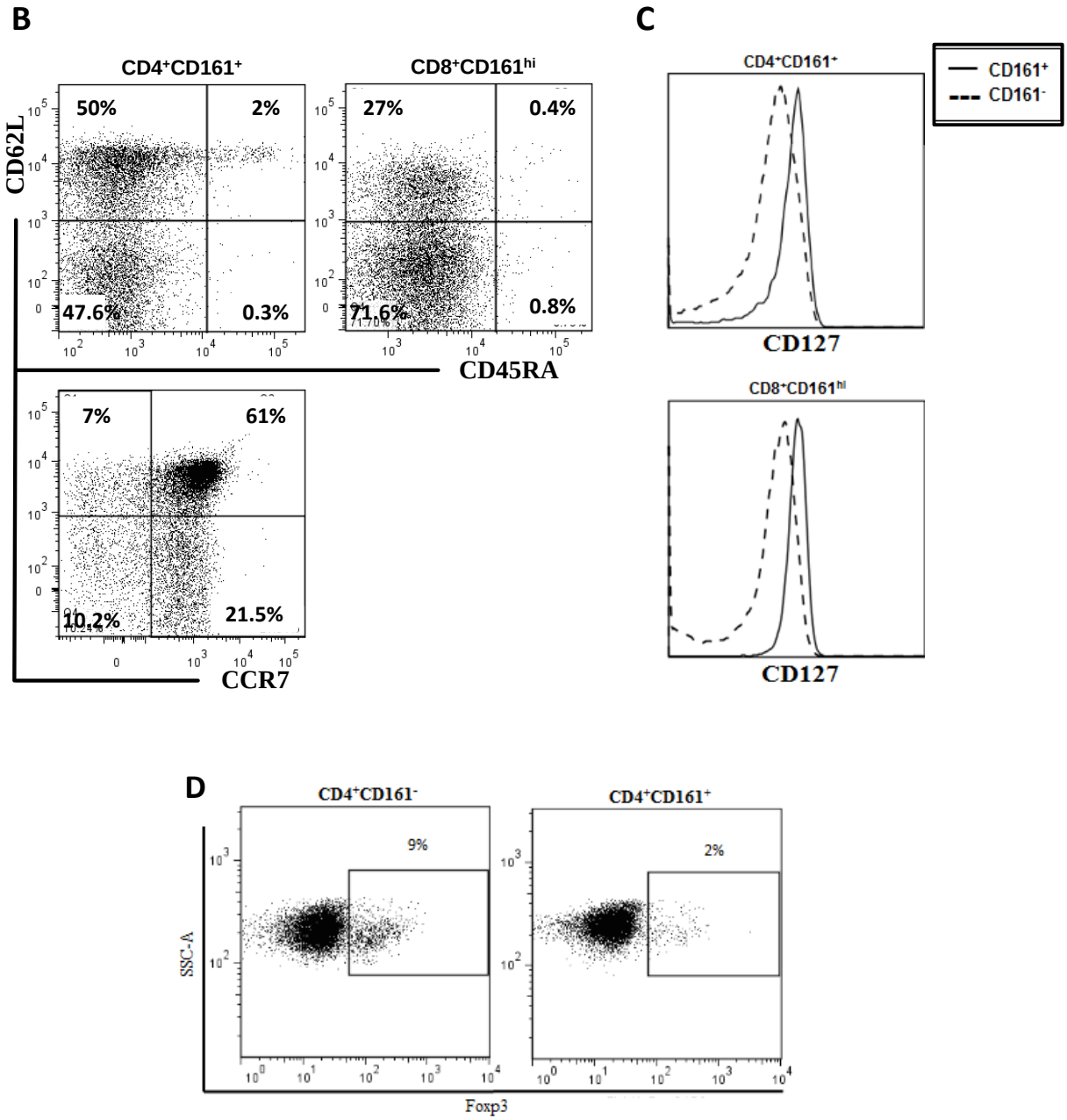
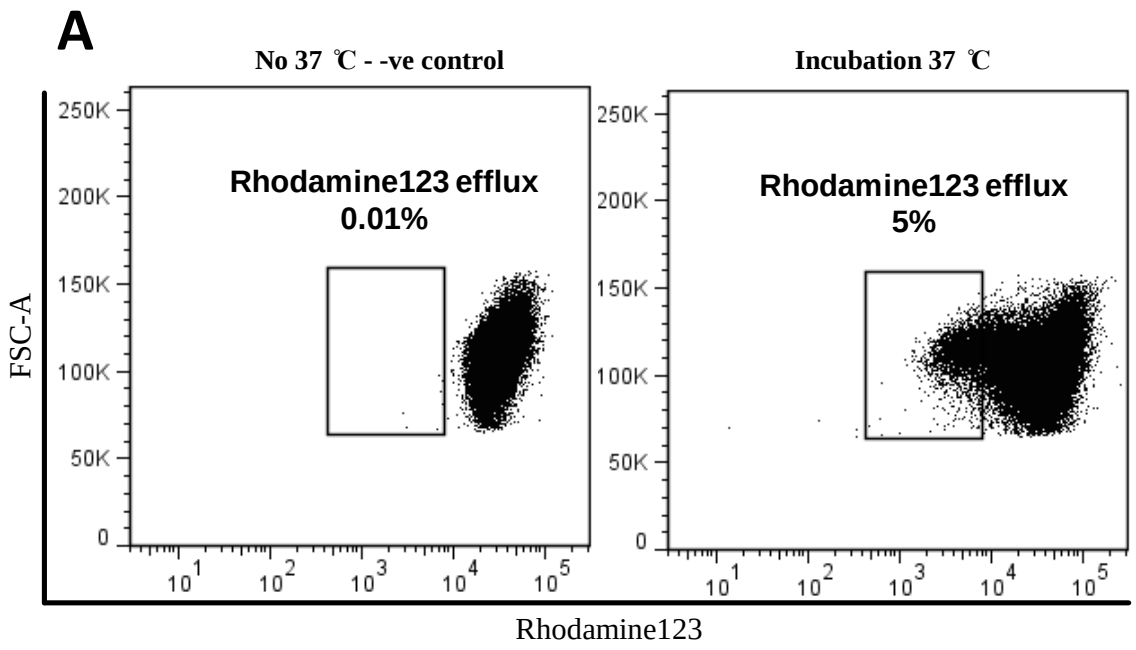


Figure 3.3Comprehensive phenotypic characterization of CD161^{+}CD4^{+} T cells using multi-parameter flow cytometry. PBMC were stained with a panel of surface markers including anti-CD16, -TCR Vα24, -CD8β, and -γδ TCR to exclude NK, NKT, CD8dim and γδ T cells respectively. **A.** Cells were gated on CD4^{+}CD161^{+} and CD8^{+} CD161^{hi} stem-like memory T cells and analysed for expression of a panel of surface markers as defined in Materials and Methods. **B,** Multi-parameter analysis of gated CD161^{+}CD4^{+} T cells identifies them to consist of a mixture effector (CD45RA^{-}CD62L^{-} CCR7^{-}) and central memory (CD45RA^{-}CD62L^{+}CCR7^{+}) phenotype. **C,** histogram comparing the mean fluorescence intensity (MFI) of IL-7Rα (CD127) expression between CD161^{+} (solid line) and total CD4^{+}T cell population (dashed line). **D,** A representative FACS plot comparing Foxp3 expression in CD4^{+}CD161^{-}**(left)** and CD4^{+}CD161^{+}**(right)** T cells is presented.

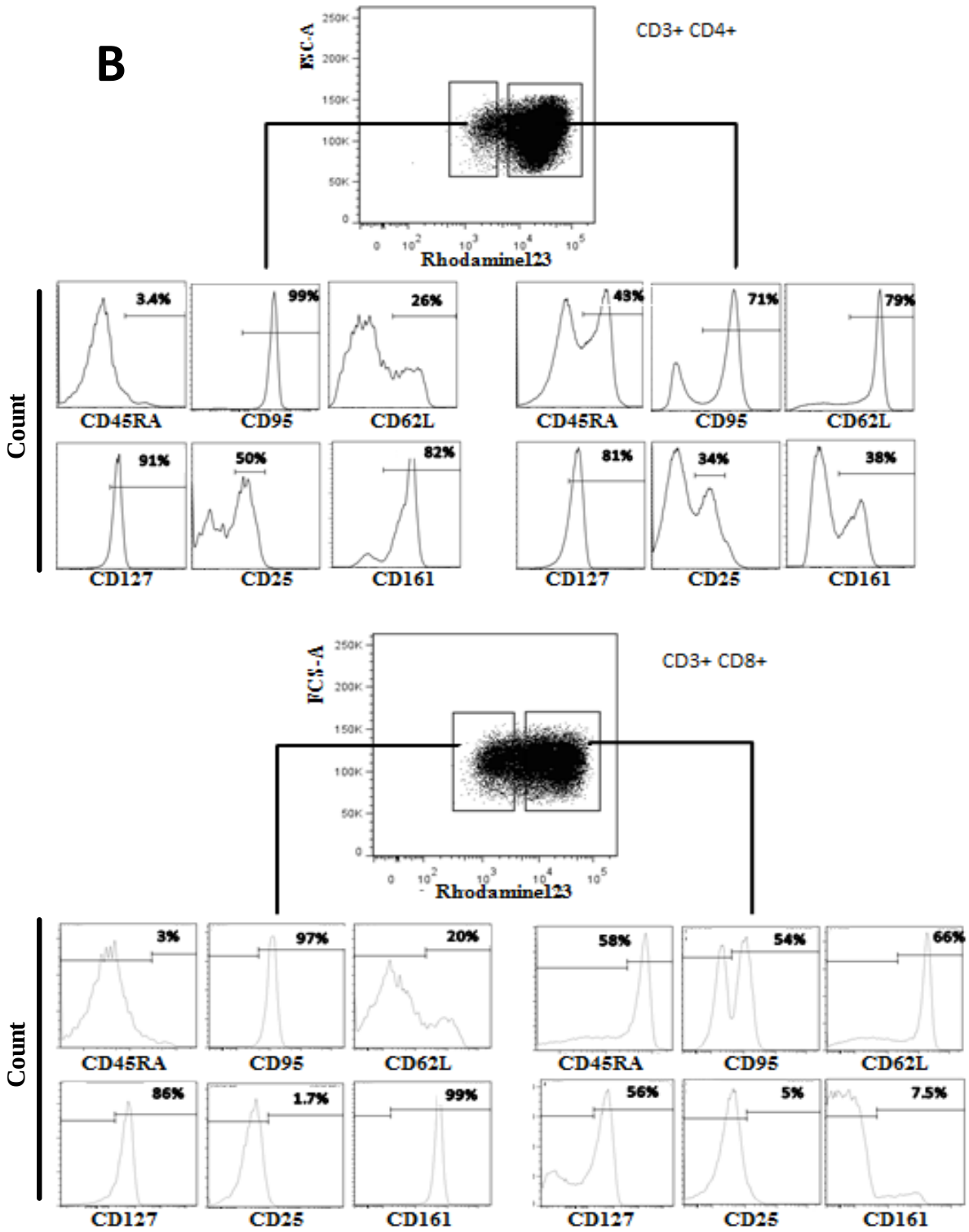
3.2.4 A subset of $CD4^+$ $CD161^+$T cell exhibit rapid multidrug efflux ability through ATP-dependent transporters and are resistant to daunorubicin lymphocytotoxicity

Overexpression of ABC-superfamily multidrug efflux proteins is one mechanism by which haematopoietic stem cells resist chemotherapy(Chaudhary and Roninson 1991) and was recently shown to play a role in the persistence of antigen-specific $CD8^+$ memory T cells after chemotherapy(Turtle et al. 2009).I investigated the existence of a stem cell-like memory subset within the $CD4^+$ T cell compartment by assessing ABCB1-mediated efflux of the fluorescent marker rhodamine-123 (Rh123). Freshly isolated PBMC were incubated with Rh123 and, using multi-parameter flow cytometry, a subset of $CD4^+$ T cells with a rapid Rh123-effluxing capacity was identified (median 3.7%, range 2.5-5%)**(Figure 4A)**. The rapidly effluxing $CD4^+$ T cells were phenotypically very similar to the recently described $CD8^+$ T cells with rapid Rh123-effluxing capacity(Turtle et al. 2009), including high expression of CD95, CD127 and CD161 **(Figure 4B and C).** I also found that the addition of vinblastine (a competitive inhibitor of ABCB1 and ABCC1), and other inhibitors with more specificity for ABCB1 such as cyclosporine A and PK11195, completely prevented or significantly reduced the efflux of Rh123 by $CD4^+$ T **(Figure 4D)**(Schinkel and Jonker 2003;Walter et al. 2004).Intriguingly, I also found a remarkable resemblance in the phenotype of Rh-123 effluxing $CD4^+$ T cells and CMV-specific memory $CD4^+$ T cells, namely, the uniform expression of CD95, low expression of CD62L and most specifically, the expression of CD161**(Figures 2A and 4B, upper panel)**, suggesting that CMV-specific $CD4^+$ T cells may also have high multi-drug efflux capacity.

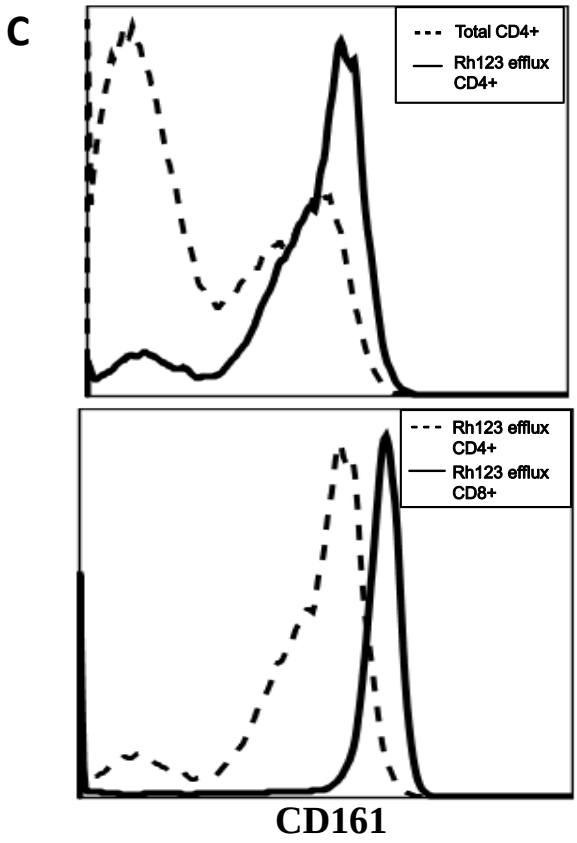
I examined if $CD161^{+}CD4^{+}$ T cells are also capable of effluxing daunorubicin through the ABC-superfamily multidrug efflux proteins. Daunorubicin is a fluorescent anthracycline used for the treatment of AML which is effluxed through the same ABC transporters that efflux Rh123. Memory $CD4^{+}CD161^{+}CD95^{+}$ T cells were capable of rapidly effluxing daunorubicin and the efflux is completely blocked by the addition of ABCB1 and ABCC1 inhibitors (**Figures 4E and 4F)**.

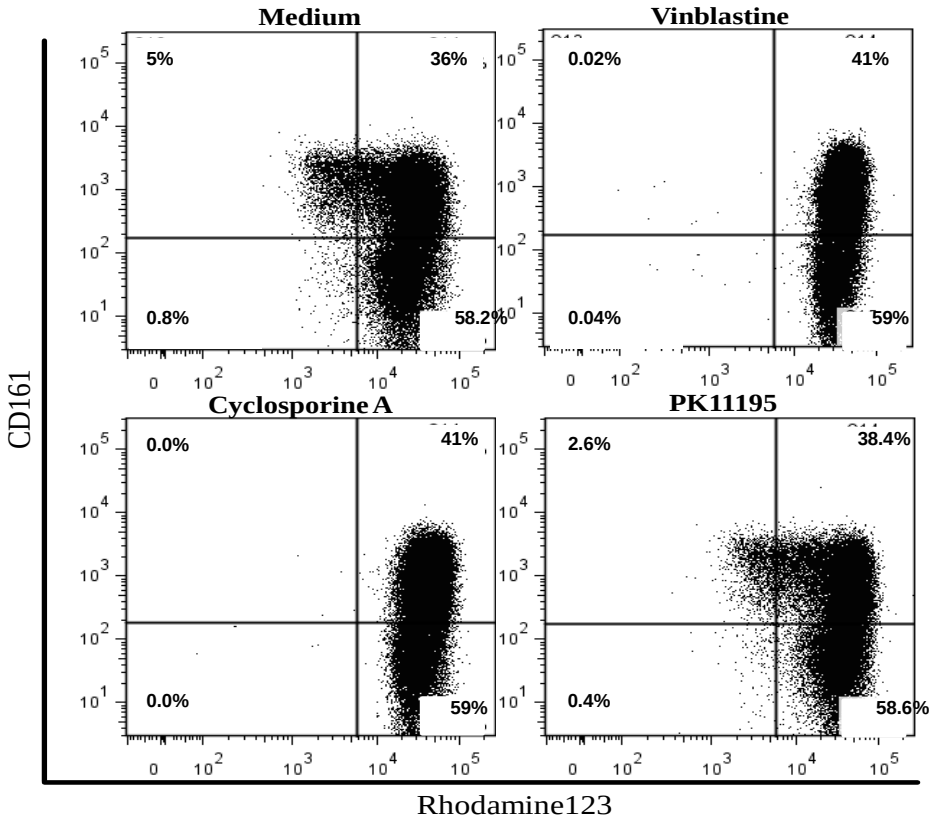


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B
CD3+ CD4+
FSC-A
Rhodamine123
Count
3.4%
99%
26%
43%
71%
79%
CD45RA
CD95
CD62L
91%
50%
82%
81%
34%
38%
CD127
CD25
CD161
CD3+ CD8+
FSC-A
Rhodamine123
Count
3%
97%
20%
58%
54%
66%
CD45RA
CD95
CD62L
86%
1.7%
99%
56%
5%
7.5%
CD127
CD25
CD161

Contd…


C
Total CD4+
Rh123 efflux CD4+
Rh123 efflux CD4+
Rh123 efflux CD8+
CD161


D
Medium
Vinblastine
Cyclosporine A
PK11195
5%
36%
0.8%
58.2%
0.02%
41%
0.04%
59%
0.0%
41%
0.0%
59%
2.6%
38.4%
0.4%
58.6%
CD161
Rhodamine123

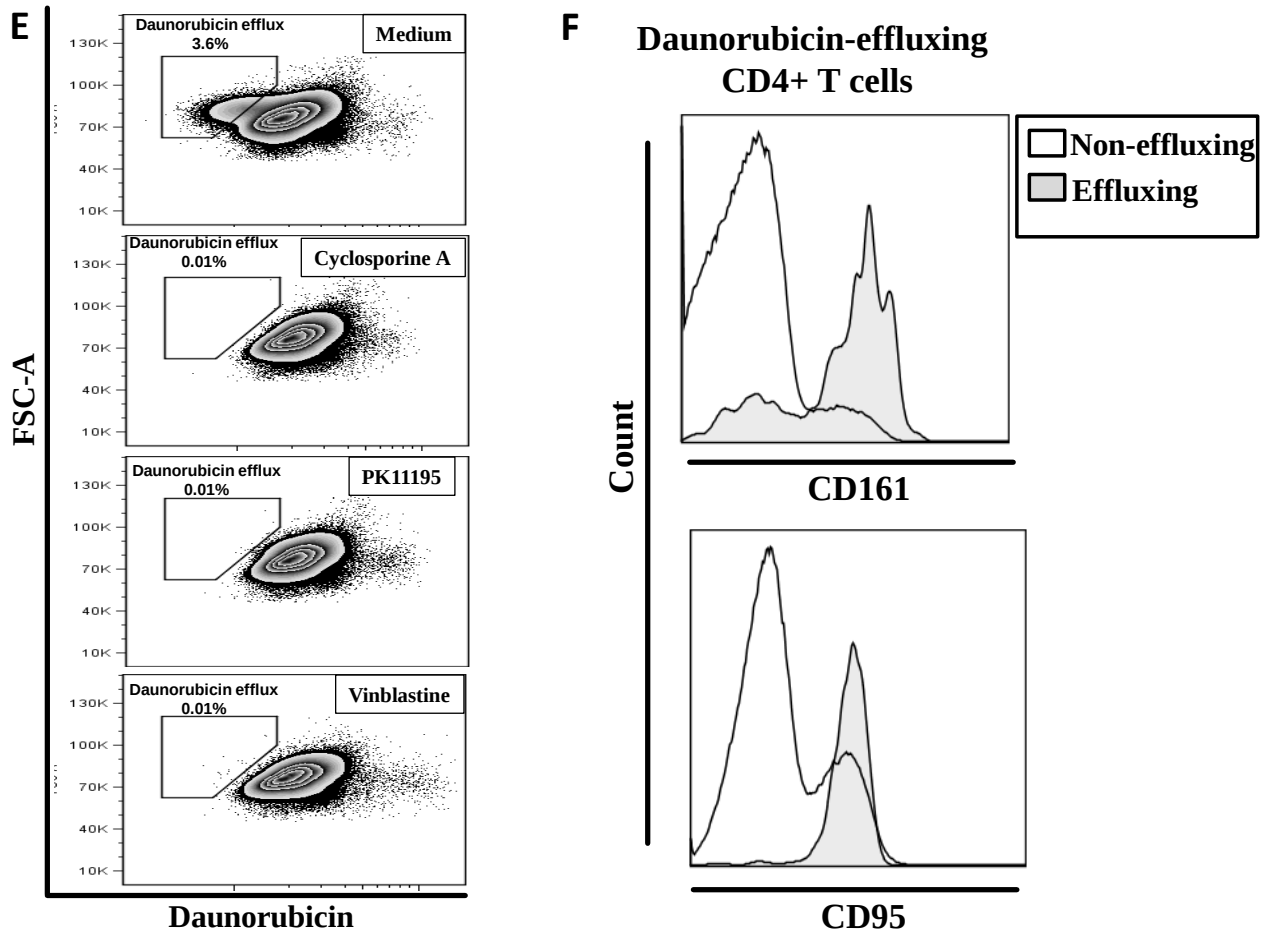


Figure 3.4: A subset of CD4$^+$ T cells efflux Rhodamine123 and daunorubicin. PBMC were incubated with rhodamine123 for 30 to 60 minutes at 37oC and multicolour FACS analysis was performed on gated CD4$^+$ Rh 123- (effluxing subset) and Rh 123$^+$ (non-effluxing subset). **A,** A representative FACS plot showing Rh123 efflux by CD4$^+$ T cells after incubation at 37°C for 30-60min (right) compared with negative control (left). **B,** histograms comparing the phenotype of Rh123$^+$ (non-effluxing, right) and Rh123- (effluxing, left) subsets within CD4$^+$ and CD8$^+$ T cells. Rh123 effluxing CD4$^+$ and CD8$^+$ subsets are enriched within the CD161$^+$ effector memory (CD95$^+$ CD45RA$^-$ CD62L$^-$) population. **C,** Histograms showing the increased expression of CD161 on Rh123-effluxing CD4$^+$ T cells relative to the total CD4$^+$ T cell population (upper panel) and comparing CD161 expression on Rh123-effluxing CD4$^+$ and CD8$^+$ T cells (lower panel) are presented. **D,** FACS plots showing the abrogation of Rh123 efflux by CD161$^+$CD4$^+$ T cells following co-incubation with inhibitors of ABCB1 and ABCC1 (cyclosporine, PK11195 and vinblastine). **E,** PBMC were co-incubated with fluorescent anthracycline (daunorubicin) for 30 to 60 min at 37oC. FACS plot shows daunorubicin efflux by CD4$^+$ T cells and complete abrogation of efflux upon addition of the ABCB1 inhibitors (cyclosporine A and PK11195) and ABCC1 (Vinblastine). **F,** Histograms show that the gated daunorubicin-effluxing CD4$^+$ T cells (grey histogram) are enriched within the CD95$^+$ CD161$^+$ memory T cell compartment compared to non-effluxing counterparts (white histogram).

3.2.5 A subset of CMV specific CD161^{+}CD4^{+} T cellswith multi-drug efflux capacity are resistant to daunorubicin lymphocytotoxicity

I next investigated if CMV specific CD161^{+}CD4^{+} T cellshave high multi-drug efflux capacity and consequently, the capacity to survive daunorubicin, a fluorescent chemotherapeutic agent effluxed through the same ABC transporters that efflux Rh123. PBMC from CMV-seropositive healthy donors were incubated with daunorubicin for 44-48 hr *in vitro.*

Using Annexin V staining, I found that daunorubicin induces apoptosis in both CD161^{-} and CD161^{+} CD4^{+} T cell subsets; however, CD161^{+}CD4^{+} T cells exhibit significantly greater resistance to the toxic effect of the drug (31.3%, ± 5.3 annexin V^{+}) compared to their CD161^{-} counterpart (46.7%, ± 5.9% annexin V^{+}), p= 0.02 **(Figures 5A and B),** supporting the existence of a drug-effluxing subset within the CD4^{+}CD161^{+} T cells. Next, I compared the frequencies of CMV-specific CD4^{+} T cells (as assessed by IFNγ production following 6 hour stimulation with a MHC class II CMV peptide library pool) before and after 2 days of *in vitro* treatment with daunorubicin. I found a greater than 2-fold increase in the frequencies of CMV specific CD4^{+} T cells after 2 days of in vitro exposure to daunorubicin compared to baseline (0.16% ± 0.03 vs. 0.07% ± 0.01, p=< 0.011) **(Figure 5C),** supporting selective resistance of CMV-specific CD4^{+} T cells to the lymphocytotoxic effect of daunorubicin *in vitro.*

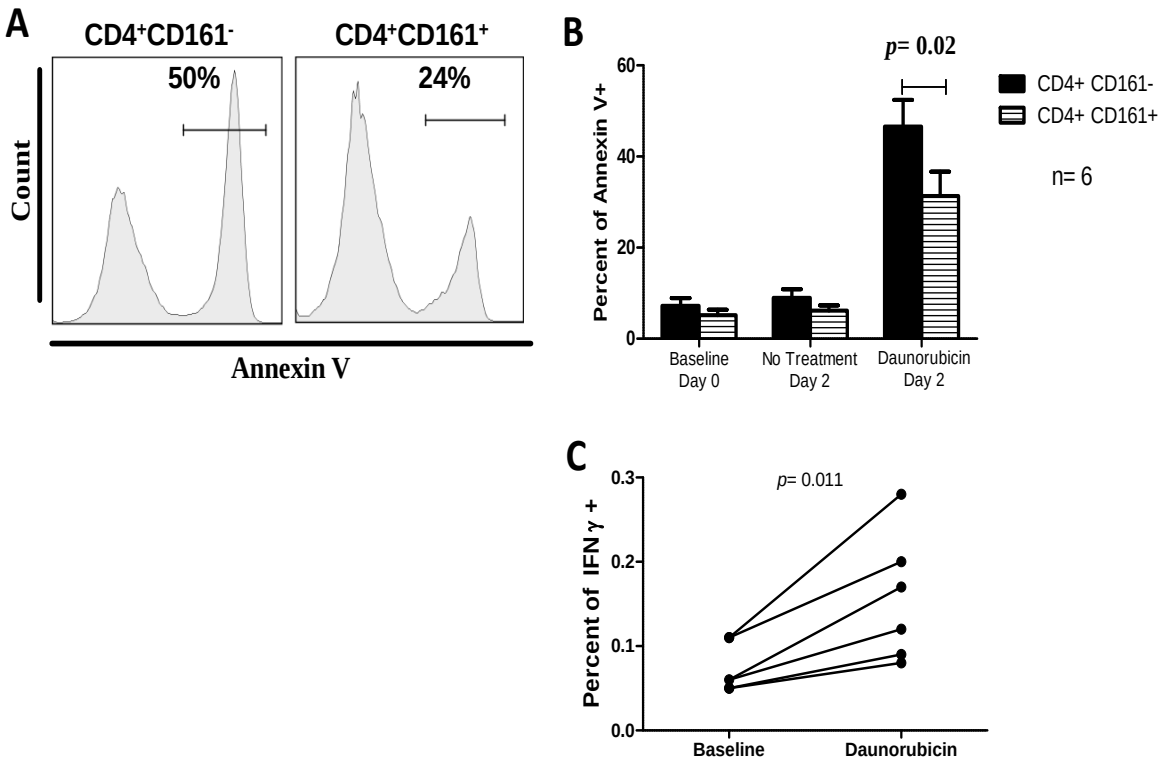


Figure 3.5: CMV-specific CD161^{+}CD4^{+} T cells efflux daunorubicin and resist apoptosis following *in vitro* exposure to daunorubicin*in vitro*. PBMC were exposed to daunorubicin *in vitro* and apoptosis was assessed by annexin V staining**. A**, Histograms show the percentage of annexin V in CD4^{+}CD161^{-} and CD4^{+}CD161^{+}. **B,** CD4^{+}161^{+} T cells were significantly more resistant to apoptosis after 2 days of *in vitro* treatment with daunorubicin (*p*=0.02) as assessed by annexin V staining compared to their CD161^{-} counterpart (n=6). **C,** Paired sample statistical analysis showing the preferential survival of CD4^{+}CD161^{+} CMV-specific T cells (IFNγ-producing) after *in vitro* treatment with daunorubicin (***p***= 0.011).

3.2.6 CD161 expressing $CD4^+$ T cells have a slow proliferation rate *in vitro*

The long-term persistence of Ag specific memory T cell during normal homeostasis may be related to their slow proliferative rate. Indeed HSCs resist chemotherapy through a number of mechanisms related both to cell quiescence and the overexpression of ABC-superfamily multidrug efflux proteins(Chaudhary & Roninson 1991;Li and Bhatia 2011). To evaluate the proliferative capacity of $CD161^+CD4^+$ T cells, I measured CFSE dilution after 5 days of polyclonal stimulation with anti-CD3/ CD28 beads *in vitro*. Similar to $CD8^+CD161^+$ stem like memory T cells, $CD161^+CD4^+$ T cells had a poorer proliferative capability compared to their $CD161^-$ counterpart (58.7% ± 5.1% vs. 91.6% ± 0.4%, $p < 0.0001$) **(Figures 6 A-B)**.

I next examined if under conditions of lymphopaenia-driven homeostasis, as seen following chemotherapy, the increased availability of cytokines such as IL-7, IL-15 or IL-21, can drive the proliferation of $CD161^+CD4^+$ T cells(Gattinoni et al. 2005).There was a significant increase in the absolute numbers of $CD4^+CD161^+$ and CMV specific $CD161^+CD4^+$ T cells in patients with AML recovering from daunorubicin-based induction chemotherapy compared to their pre-treatment samples (174.5 ± 28.3 vs. 89.5 ± 25.8 cells/μL, $p= 0.026$, and 2.6 ± 1.6 vs. 0.4 ± 0.2 cells/μL, $p= 0.0625$, respectively), (n=3). These data suggest that $CD161^+CD4^+$ T cells with rapid efflux capacity survive chemotherapy *in vivo* and repopulate the memory compartment, thereby contributing to the maintenance of viral-specific memory after recovery from lymphocytopaenia.

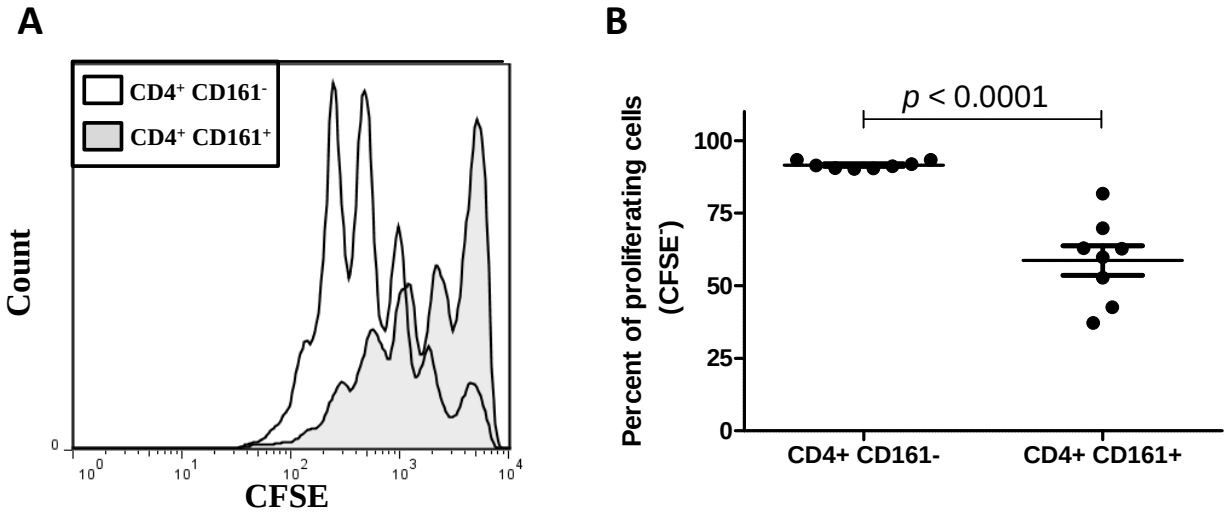


Figure 3.6:Proliferation of $CD4^+CD161^+$ T cells. PBMC were loaded with CFSE and stimulated with anti-CD3/28 beads for 5 days. **A,** A representative histogram showing the relatively lower proliferative profile of $CD4^+CD161^+$ (**grey histogram**) compared to $CD4^+CD161^-$ T cells is presented (**white histogram**). **B,** Data from 8 experiments comparing CFSE dilution by $CD4^+CD161^+$ vs. $CD4^+CD161^-$ T cells. CD161 expressing $CD4^+$ T cells have a significantly lower proliferative capacity compared to their $CD161^-$ counterpart (***p*** <0.0001).

3.2.7 CD161 is a marker for long-lived Ag specific memory T cell

To assess if CD161 is a marker of long-lived memory $CD4^+$ T cells, I analysed the phenotype of Flu-specific $CD4^+$ T cells in paired samples collected at 4 weeks and 2 years following Flu vaccination in 4 healthy donors(de et al. 2011). Vaccination resulted in induction of Flu-specific $CD4^+$ T cells in all healthy controls, as assessed by IFNγ production following overnight stimulation with or without seasonal influenza vaccine (A/Brisbane/59/2007(H1N1)*in vitro*. Although the frequencies of Flu-specific $CD4^+$ T cells remained stable and were not significantly different at 4 weeks and 2 years following vaccination (0.15 ± 0.05 vs. 0.21 ± 0.04 N=4, p=0.32) **(Figure 7A),** the proportion of Flu-specific $CD4^+$ T cells that co-expressed CD161 was significantly greater at 2 years, compared to 4 weeks post-vaccination, (72.8 ± 3.1 vs. 49.3% ± 3.5% p= 0.0046) (**Figure 7B),** suggesting that CD161 is a marker for long-lived viral-specific memory T cells.

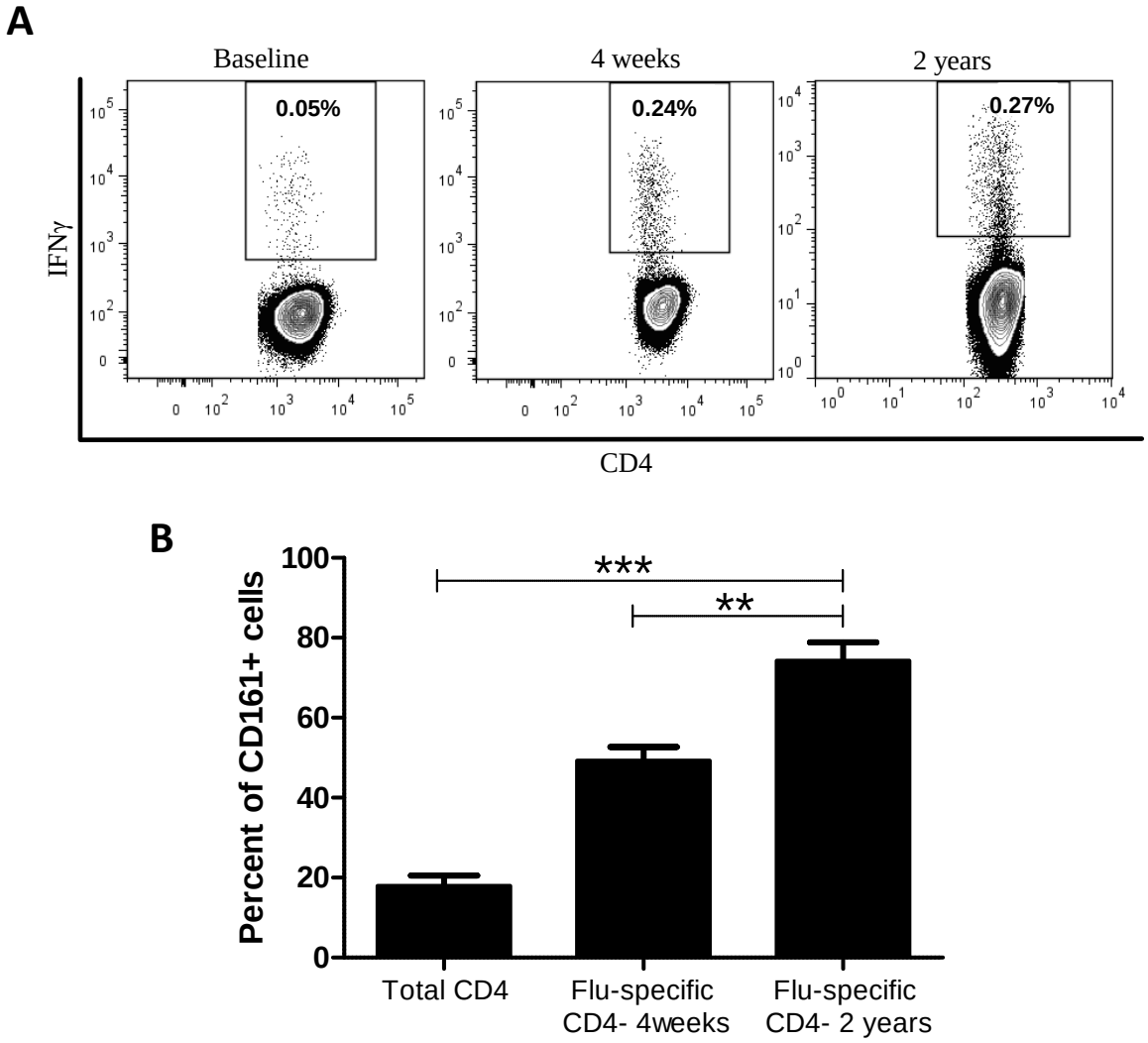


Figure 3.7: Kinetic of Flu-specific CD161^{+}CD4^{+} T cells following vaccination. PBMC collected from healthy donors 4 weeks and 2 years following influenza vaccination were stimulated *in vitro* with influenza antigen and IFNγ-producing CD4^{+} T cells were enumerated. **A,** a representative experiment to show the frequencies of IFNγ-producing Flu-specific CD4^{+} T cells before vaccination (**left panel**), 4 weeks (**middle panel**) and 2 years (**right panel**) post-vaccination. **B,** IFNγ-producing Flu-specific CD4^{+} T cells have significantly higher expression of CD161 compared to the total CD4^{+} T cell population(*** ***p*** < 0.0001). The highest expression of CD161 is observed on Flu-specific CD4^{+} T cells 2 years following influenza vaccination, (** ***p***= 0.0046).

3.3 Discussion

Following virus infection in immunocompetent individuals, Ag-specific T cells pass through three distinct phases: clonal expansion, contraction (also called the death phase), and memory(Kaech et al. 2002;Pepper and Jenkins 2011;Sprent and Surh 2002). Memory T cells confer immediate protection as well as the capacity to mount a more rapid and effective secondary immune response. Under circumstances where the immune system is compromised or destroyed, for instance following treatment with a number of lymphocytotoxic chemotherapeutic agents, patients still appear to be protected from viral infections. This strongly suggests that a number of mechanisms have evolved to maintain long-term T cell memory. A recent report described a distinct subset of human viral-specific stem-like memory $CD8^+$ T cells, identified on the basis of their drug efflux capacity and distinct phenotype,(Turtle et al. 2009) that persist after cytotoxic chemotherapy. Since $CD4^+$T cells play an equally important role in viral immunity, I hypothesized that a similar subset of drug-effluxing $CD4^+$ stem-like memory T cells may also exist.

In this study the persistence of CMV-specific $CD4^+$ T cell in the peripheral blood of patients with AML after recovery from remission induction chemotherapy was shown. Interestingly, CMV-specific $CD4^+$ T cell frequencies after recovery from lymphocytotoxic chemotherapy were significantly higher than at presentation. Although it could be argued that the lower frequencies of CMV-specific $CD4^+$ T cells in AML patients at diagnosis may be related to the underlying neoplasia(Costello et al. 2002;Le et al. 2009), their frequency and function (as assessed by their ability to produce IFNγ and TNFα following *in vitro* stimulation with an overlapping CMV peptide library) was not significantly different to that seen in healthy donors.

Therefore, it is likely that the proportional increase in CMV-specific $CD4^+$ T cells after recovery from chemotherapy-induced lymphopaenia is related to their preferential survival and expansion.

Multiple mechanisms including overexpression of ABC-superfamily multidrug efflux proteins and cell quiescence(Li & Bhatia 2011) are employed by HSC (Chaudhary & Roninson 1991;Li & Bhatia 2011) as well as malignant cells (Dean et al. 2005;Li & Bhatia 2011) to resist chemotherapy. Similar mechanisms have also been reported to explain the persistence of stem-like $CD8^+$ memory T cells after chemotherapy. In this study, I identified a sub-population of memory $CD4^+$ T cells that were capable of effluxing the fluorescent substrate Rh123 as rapidly as $CD8^+$ stem-like memory T cells. Daunorubicin, a fluorescent anthracycline employed in the treatment of AML, is effluxed through the same ABC transporters that efflux Rh123. I showed that the high ABCB1-mediated drug efflux capacity of $CD4^+CD161^+$ memory T cells facilitates their resistance to daunorubicin and, that this resistance is abrogated by the addition of competitive inhibitors of ABCB1 and ABCC1(Schinkel & Jonker 2003;Walter et al. 2004).

The drug effluxing $CD4^+$T cells in our study had a memory phenotype and were defined as $CD95^+$, $CD45RA^-$, $CD127^{hi}$, $CD28^+$, $CD25^{int}$. The majority of these cells also expressed CD161 (NKR-P1) primarily known as an NK marker(Lanier, Chang, & Phillips 1994b). However, whereas in $CD8^+$ T cells two populations of CD161 expressing T cells could be defined, a $CD8^+CD161^{hi}$ and a $CD8^+CD161^{int}$ subset, among $CD4^+$ T cells, the proportion of $CD161^{hi}$ cells was very low and the expression of CD161 was at intermediate level. $CD161^+CD4^+$ T cells were phenotypically different from the naïve-like CD95 expressing T memory stem cells described by Gattinoni et al.(Gattinoni et al. 2011).I defined human memory stem-

like $CD4^+$ T cells on the basis of their drug efflux capacity, a property shared with HSCs, and used previously to define human memory stem-like $CD8^+$ T cells(Chaudhary & Roninson 1991;Turtle et al. 2009). Interestingly, there is a remarkable phenotypic similarity between $CD4^+CD161^{int}$ T cells with drug effluxing ability and the human $CD8^+CD161^{hi}$ stem-like memory T cells described by Turtle et al(Turtle et al. 2009). Similar to their $CD8^+$ counterpart, effluxing $CD161^+CD4^+$ T cells proliferated poorly in response to anti-CD3/CD28 stimulation *in vitro*, while propagating in response to homeostatic signals and repopulating the memory compartment in AML patients recovering from chemotherapy-induced lymphocytopaenia. A recent report that antigen-specific memory T cells express the anti-apoptotic marker Bcl-2,(Antoine et al. 2012) also involved in HSC survival, suggests that this subset of T cells may resist insult by cytotoxic agents through a number of mechanisms including drug efflux, low proliferative rate and expression of anti-apoptotic markers(Strasser et al. 1991;Strasser et al. 1994).

Altogether, these results indicate that like HSC and $CD8^+$ memory T cells, a subset of $CD4^+$ memory T cells have drug-efflux capacity and are capable of surviving lymphocytotoxic insult. The identification of CD161 as a specific antigenic marker for stem-like memory $CD4^+$as well as $CD8^+$ T cells(Turtle et al. 2009) suggests a role for CD161 expressing T cells in long-term protection against pathogens. It is likely that CD161 expressing $CD4^+$ T cells comprise a mixed cell population, some of which are endowed with stem cell like attributes. Indeed in our study only a subset of $CD161^+CD4^+$ T cells displayed Rh-effluxing capacity **(Figure 4c)**. CD161 was recently identified as a hallmark of Th17 cells.(Cosmi et al. 2008) CD161 is also expressed on MAIT cells(Dusseaux et al. 2011) and on a subset of $CD8^+$ T cells with specificity for tissue localizing viruses such as hepatitis C and hepatitis B(Billerbeck et al. 2010).Here the enrichment of$CD161^+CD4^+$ T cells are enriched within the

CMV-specific T cell repertoire in AML patients and normal controls was shown. Furthermore, in a cohort of healthy donors who mounted a $CD4^+$ T cell response to vaccination with seasonal influenza, I observed that the proportion of CD161 expressing Flu-specific $CD4^+$ T cells increased over time. Upon stimulation with viral antigens, viral-specific CD161 expressing $CD4^+$ T cells displayed a Th1 phenotype and failed to produce IL-17, indicating that these cells are in fact distinct from CD161 expressing Th17 cells(Cosmi et al. 2008).

Collectively, these results support a role for $CD161^+CD4^+$ T cells in the maintenance of long-lived memory to pathogens. These data have significant implications for the development of the next generation of T cell based immunotherapies against pathogens and tumours. The long-term survival capacity of CD161 expressing T cells and their drug-effluxing capacity make them especially attractive for adoptive therapy against cancer. Thus, development of strategies to generate antigen-specific CD161 expressing stem cell like memory T cells might offer a highly effective approach to immunotherapy.

CHAPTER 4. DIFFERENTIAL EFFECT OF DEXAMETHASONE, CALCINEURIN INHIBITORS AND MTOR INHIBITORS ON $CD161^{+}CD4^{+}$ T CELLSAND $FOXP3^{+}CD4^{+}$ REGULATORY T CELLS

4.1 Introduction

A subset of antigen-specific memory $CD8^{+}$ T cells with stem-like properties were recently described. This subset was capable of surviving cytotoxic drugs through mechanisms shared with haematopoietic stem cells, namely the expression of ATP binding cassette multidrug efflux proteins,(Chaudhary& Roninson 1991;Schinkel & Jonker 2003) and provided protection against infectious pathogens in AML patients(Turtle et al. 2009). Likewise, in **chapter 3,** I described the existence of a similar population within the memory $CD4^{+}$ helper T cell compartment. Multidrug effluxing $CD8^{+}$ and $CD4^{+}$ T cells were defined by the expression of the NK cell marker CD161 (NKR-P1)(Lanier, Chang, & Phillips 1994b).

ATP binding cassette (ABC) transporters belong to a superfamily of transmembrane proteins. By utilizing the ATP hydrolysis energy, these transporters facilitate the translocation of a variety of substrates including a number of drugs, across the plasma membrane.(Jones and George 2004) Almost fifty ABC transporters have been discovered so far and ABCB1, also known as P-glycoprotein, is one of the most extensively studied transporters.(Mizutani et al. 2008) This is the major transporter through which stem cells as well as cancer cells survive cytotoxic drugs

(Chaudhary & Roninson 1991;Gottesman et al. 2002).A wide range of inhibitors with different specificity for ABC transporters have been introduced(Falasca and Linton 2012;Walter et al. 2004). Noteworthy, inhibition of the ABCB1 transporter has been exploited as a mechanism to overcome drug resistance in leukemic cells (Walter et al. 2004).

Prior to allogeneic stem cell transplantation, patients undergo a preparative conditioning regimen which includes prophylaxis for GvHD such as calcineurin inhibitors(Nash et al. 2000). Moreover, once acute GvHD is diagnosed, corticosteroids are immediately used as a standard first line intervention to treat or at least ameliorate GvHD symptoms. However, a great proportion (~60%) of patients fails to respond to corticosteroids(MacMillan et al. 2002;Martin et al. 1990;Van Lint et al. 2006) necessitating second line treatment(Benito et al. 2001;Hoda et al. 2010). Of note, some of the drugs used for the prevention or treatment of GvHD, including corticosteroids, are substrates for ABC transporters(Schinkel & Jonker 2003).

In this chapter I hypothesise that $CD161^{+}CD4^{+}$ T cells will efflux immunosuppressive drugs used for GvHD prophylaxis and treatment using the MDR pump complex. Thus, steroid-refractory GvHD could be explained by the ability of this subset of helper T cells to escape the lymphocytotoxic effect of these drugs. I also test if the addition of ABC transporter inhibitors T cells treated with steroids will induce further apoptosis *in vitro.*

4.2 Results

4.2.1 Selection of P-glycoprotein (ABCB1) substrates

First, three different drugs which are primarily used in GvHD prophylaxis or treatment were selected including calcineurin inhibitors e.g. tacrolimus (Tacro), mTOR inhibitors e.g. rapamycin (Rapa) and corticosteroids e.g. dexamethasone (Dex) (all from Sigma Aldrich, UK). These drugs mediate their immunosuppressive activity through different modes of action. Tacrolimus and rapamycin inhibit T cell proliferation whereas corticosteroid (e.g. dexamethasone) are lymphocytotoxic and induce apoptosis(Distelhorst 2002). All these three drugs are substrates of ABCB1 multidrug efflux transporter(Schinkel & Jonker 2003). Since the mode of action for each drug is different, I first evaluated the optimal *in vitro* conditions for their use.

4.2.2 Titration of drugs (ABCB1 substrates); Dexamethasone, Tacrolimus, and Rapamycin

Dose titration experiments for Dexamethasone, Tacrolimus, and Rapamycin were performed to determine the optimal dose for *in vitro* T cell proliferation and viability assays. Since each drug has a different mode of action, Annexin V and CFSE assays were used to measure apoptosis (cell death) and proliferation, respectively. Titration experiments were done on PBMC from three healthy controls.

PBMC were incubated with or without anti-CD3/CD28 beads (Dynabeads, Invitrogen, UK) in the presence or absence of the test drugs at different concentrations for 5 days (120 hours). T cell proliferation and viability were assessed on a daily basis.

4.2.2.1 Dexamethasone (Dex):

Dexamethasone is a glucocorticoid with anti-inflammatory properties. In addition, it is lymphocytotoxic and induces apoptosis in T lymphocytes as well as lymphoid leukaemia cells(Distelhorst 2002;Frankfurt and Rosen 2004;Herold et al. 2006).Generally, it is more potent than prednisolone, which is widely used in the transplant clinic to treat GvHD symptoms(Van Lintet al. 2006).

To determine the impact of dexamethasone on T cell viability and proliferation, PBMC were incubated with increasing concentrations of dexamethasone *in vitro*; 10^{-6}, 10^{-5} and 10^{-4} M (Blauer et al. 1991;Orlikowsky et al. 2001)and daily Annexin V and CFSE assays for 5 days were performed**(Figures 4.1 and 4.2)**. I determined that, using the conditions outlined above, day three (Day 3) is the optimal time to measure dexamethasone-induced T cell apoptosis **(Figure 4.3A).**

Dexamethasone induced apoptosis in $CD4^+$ T cells when compared to the negative control at 3 tested concentrations **(Figure 4.1 and 4.3B)** and suppressed T cell proliferation in a dose dependant manner as depicted by CFSE staining **(Figure 4.2)**. Based on these preliminary data, I chose a dexamethasone dose of 10^{-5} M (10 µmol/L "µM" ~ 3.9µg/ml) as the concentration to be used in my *in vitro* experiments.

4.2.2.2 Rapamycin (Rapa):

Rapamycin (also known as Sirolimus) exerts its immunosuppressive activity through inhibition of the mTOR (mammalian target of rapamycin) pathway. Unlike calcineurin inhibitors, which inhibit IL-2 secretion, rapamycin prevents T cell proliferation through inhibiting the response to IL-2.mTOR inhibitors are occasionally prescribed as second line intervention for steroid-refractory aGvHD(Dignan et al. 2012). In accordance with previously published *in vitro* studies that used rapamycin at concentrations ranging from 0.1 to 10 μM, I evaluated three concentrations of rapamycin in my experiments including 0.1, 1 and 10 μM(Uss et al. 2007).

As expected, there was no significant difference in the proportion of apoptotic cells (Annexin V^+) in $CD4^+$ T cells treated with or without rapamycin **(Figure 4.1 and 4.3A)**; however, at the concentration of 10 μM, rapamycin was more likely to be toxic to T cells. In contrast, all 3 concentrations inhibited $CD4^+$ T cell proliferation as assessed by CFSE assay, **(Figure 4.2)**. Therefore, I selected a rapamycin concentration of 0.1 μM to avoid non-specific drug toxicity.

4.2.2.3 Tacrolimus (Tacro):

Tacrolimus (also known as FK506) is a member of calcineurin inhibitors. It interferes with T cell activation through inhibition of IL-2 transcription and signal transduction(Thomson et al. 1995).In AHSCT, Tacrolimus is commonly used in GvHD prophylaxis(Dignan et al. 2012). Concentrations used for *in vitro* experiments range from 1 to 25 ng/mL (1 to 30 nM)(Uss et al. 2007). Therefore, I examined tacrolimus

activity at 1, 10 and 50 nM. The induction of apoptosis by tacrolimus was minimal when compared with no treatment control **(Figure 4.1 and 4.3A).** On the other hand, its ability to inhibit T cell proliferation was most significant at a dose of 25-50 nM **(Figure 4.2)**. Therefore, I chose a tacrolimus dose of 25nM of for my subsequent experiments.

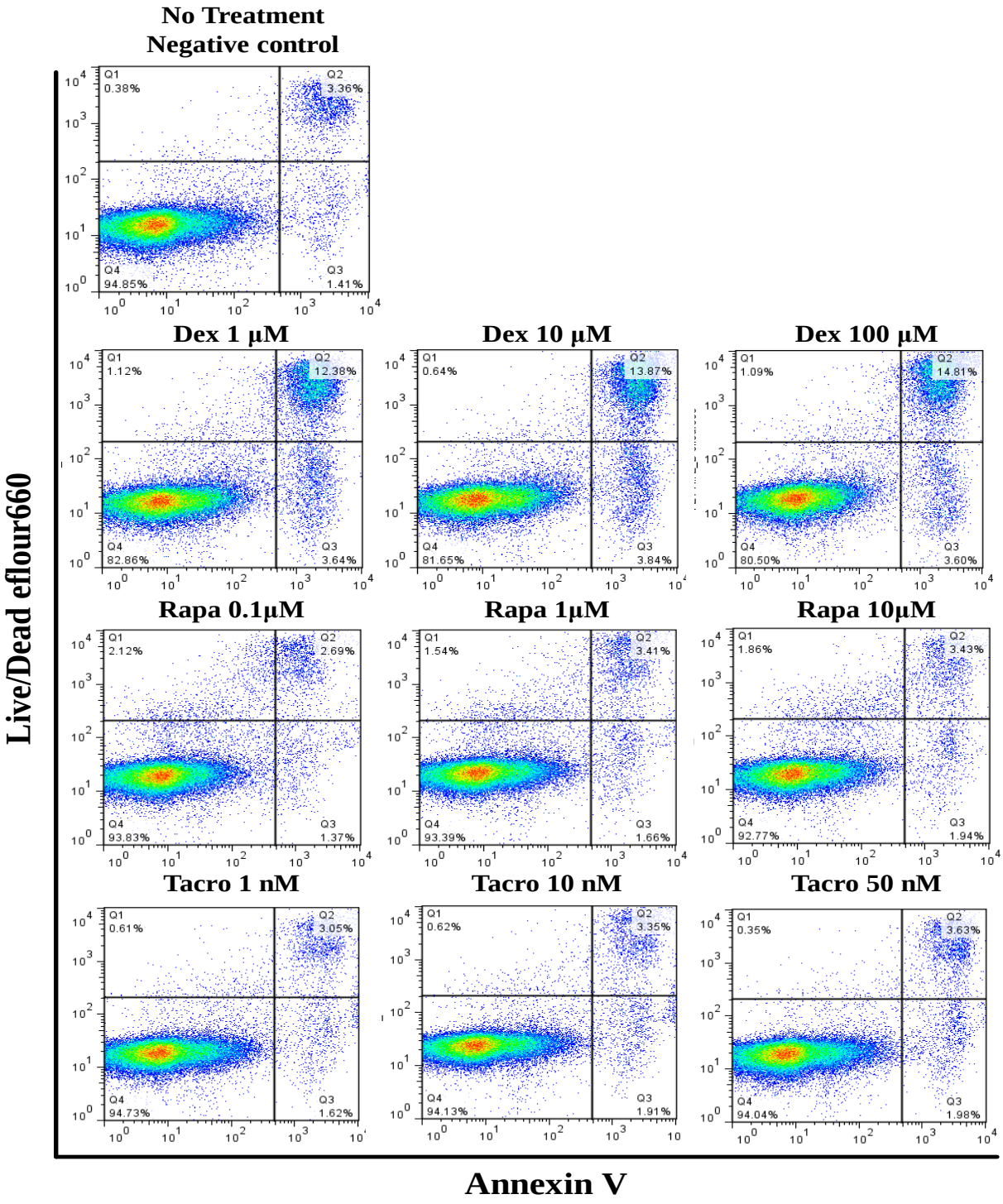


Figure 4.1: A representative drug titration (apoptosis experiment)(n=3) Fresh PBMCs were cultured for 5 days in the presence or absence of different concentrations of immunosuppressive drugs. Dotplots showing induction of apoptosis in $CD4^+$ T cells in response to different concentrations of dexamethasone, rapamycin and tacrolimus. Apoptosis was measured by Annexin V and Live/Dead staining (eflour660).

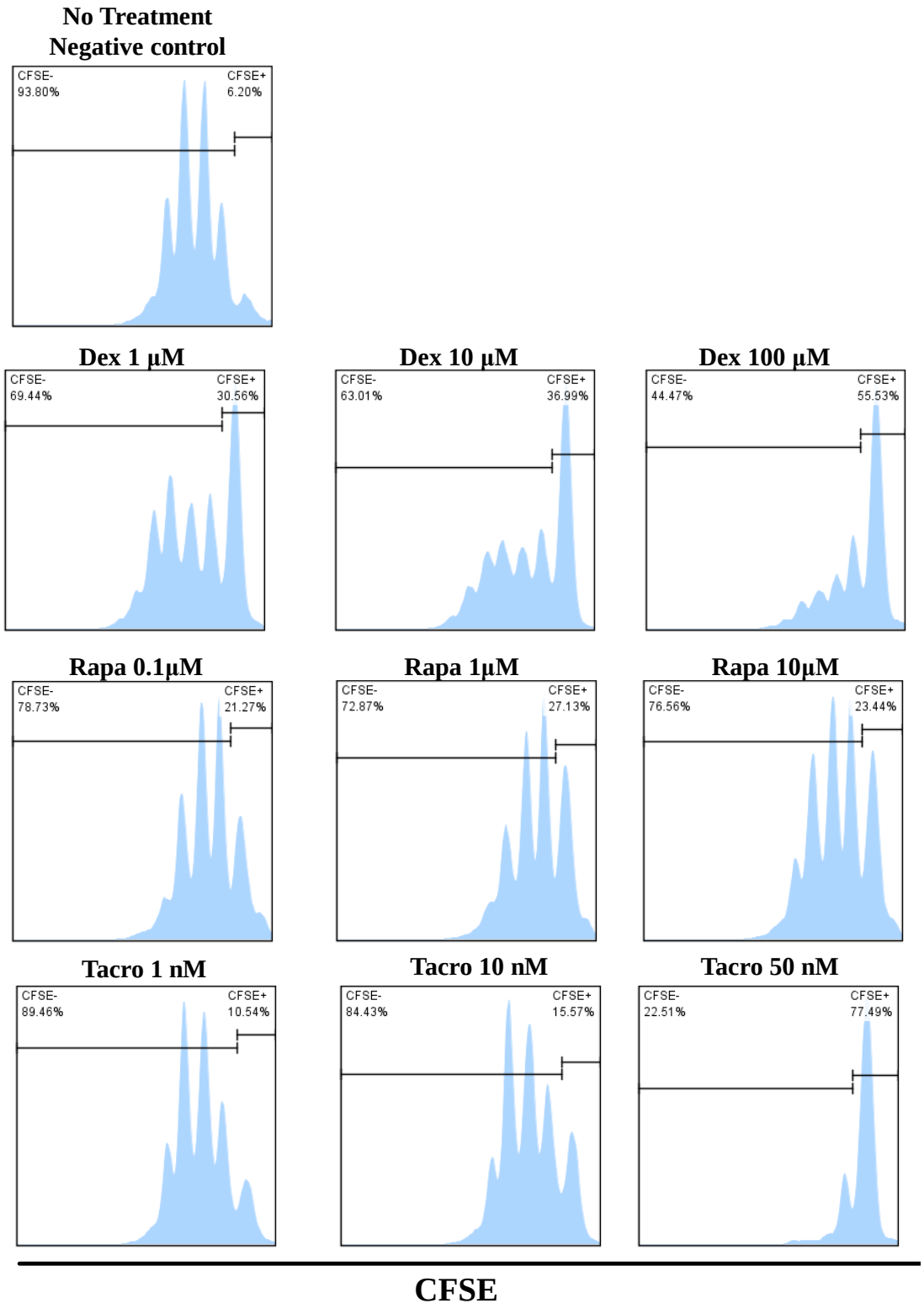


Figure 4.2: A representative drug titration (proliferation experiment) (n=3). Fresh PBMCs were cultured for 5 days in presence or absence of different concentrations of immunosuppressive drugs. Histograms showing the proliferation of $CD4^+$ T cells using CFSE staining in presence or absence of different concentrations of dexamethasone, rapamycin and tacrolimus.

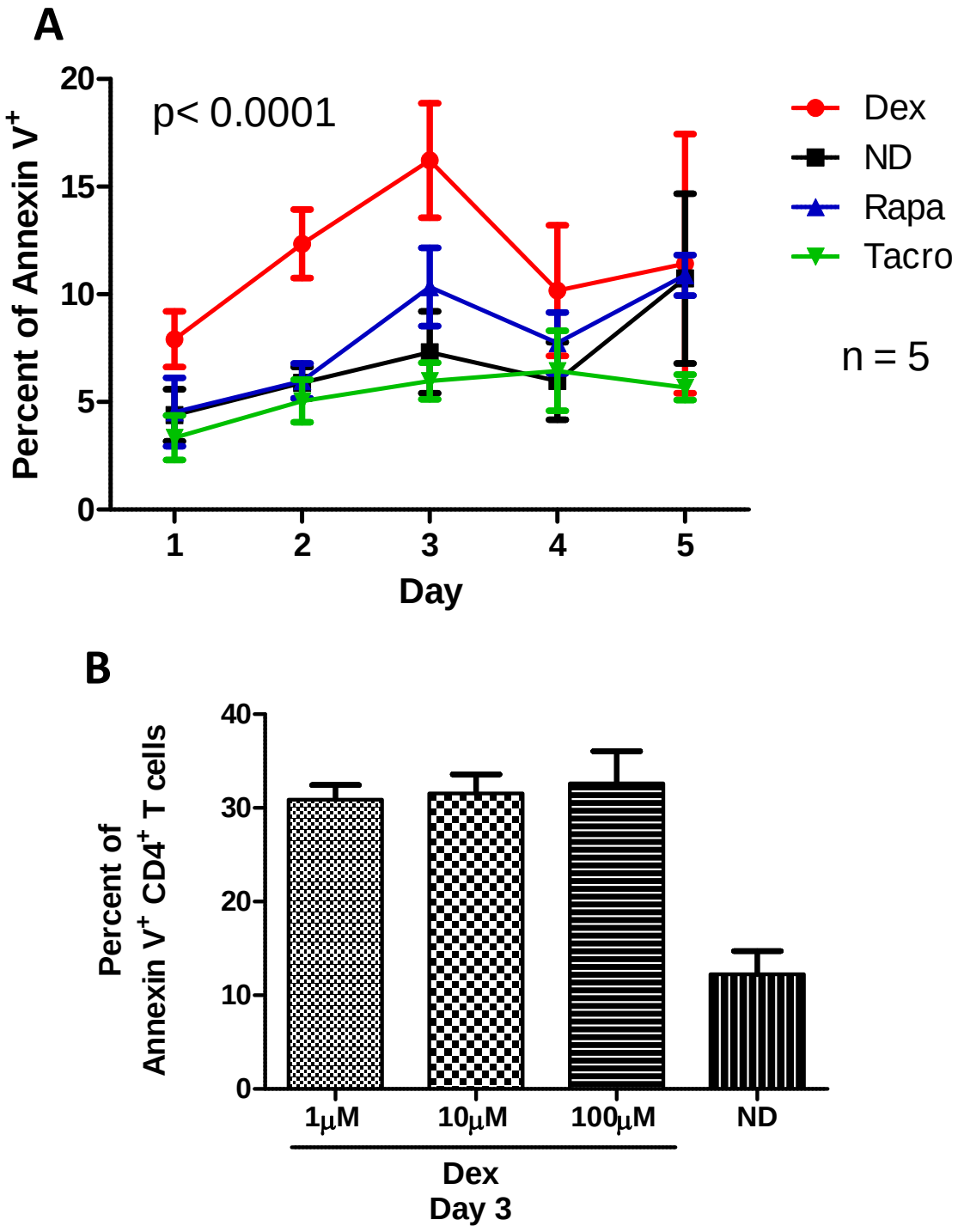


Figure 4.3: Apoptosis induction by drug treatment (n=5) A, PBMC were cultured for 5 days in the presence or absence of dexamethasone (**red line**), rapamycin (**blue line**), tacrolimus (**green line**) or no drug (**black line**) and apoptosis was evaluated using Annexin V staining. Results are presented as mean ±SEM. Two way ANOVA was used **(*p*= <0.0001)**. **B,** Bar chart showing Annexin V^+ apoptotic cells within $CD4^+$ T cells after three days of culture with or without dexamethasone at different concentrations; all 3 concentrations of dexamethasone induced apoptosis of CD4+ T cells (***p*=ns**). (***p*=ns**).

4.2.3 $CD161^{+}CD4^{+}$ T cells are more resistant to drug-induced apoptosis than $CD161^{-}$ T cells.

In the previous chapter (**section 3.2.4),** I showed that $CD161^{+}CD4^{+}$ T cells are capable of effluxing drugs through the ABC multidrug efflux proteins. This property gives this subset of $CD4^{+}$ T cells the ability to survive the lymphocytotoxic effect of some chemotherapeutic agents such as daunorubicin. To investigate if immunosuppressive drugs also act as ABCB1 substrates, I examined the ability of $CD161^{+}CD4^{+}$ T cells to survive the lymphocytotoxic effect of these drugs. Dexamethasone induced significant apoptosis of unstimulated $CD4^{+}$ T cells, measured by Annexin V staining compared with the negative control (16.2% ± 2.6% vs. 7.3% ± 1.9%, **p=0.034**) **(Figure 4.4 A)**. However, $CD161^{+}CD4^{+}$ T cells appeared to exhibit more resistance to the lymphocytotoxic effect of dexamethasone compared to their $CD161^{-}$ counterpart, although the difference did not reach statistical significance(9% ± 1% vs. 11% ± 1.1%, p= **0.092**) **(Figure 4.4 B).** In contrast, tacrolimus and rapamycin did not induce significant apoptosis in $CD4^{+}$ T cells compared to the negative control (6% ± 0.8% vs. 7.3% ± 1.9%; **p=0.55**and 10.3% ± 1.8 vs. 7.3% ± 1.9%; **p=0.3**, respectively) **(Figure 4.4 A)**.

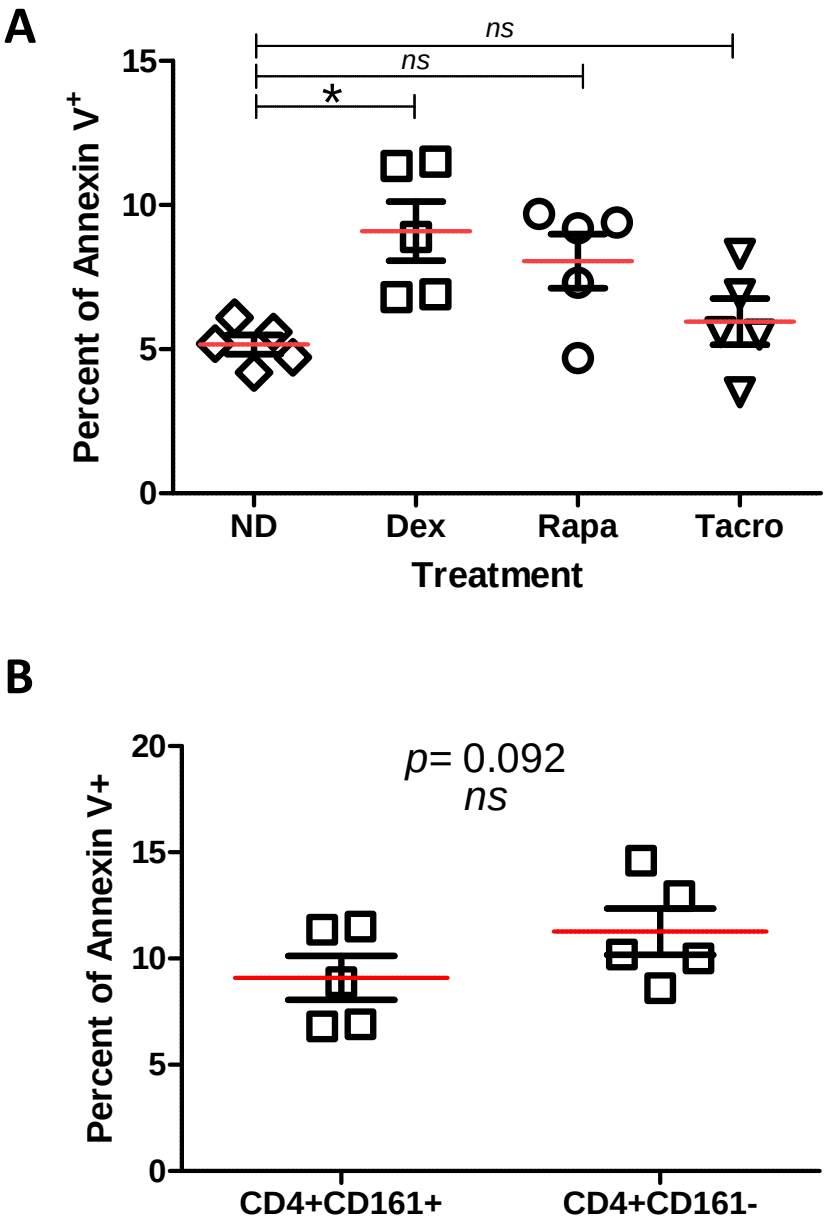


Figure 4.4: Percentage of Annexin V$^+$ apoptotic cells (n=5). A, Dot chart showing Annexin V$^+$ apoptotic cells within CD4$^+$ T cells in the presence or absence of dexamethasone **(* *p*=0.034)**, rapamycin and tacrolimus**. B,** Dot chart showing the difference in Annexin V percentage (apoptosis) between CD161$^+$CD4$^+$ T cells and their CD161$^-$ counterparts in response to dexamethasone (***p*=0.092**). One way ANOVA was used to test significance.

4.2.4 $CD161^{+}CD4^{+}$ T cells proliferate preferentially in the presence of Dexamethasone and Tacrolimus, but not Rapamycin

I next examined the impact of dexamethasone, tacrolimus and rapamycin on the frequencies of $CD161^{+}CD4^{+}$ and $CD161^{-}CD4^{+}$T cells following anti-CD3/CD28 stimulation. I calculated the percentage increase in$CD161^{+}CD4^{+}$ T cells following stimulation using the following formula:

$$\boldsymbol{x} = \left[\left(\frac{\%\ \boldsymbol{of\ CD4 + CD161 + at\ Day5}}{\%\ \boldsymbol{of\ CD4 + CD161 + at\ \ Day\ 0}}\right)\boldsymbol{x100}\right] - \mathbf{100}\%$$

A selective expansion of$CD161^{+}CD4^{+}$ T cells was observed when stimulated with anti-CD3/28 in the presence of dexamethasone when compared to negative (no drug) control (percentage increase 31.3% ± 17.8 vs. -21% ± 7.9%, ***p*= 0.018**). Likewise, in the presence of tacrolimus I saw a selective induction of$CD161^{+}CD4^{+}$ T cells (percentage increase 23.6% ± 3% vs. -21% ± 7.9%, ***p*= 0.0001**). In contrast, I did not observe a significant impact of rapamycin on the proliferation of $CD161^{+}CD4^{+}$ T cells in culture (percentage increase -15% 6.8% vs. -21% 7.9%,***p*=ns**) (**Figure 4.5A and B).**

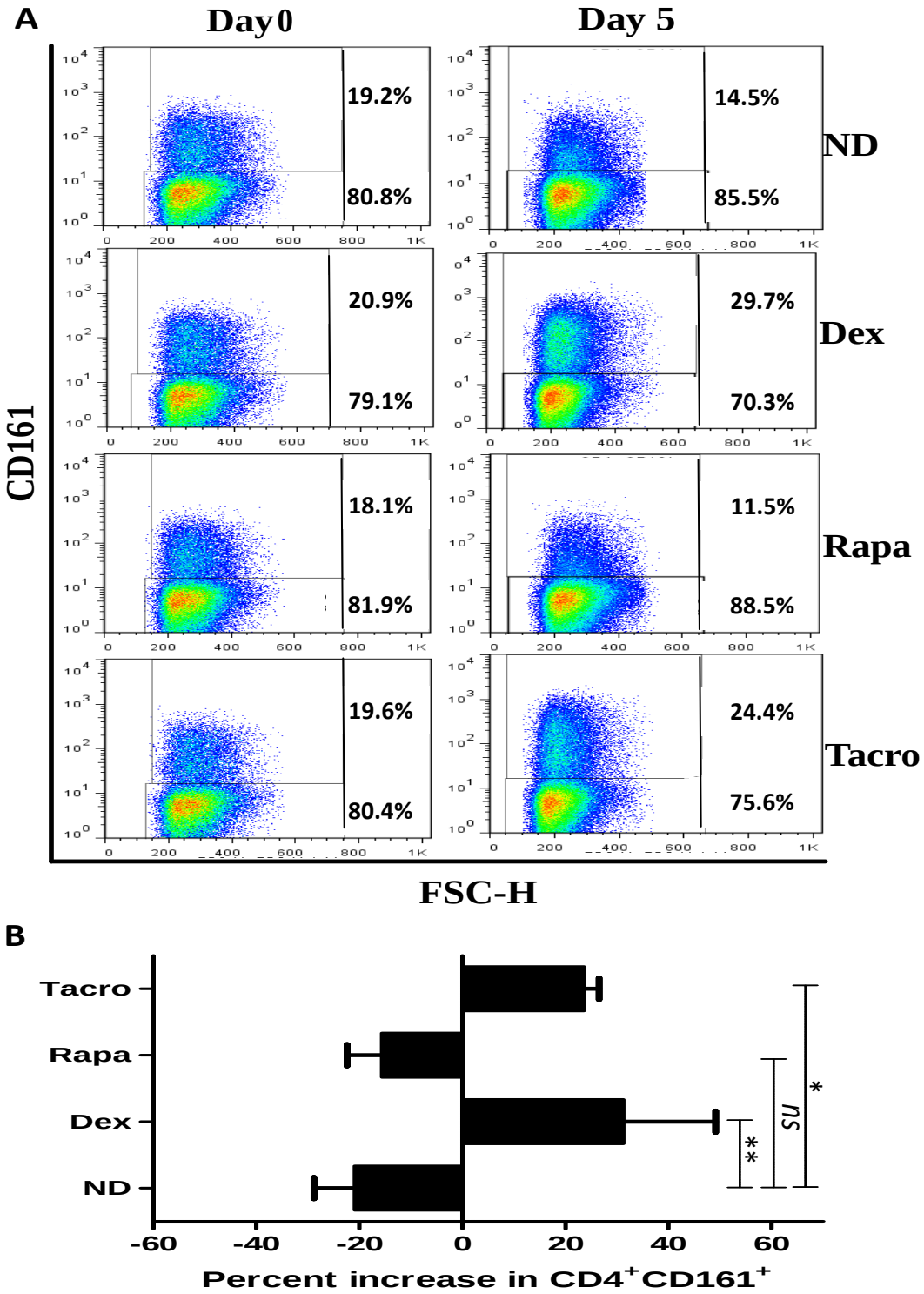


Figure 4.5: Representative experiment of $CD161^{+}CD4^{+}$ T cells expansion (n=8). PBMC were stimulated with anti-CD3/28 beads for 5 days in the presence or absence of dexamethasone, rapamycin and tacrolimus**. A,** Dot plots showing the percentage of $CD161^{+}CD4^{+}$ T cells after 1 and 5 days of culture in the presence or absence of dexamethasone, rapamycin and tacrolimus. Percentage increase was calculated by dividing the percentage of $CD161^{+}CD4^{+}$ T cells at day 5 (**right panel**) by the frequencies of $CD161^{+}CD4^{+}$ T cells at baseline (**left panel**). **B,** Bar chart showing the percentage increases in $CD161^{+}CD4^{+}$ T cells in response to drugs treatment. Results were shown as mean ± SEM. One way ANOVA was used.

I next investigated if $CD4^+CD161^+$ T cells have a greater ability to survive and expand in the presence of immunosuppressive drugs compared to their $CD161^-$ counterpart. I therefore calculated the $CD161^+CD4^+$ T cell proliferation ratio for each condition by dividing the percentage of $CD4^+CD161^+CFSE^{lo}$ cells by $CD4^+CD161^-CFSE^{lo}$ at day 5 in the presence or absence of the drug. In keeping with previously published data (Turtle et a. 2009), I found that in the absence of drugs, $CD4^+CD161^+$ T cells stimulated with anti-CD3/CD28 have an inherently slower proliferative response than $CD4^+CD161^-$ T cells **(section 3.2.6)**, i.e. they are more quiescent. However, in the presence of dexamethasone, $CD161^+CD4^+$T cells proliferated more significantly than $CD161^-$ $CD4^+$T cells compared to no drug control (1.17 ± 0.15 vs. 0.85 ± 0.08, **p= 0.045**). This phenomenon was even more pronounced in the presence of tacrolimus(1.26 ± 0.14, **p= 0.015**) but not rapamycin (0.9 ± 0.04, **p=0.2**) **(Figure 4.6 A&B)**, suggesting that $CD161^+$ $CD4^+$ T cells, through their rapid effluxing properties, may have the ability to resist these drugs in vitro.

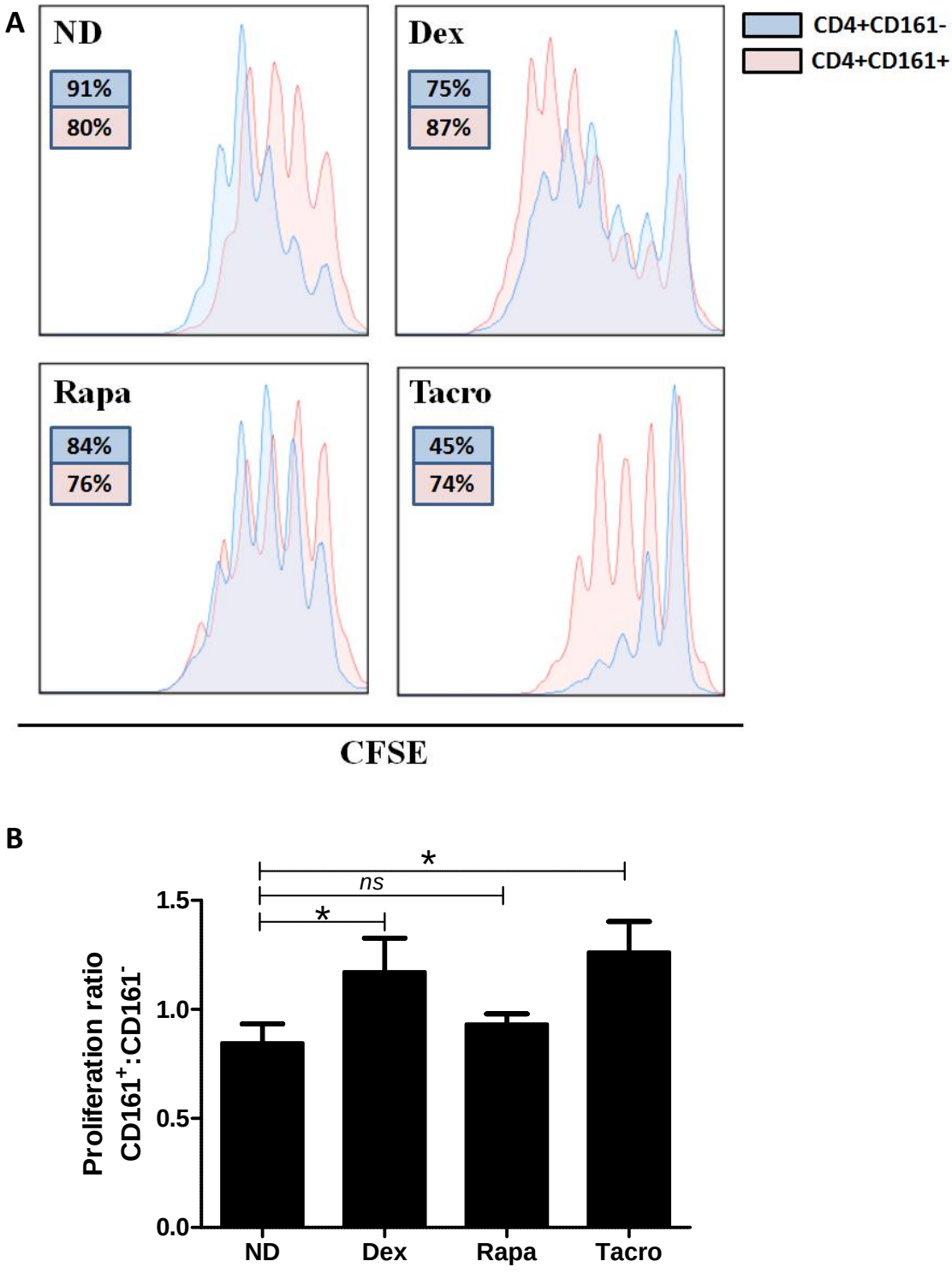


Figure 4.6: Representative $CD4^+$ T cells proliferation assay (n=5).A, Histograms showing the difference in proliferation between of $CD4^+CD161^+$ (Pink) and $CD161^-$ (Blue) T cells in absence (ND) or presence of dexamethasone (Dex), rapamycin (Rapa) and tacrolimus (Tacro). Proliferation ratio was calculated by dividing the percentage of $CD161^+CFSE^-$ by $CD161^-CFSE^-$. **B,** Bar chart showing the difference in proliferation ratio in presence or absence of dexamethasone, rapamycin and tacrolimus. Results were shown as mean ± SEM. One way ANOVA was used to test significance.

4.2.5 IFNγ-production $CD161^{+}CD4^{+}$ T cells selectively expand in the presence of Rapamycin

The role of IFNγ-producing T cells (helper T cells type1 "Th1") in GvHD has been contradictory (Ferrara, Levine, Reddy, & Holler 2009;Yang et al. 1998). However, their contribution to the onset of acute GvHD has been well described(Ferrara and Reddy 2006;Fowler et al. 1994;Nikolic et al. 2000). Takahashi and colleagues have recently shown that $CD4^{+}$ T cells co-expressing $CD161^{+}$ produce more IFNγ(Takahashi, Dejbakhsh-Jones, & Strober 2006). Therefore, I examined whether the proliferating population of $CD161^{+}CD4^{+}$ T cells also produce more IFNγ in the presence of Dex, Tacro or Rapa.

To address this, PBMC were stimulated with anti-CD3/28 beads in the presence or absence of the selected drugs for 5 days. The kinetics of IFNγ-producing$CD161^{+}CD4^{+}$ T cell expansion in the presence or absence of dexamethasone, rapamycin and tacrolimus were assessed on a daily basis **(Figure 4.7B)**. The fold increase in IFNγ-producing$CD161^{+}CD4^{+}$ T cell was calculated by dividing the percentage of $IFN\gamma^{+}CD161^{+}CD4^{+}$ T cells at day 5 by $IFN\gamma^{+}CD161^{+}CD4^{+}$ T cell at baseline. Treatment with dexamethasone did not have a significant impact on the expansion of $IFN\gamma^{+}CD161^{+}CD4^{+}$ T cells compared to no drug control (Fold increase0.9 ± 0.09 vs. 1.24 ± 0.16, ***p*=0.12**). As expected, tacrolimus impaired IFNγ production by $CD161^{+}CD4^{+}$ T cells compared to controls (Fold increase 0.3 ± 0.07 vs. 1.24 ± 0.16, ***p*=0.0001**). Rapamycin on the other hand, facilitated the selective expansion of IFNγ-producing$CD161^{+}CD4^{+}$ T cells(Fold increase1.8 ± 0.17 vs. 1.24 ± 0.16, ***p*= 0.027**) **(Figure 4.7 A&C)**.

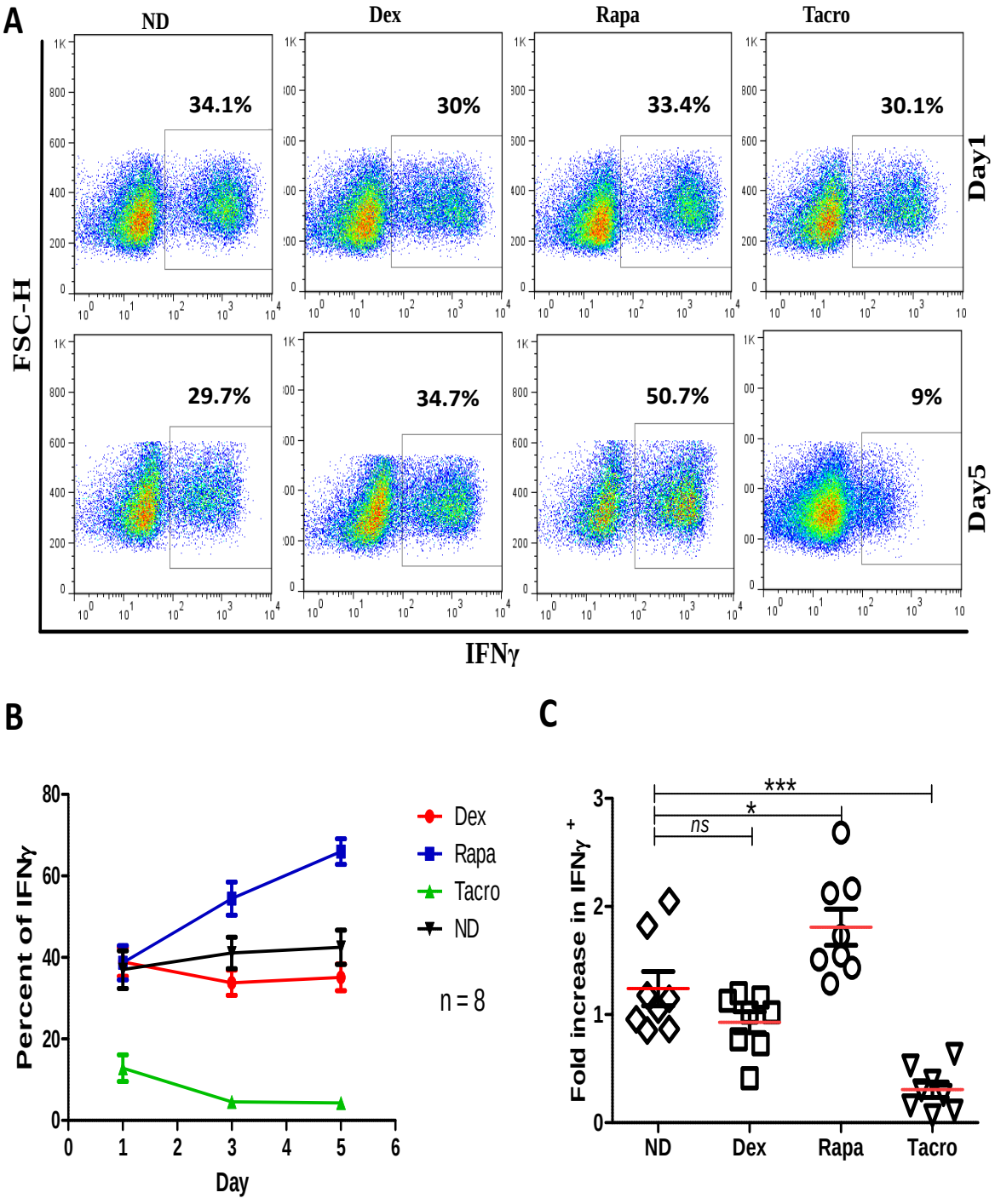


Figure 4.7:IFNγ production by CD161$^+$CD4$^+$ T cells in the presence or absence of dexamethasone, rapamycin and tacrolimus (n=8).A, Dot plots showing the percentage of CD4$^+$CD161$^+$ IFNγ$^+$ T cells after 1 and 5 days of culture. Fold increase was calculated by dividing the percentage of CD4$^+$CD161$^+$IFNγ$^+$ at day 5 (**lower panel**) by CD4$^+$CD161$^+$IFNγ$^+$ at baseline (pre-expansion) (**upper panel). B,** Line chart showing the time-based change in the percentage of CD4$^+$CD161$^+$IFNγ$^+$ T in response to drug treatment. **C,** Dot chart showing the fold increase in IFNγ-producingCD161$^+$CD4$^+$ T cells in presence or absence of GvHD drugs. Results are shown as mean ± SEM. Two and one way ANOVA was used to test significance.

4.2.6 IL-17 producing CD161+CD4+ T cells selectively expand in the presence of Dexamethasone and Rapamycin, but not Tacrolimus

$CD161^{+}CD4^{+}$ T cells were recently shown to be the precursor of IL-17-producing cells, (Th17)(Cosmi et al. 2008). Additionally, recent studies have discussed the role of Th17 cells in GvHD pathogenesis(Bossard et al. 2012;Dander et al. 2009;Ratajczak et al. 2010). Therefore, I examined whether the significant increase in the frequencies of $CD161^{+}CD4^{+}$ T cells in the presence of dexamethasone and tacrolimus, is associated with an increase in IL-17 production.

The frequency of IL-17-producing $CD161^{+}CD4^{+}$ T cells was measured using intracellular flow cytometry. **Figure 4.8 A&B** shows the sequential change in the frequencies of IL-17-producing $CD161^{+}CD4^{+}$ T cells *in vitro* following anti-CD3/CD28 stimulation in the presence or absence of immunosuppressive drugs. I calculated the fold increase in IL-17-producing $CD161^{+}CD4^{+}$ T cells after stimulation with anti-CD/CD28 beads for 5 days compared to baseline. Interestingly, there was a significant fold increase in IL-17-producing $CD161^{+}CD4^{+}$ T cells in the presence of dexamethasone compared to no drug control (5.2 ± 0.7 vs. 1.9 ± 0.3, **p=0.0008**).

Similarly, there was a significant fold increase in IL-17-producing $CD161^{+}CD4^{+}$ T cells in the presence of rapamycin compared to no drug control (4.4 ± 0.9 vs. 1.9 ± 0.3, **p=0.022**). In contrast, tacrolimus impaired IL-17 production in $CD161^{+}CD4^{+}$ T cells compared to the no drug control group (0.37 ± 0.07vs. 1.9 ± 0.3, **p=0.0002**) **(Figure 4.9)**. These data suggest that IL-17 producing CD4+CD161+ T cell have to ability to proliferate and expand in the presence of immunosuppressive and lymphocytotoxic agents such as rapamycin and corticosteroids.

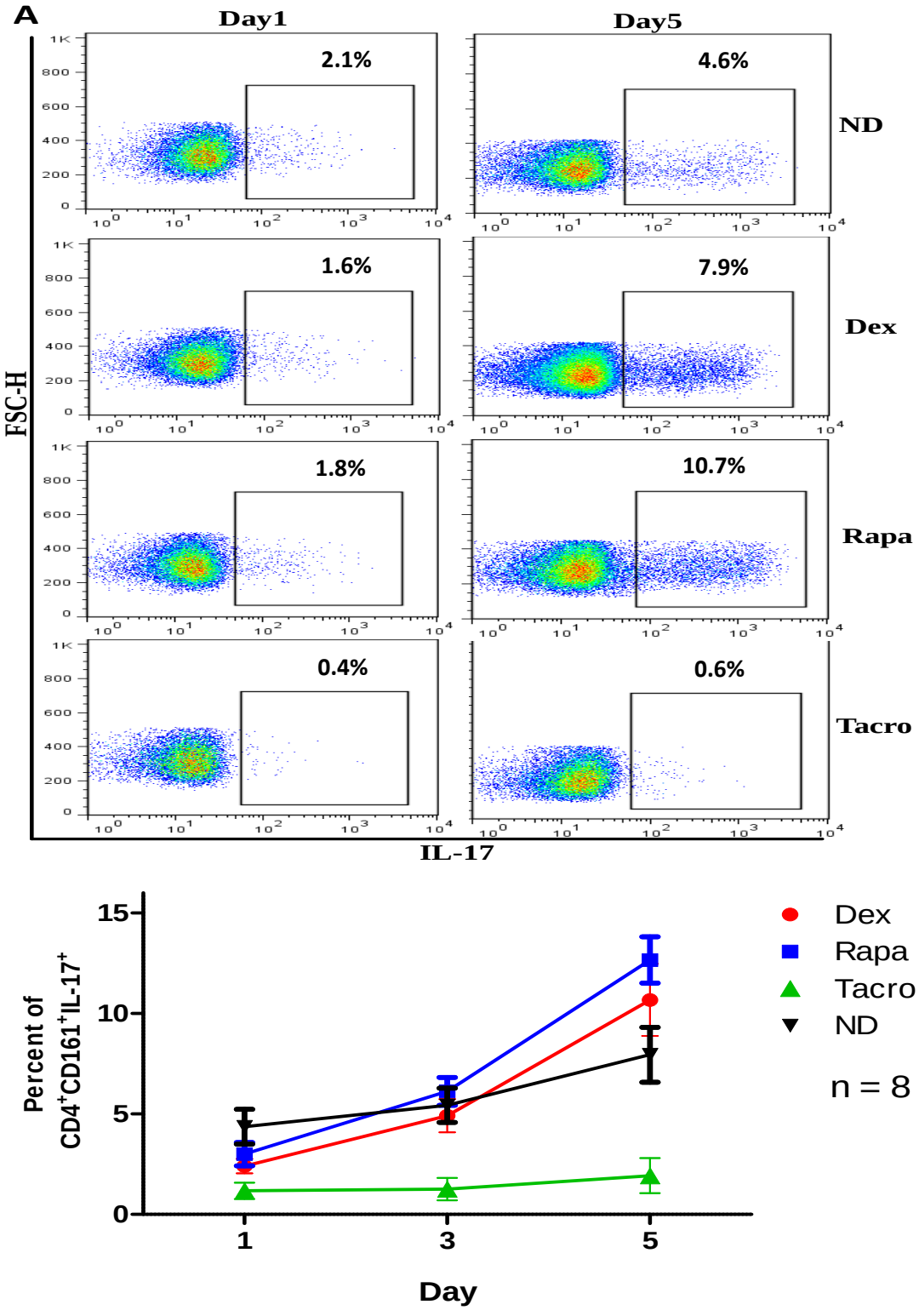


Figure 4.8IL-17 production by CD161$^+$CD4$^+$ T cells in the presence or absence of dexamethasone, rapamycin and tacrolimus (n=8). A, Dot plots showing the percentage of CD4$^+$CD161$^+$IL-17$^+$ T cells after 1 and 5 days of culture. Fold increase was calculated by dividing the percentage of CD4$^+$CD161$^+$IL-17$^+$ T cells at day 5 **(right panel)** CD4$^+$CD161$^+$IL-17$^+$ T cells percentage at baseline (Day 0) **(left panel). B,** Line chart showing the kinetics of CD4$^+$CD161$^+$IL-17 T cell expansion in response the presence or absence of dexamethasone, tacrolimus or rapamycin. Results are shown as mean ± SEM. Two way ANOVA was used to test significance.

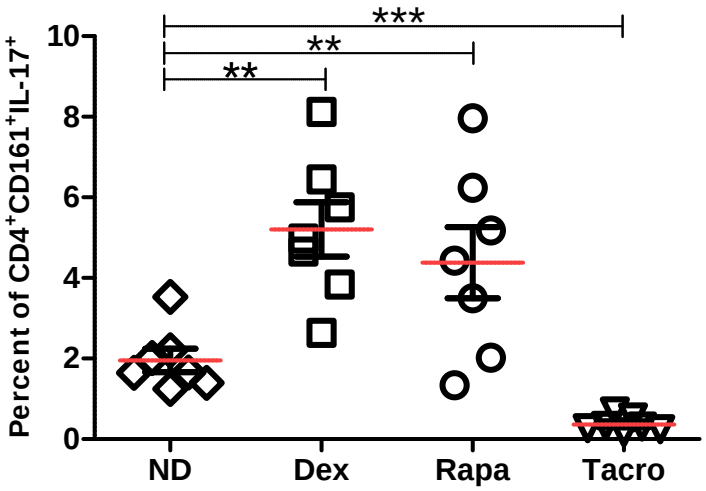


Figure 4.9Fold increase in IL-17-producing CD161$^+$CD4$^+$ T cells(n=7). Dot chart showing the fold increase in IL-17-producing CD161$^+$CD4$^+$ T cells in presence or absence of dexamethasone, rapamycin and tacrolimus. Results are shown as mean ± SEM. One way ANOVA was used to test significance.

4.2.7 Dexamethasone induces a reduction in CD4$^+$Foxp3$^+$ regulatory T cells

Regulatory T cells have indisputably established their role in immune tolerance. In AHSCT, for instance, Foxp3$^+$ Treg has been demonstrated in several human and murine studies to play a key role in preventing GvHD occurrence **(section 1.5).**In addition, disruptions in the ratio of Treg/Teff correlate with GvHD onset and severity.

The preferential expansion of CD4$^+$CD161$^+$IL-17-producing T cells in the presence of dexamethasone led me to question whether this might impact on the balance between Th17 and Treg cells. To address this question, the frequencies of CD4$^+$Foxp3$^+$ Tregs were evaluated, in the presence or absence of dexamethasone, tacrolimus and rapamycin **(Figure 4.10).**

The fold increase in $CD4^+Foxp3^+$ T cells was calculated by dividing $CD4^+Foxp3^+$ T cells at day 5 by frequencies at baseline. After 5 days of culture, the frequencies of $CD4^+Foxp3^+$ T cells did not change significantly in the absence of co-incubation with immunosuppressive drugs. Co-incubation with rapamycin had only a minor impact on $CD4^+Foxp3^+$ T cells compared to no drug control (0.72 ± 0.07 vs. 1.3 ± 0.08, ***p*=ns**). In contrast, in the presence of dexamethasone there was a significant reduction in the frequencies of $CD4^+Foxp3^+$ T cells compared to no drug control (fold increase 0.09 ± 0.01 vs. 1.3 ± 0.08, ***p*< 0.0001**).Likewise, tacrolimus reduced the frequencies of $CD4^+Foxp3^+$ T cells (fold increase 0.53 ± 0.13 vs. 1.3 ± 0.08, ***p*< 0.05**)**(Figure 4.11)**. This reduction in Treg frequencies is likely due to dexamethasone-induced apoptosis of Tregs. These data suggest that dexamethasone may have a detrimental effect on CD4+Foxp3+ T cells *in vitro* and possibly *in vivo*.

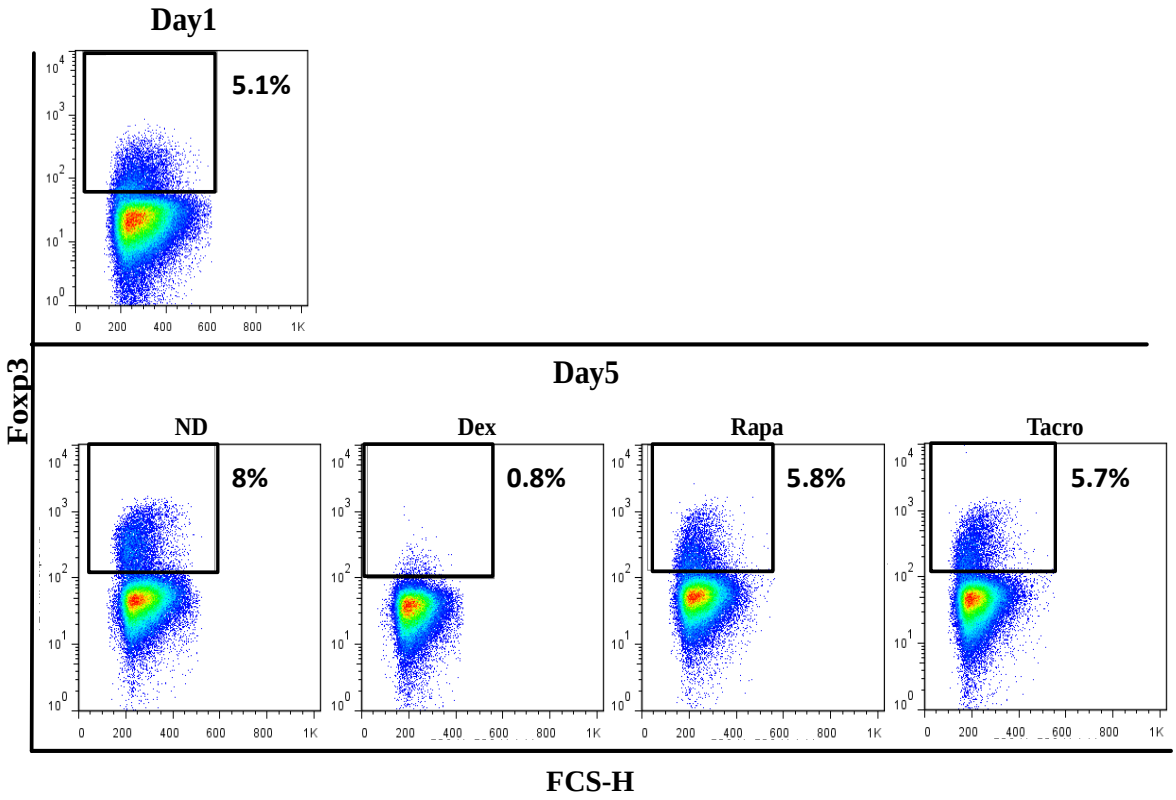


Figure 4.10: Representative FACS plot depicting Foxp3 expression on total $CD4^+$ T cells. Dot plots showing the percentage of $CD4^+Foxp3^+$ T cells after 1 and 5 days of *in vitro* culture with or without dexamethasone, rapamycin and tacrolimus.

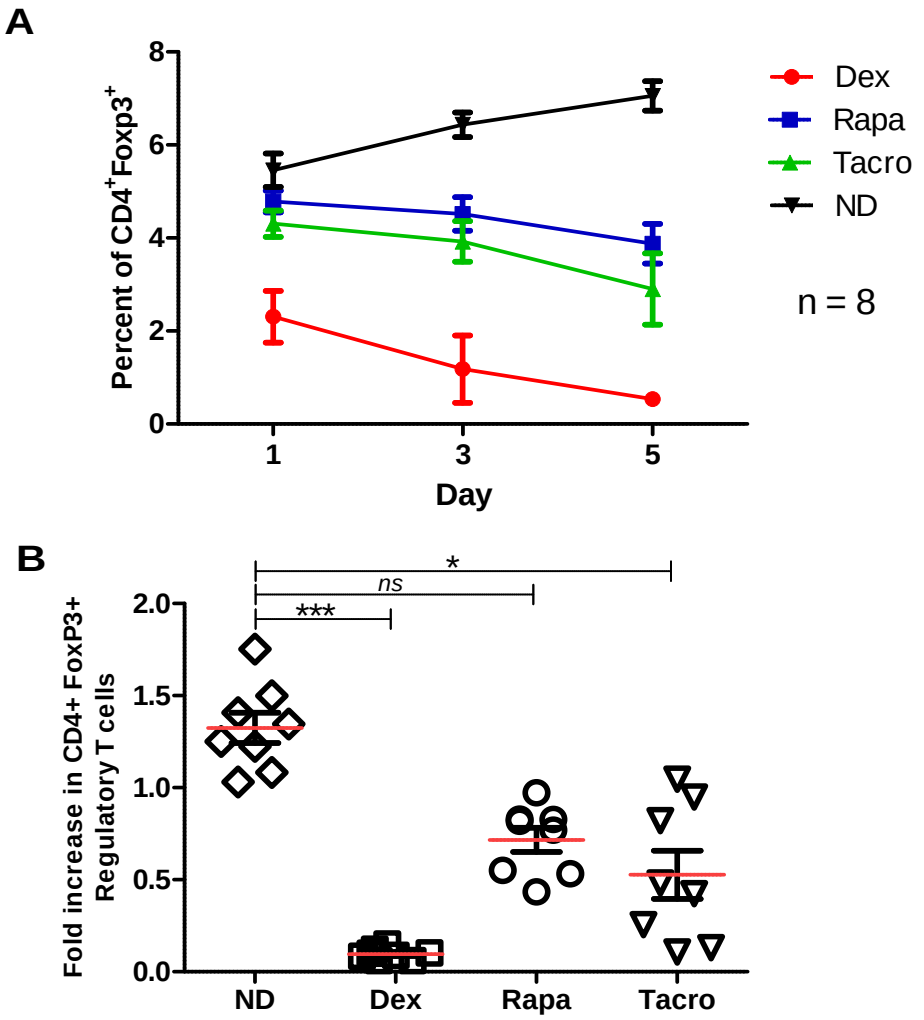


Figure 4.11:Expression of Foxp3 in the presence or absence of dexamethasone, rapamycin and tacrolimus (n=8). A, Line chart showing the time-based change in the percentage of $CD4^{+}Foxp3^{+}$ T cells presence of dexamethasone (**red line**), rapamycin (**blue line**), tacrolimus (**green line**) and no drug (**black line**). **B,** Dot chart showing the fold increase in the expression of Foxp3 on $CD4^{+}$ T cells in the presence or absence of dexamethasone, tacrolimus or rapamycin**.** Results were shown as mean ± SEM**.** Two and one way ANOVA were used to test significance, respectively.

4.2.8 ABCB1 and ABCC1 inhibitors synergise with Dexamethasone to induce apoptosis of $CD161^{+}CD4^{+}$ T cells and reduce IL-17 production

I have formerly described in **section 3.2.4** the drug-effluxing ability of $CD161^{+}CD4^{+}$ T cells through the ABCB1 transporter. Since dexamethasone is a substrate of ABCB1, I hypothesized that blocking these transporters using the previously mentioned ABC transporter inhibitors (cyclosporine, vinblastine and PK11195) may inhibit the efflux of dexamethasone by$CD161^{+}CD4^{+}$ T cells and induce their apoptosis. To test this hypothesis, PBMC were cultured for 1, 3 and 5 days in the presence or absence of dexamethasone with or without the ABCB1 and ABCC1 inhibitors. Cells were either un-stimulated or stimulated with anti-CD3/28 beads.

The percentage of apoptotic cells in $CD161^{+}CD4^{+}$ T cells in the different culture conditions was examined**.** As shown in **Figure 4.12**, whereas the specific inhibitors of ABCB1, PK11195 and cyclosporine, both failed to increase dexamethasone-induced apoptosis, the addition of vinblastine, a competitive inhibitor of ABCB1 and ABCC1, to the culture significantly enhanced dexamethasone-induced apoptosis after 5 days of co-culture, $\boldsymbol{p = 0.037}$, suggesting that both ABCB1 and ABCC1 may be involved in efflux of dexamethasone in $CD161^{+}$ $CD4^{+}$ T cells.

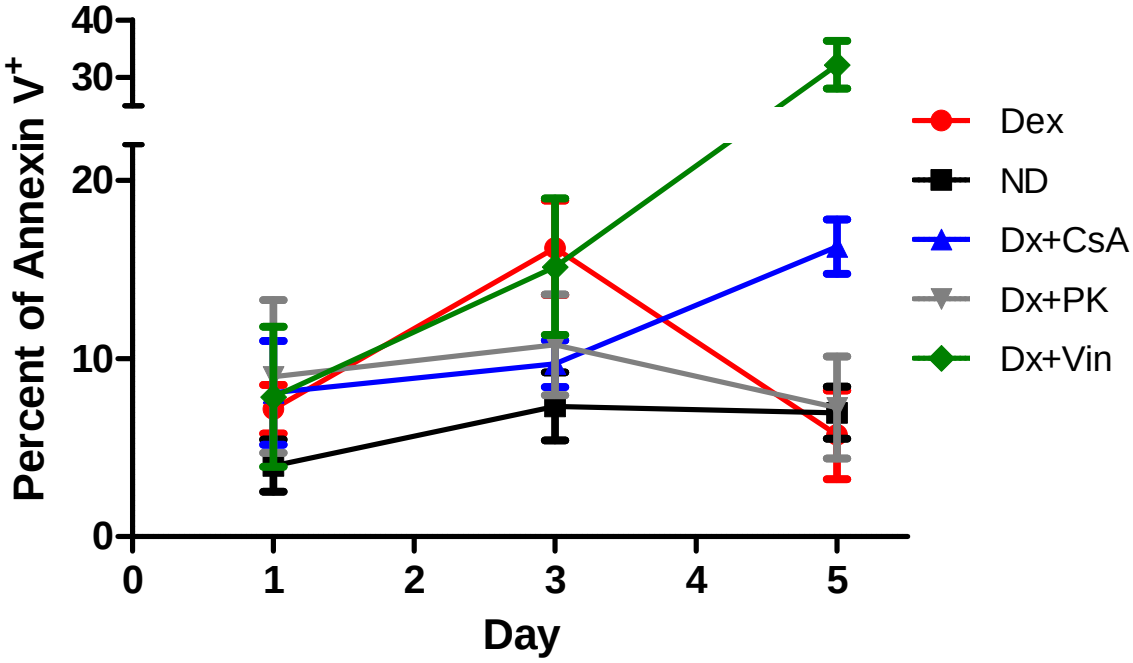


Figure 4.12:Apoptosis induction by drug treatment (n=3) Line chart showing the kinetics of drug-induced apoptosis in the absence(no drug, **black line**) or presence of dexamethasone (**red line**)with or without cyclosporine (CsA)(**blue line**), PK11195 (PK)(**grey line**) (ABCB1 inhibitor) and vinblastine (Vin)(**green line**) (ABCB1 and ABCC1 inhibitor). Results are presented as mean ± SEM. Two way ANOVA was used to test significance.

The ability of $CD161^{+}CD4^{+}$ T cells to proliferate and expand in the presence of dexamethasone was demonstrated earlier in **section 4.2.4**. I sought to examine if specific ABC inhibitors affect the ability of $CD161^{+}CD4^{+}$ T cells to proliferate in the presence of dexamethasone. As shown in **Figure 4.13**, combining dexamethasone with PK11195 or cyclosporine non-significantly reduced the ability of $CD161^{+}CD4^{+}$ T cells to expand in the presence of dexamethasone (20.8% ± 8.7 vs. 31.3% ± 17.8 with dexamethasone alone, **p= 0.36**) and (9% ± 6.1 vs. 31.3% ± 17.8, **p= 0.24**). The addition of vinblastine completely abrogated the ability of $CD161^{+}CD4^{+}$ T cells to

proliferate in the presence of dexamethasone(-24.5% ± 6.2 vs. 31.3% ± 17.8, **p=0.049**),supporting the notion that CD4+CD161+ T cells may overcome the immunosuppressive effect of dexamethasone by effluxing the drug through ABCB1 and ABCC1 transporters.

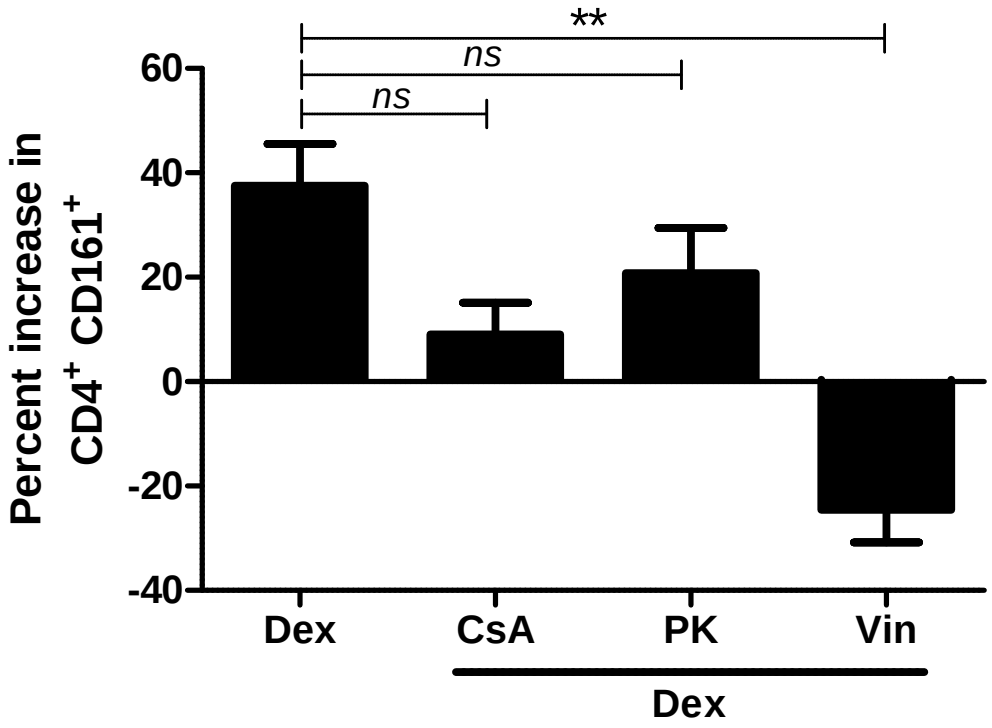


Figure 4.13:Change in the percentage of $CD4^+$ $CD161^+$ T cells in the presence of dexamethasone and ABC transporter inhibitors(n=3). Bar chart showing the percentage proliferating $CD161^+CD4^+$ T cells in co-culture in the presence of dexamethasone, with or without cyclosporine, PK11195 and vinblastine. Percent increase was calculated by dividing the percentage of $CD4^+CD161^+$ at day 5 by percentage of $CD161^+CD4^+$ T cells at day 0. Results were presented as mean ± SEM. One way ANOVA was used to test significance.

I next examined if the addition of specific ABC inhibitors to dexamethasone can suppress IL-17 producing by$CD161^+CD4^+$ T cells. Both vinblastine and cyclosporine inhibited the ability of $CD161^+CD4^+$ T cells to produce IL-17 in the presence of dexamethasone (0.51% ± 0.15and 0.6% ± 0.4, vs. 4.7% ± 0.77 with dexamethasone alone, **p= 0.010 and p= 0.012** respectively).

Similarly, PK11195 inhibited the ability of $CD161^{+}CD4^{+}$ T cells to produce IL-17 in the presence of dexamethasone; however insignificantly (2.1% ± 0.8 vs. 4.7% ± 0.77 with dexamethasone alone, **p= 0.09**) (**Figure 4.14)**.

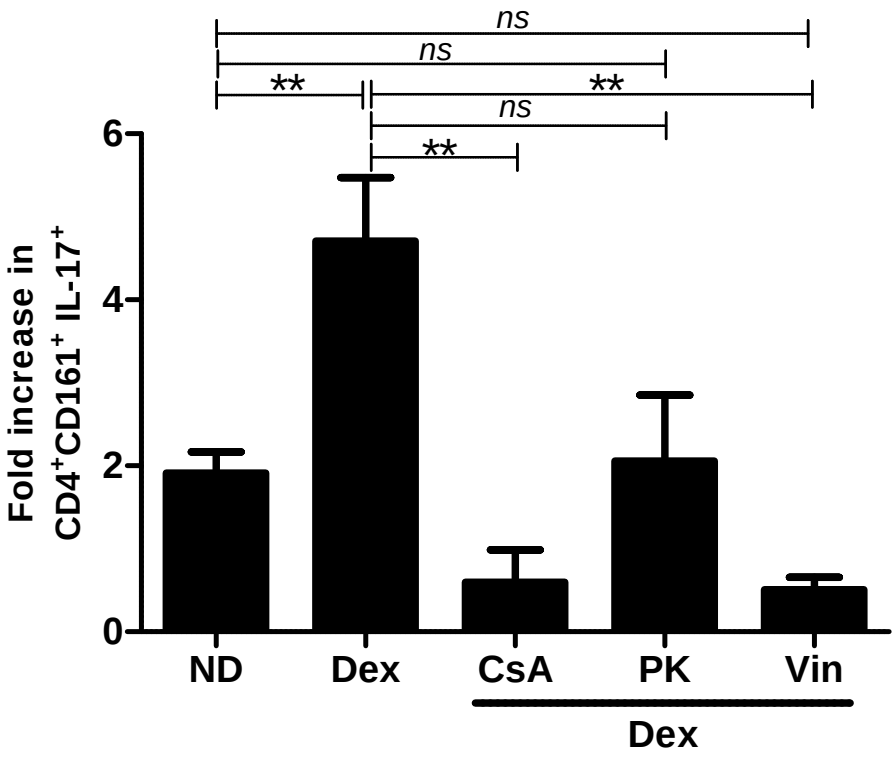


Figure 4.14:Fold increase in IL-17-producing $CD161^{+}CD4^{+}$ T cells(n=3). Bar chart showing the fold increase in IL-17-producing $CD161^{+}CD4^{+}$ T cells in presence or absence of dexamethasone with or without cyclosporine, PK11195 and vinblastine. Percent increase was calculated by dividing the percentage of $CD4^{+}CD161^{+}$ IL-17^{+} at day 5 by $CD4^{+}CD161^{+}$ IL-17^{+} at day 0. Results are shown as mean ± SEM. One way ANOVA was used to test significance.

I showed in section **4.2.5** that there is no impact of *in vitro* culture with dexamethasone on the ability of $CD161^{+}CD4^{+}$ T cells to produce IFNγ (**Figure 4.7).** Similarly, there was no significant effect of ABC transporter inhibition on IFNγ-producing$CD161^{+}CD4^{+}$ T cells**(Figure 4.15)**.

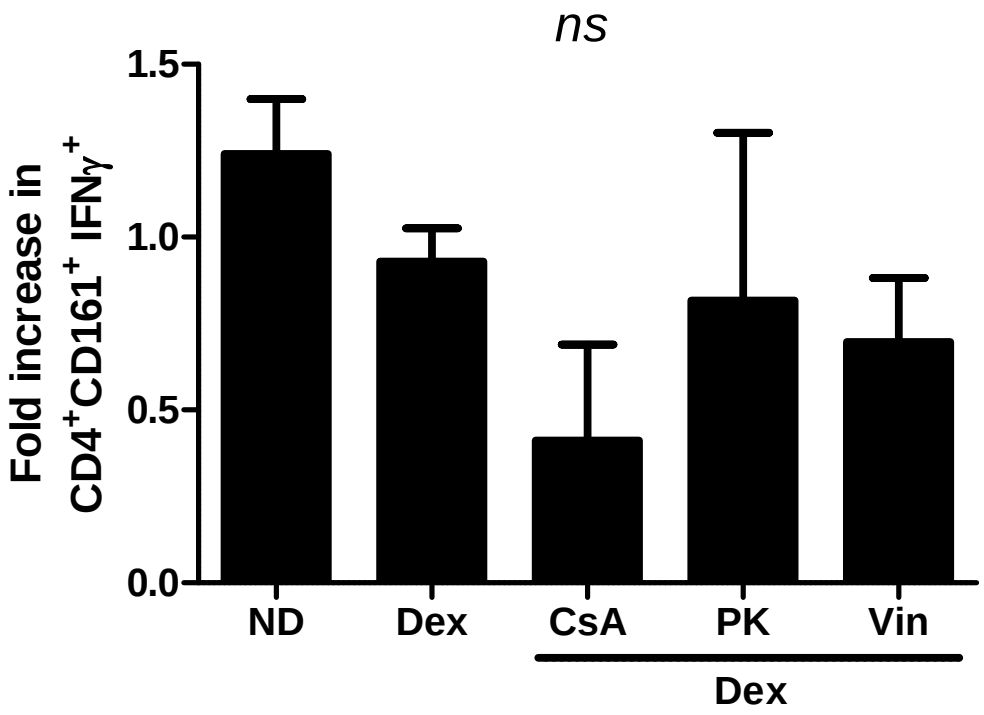


Figure 4.15:Fold increase in IFNγ-producing$CD161^{+}CD4^{+}$ T cells(n=3). Bar chart showing the fold increase in IFNγ-producing$CD161^{+}CD4^{+}$ T cells in presence or absence of dexamethasone with or without cyclosporine, PK11195 and vinblastine. Percentage increase was calculated by dividing the percentage of $CD4^{+}CD161^{+}$ $IFN\gamma^{+}$ at day 5 $CD4^{+}CD161^{+}$ $IFN\gamma^{+}$ at day 0. Results were shown as mean ± SEM. One way ANOVA was used to test significance.

Finally, I checked if dexamethasone-induced reduction of Tregs can be reversed by co-culture with ABC transporter inhibitors. PBMC were cultured in the presence of dexamethasone for 5 days with or without ABC inhibitors and the frequencies of Tregs were assessed by Foxp3 staining. The addition of cyclosporine A and PK11195 inhibitors (specific ABCB1 inhibitors) did not significantly affect the frequencies of Tregs compared to dexamethasone alone, suggesting that these cells may not have the machinery for rapid drug efflux. Similarly, the addition of vinblastine (ABCB1 and ABCC1 competitive inhibitor) to dexamethasone did not fully restore $Foxp3^{+}CD4^{+}$T cell frequencies to control levels (0.5 ± 0.2 vs. 1.32 ± 0.08, **p= 0.001**)**(Figure 4.16)**.

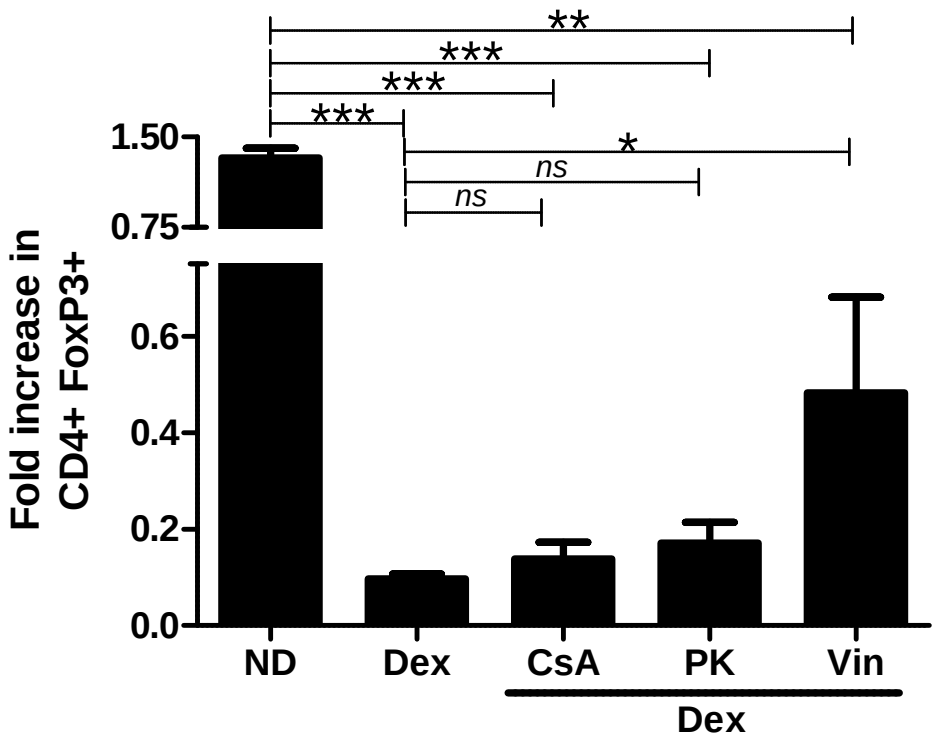


Figure 4.16:Expression of Foxp3 on $CD4^{+}$ T cells in the presence or absence of dexamethasone with or without cyclosporine, PK11195 and vinblastine. (n=3). Bar chart showing the fold increase in the expression of Foxp3 on $CD4^{+}$ T cells**.** Percent increase was calculated by dividing the percentage of $CD4^{+}Foxp3^{+}$ T cells at day 5 $CD4^{+}Foxp3^{+}$ T cells at day 0. Results are shown as mean ± SEM**.** One way ANOVA was used to test significance.

4.3 Discussion

Acute GvHD is the most frequent complication of AHSCT and it is mediated by the T cell content in the donor graft(Shlomchik 2007). A number of pharmacological approaches, mainly targeting T cells, have been employed to control this deleterious outcome(Dignan et al. 2012). In this study, I showed that, in keeping with the findings in **chapter 3**, $CD4^+CD161^+$ T cells can resist apoptosis induction by lymphocytotoxic agents *in vitro*. Notably, this resistance was associated with an alteration in their functional profile. The fact that these drugs are known to be substrates of ABC transporters may explain this phenomenon.

Corticosteroids remain the mainstay for the treatment of acute GvHD(Deeg 2007). A previous study reported the existence of a population of steroid-resistant $CD4^+$ T cells within the $CD4^+CD25^{int}$ T cell compartment. These cells had the ability to proliferate when co-cultured with steroids *in vitro*(Lee et al. 2007). In keeping with this study, I showed that whereas dexamethasone induced apoptosis and impaired $CD4^+$ T cell proliferation *in vitro,* a subset of $CD4^+$ T cells co-expressing CD161 exhibited more resistance to the lymphocytotoxic effect of dexamethasone *in vitro.*$CD161^+CD4^+$ T cells in my study had an effector memory phenotype (**Chapter 3**) and were enriched within the $CD25^{int}$ population. In accordance with these data, I herein further characterized the population of steroid resistant T cells, based on CD161 co-expression. The surviving $CD161^+CD4^+$ T cells were characterised by IL-17, but not IFNγ production, suggesting that these cells are in fact Th17 cells. As shown in **Chapter 3**, CD161 expression is a marker for rapidly-effluxing memory T

cells. Since dexamethasone is a substrate of the ABC transporter complex (Gottesman, Fojo, & Bates 2002), it is likely that the preferential survival and proliferation of $CD161^{+}CD4^{+}$ T cells in the presence of dexamethasone *in vitro* could be a consequence of rapid drug efflux by $CD4^{+}CD161^{+}$ T cells. Moreover, dexamethasone induced a reduction in the frequency of $CD4^{+}Foxp3^{+}$ regulatory T cells. These data further support my hypothesis that steroid refractoriness may be, at least partly, governed by the ability of a subset of Th17 cells to efflux steroids through the ABC transporter complex. As the majority of $CD4^{+}Foxp3^{+}$ T cells are $CD161^{-}$, they are unlikely to exhibit drug efflux ability, which may in turn explain their increased susceptibility to dexamethasone treatment *in vitro.* Based on my data and in keeping with previous studies, steroid treatment may affect the balance between Th17 and regulatory T cells. These data suggest a role of $CD4^{+}CD161^{+}$IL-17^{+} T cells in steroid-refractory GvHD.

Tacrolimus, a calcineurin inhibitor, is used for GvHD prophylaxis following allogeneic stem cell transplantation. In this study, I showed that tacrolimus inhibited the proliferation of $CD4^{+}CD161^{-}$ T cells to a greater extent than $CD4^{+}CD161^{+}$ T cells, suggesting that $CD161^{+}CD4^{+}$ T cells may efflux tacrolimus through the MDR pump. As expected, tacrolimus inhibited the production of IFNγ and IL-17 with no significant difference in the $CD161^{+}$ and $CD161^{-}$ populations. Unlike dexamethasone, tacrolimus did not affect the survival of $Foxp3^{+}$ Treg, indicating that a mechanism of action of this drug may be through shifting the balance of Th17/Treg ratio towards tolerance induction.

Rapamycin has been used as a second line treatment for steroid-refractory GvHD with some success. In my study rapamycin inhibited proliferation of $CD161^+$ and $CD161^-$ $CD4^+$ T cells to the same extent, suggesting that rapamycin may not be effluxed to the same extent by $CD4^+CD161^+$ T cells. Furthermore, rapamycin did not impact on Treg frequencies *in vitro*. A previous study suggested that rapamycin favours the expansion of regulatory T cells and prevents the differentiation of T cells towards a Th17 phenotype(Battaglia, Stabilini, & Roncarolo 2005;Coenen et al. 2007;Kopf et al. 2007). Altogether, these data suggest that rapamycin may exert activity in aGvHD through maintenance of regulatory T cells and inhibiting the proliferation of $CD161^+CD4^+$ T cells.

Blocking ABC transporters has been exploited as a strategy to overcome the resistance of cancer cells to chemotherapeutic agents(Walter et al. 2004). Here, I used inhibitors of ABCB1 and ABCC1 to minimize the efflux of dexamethasone by $CD4^+CD161^+$ T cells, and hence, overcome the steroids refractoriness of these cells. The addition of these inhibitors resulted in a reduction in IL-17 production from$CD4^+CD161^+$ T cells. Whereas the specific inhibitors of ABCB1, PK11195 and cyclosporine, both failed to increase dexamethasone-induced apoptosis, the addition of vinblastine, a competitive inhibitor of ABCB1 and ABCC1, to the culture significantly enhanced dexamethasone-induced apoptosis after 5 days of co-culture, suggesting that both ABCB1 and ABCC1 may be involved in dexamethasone efflux by $CD4^+CD161^+$ T cells. Interestingly, the addition of inhibitors did not reverse dexamethasone-induced reduction in the frequencies of regulatory T cells, suggesting that these cells may not have the machinery for rapid drug efflux.

Overall, these data support the use of ABC transporters inhibitors in combination with steroids to overcome the resistance of $CD161^{+}CD4^{+}$ T cells to corticosteroids and to restore the effector/regulatory balance in an attempt to overcome steroid-refractory GvHD.

CHAPTER 5. RATIONALE FOR THE COMBINATION OF CORTICOSTEROIDS WITH CALCINEURIN AND mTOR INHIBITORS

5.1 Introduction

In **chapter 4**I studied the *in vitro* effect of immunosuppressive drugs namely, dexamethasone, tacrolimus and rapamycin, all of which are ABCB1 substrates, on T cell subsets**(Table 5.1).**

Parameter	Dexamethasone	Rapamycin	Tacrolimus
CD4+ CD161+ %	⬆⬆⬆	—	⬆⬆⬆
IL-17	⬆⬆⬆	⬆⬆⬆	⬇⬇⬇
IFNγ	—	⬆⬆⬆	⬇⬇⬇
FoxP3	⬇⬇⬇	⬇⬇	⬇⬇

Table 5.1: Summary of the effect of co-culture with dexamethasone, rapamycin and tacrolimus on the frequencies of $CD4^+$ $CD161^+$ T cells, Th17 and Tregs *in vitro.*

Tacrolimus minimizes the risk of graft rejection in solid organs transplantation (Anon 1994;Woodle et al. 1996) and is used for GvHD prophylaxis in stem cell transplantation(Nash et al. 1996). Tacrolimus is better tolerated than cyclosporine in the AHSCT setting as the latter appears to confer more nephro- and neuro-toxicity(Nash et al. 1992).

Corticosteroids are the standard first line treatment for acute GvHD(Deeg 2007). In clinical practice, aGvHD patients who fail to respond to steroids within three to five days of treatment are considered steroid-refractory. In fact, only 35% to 40% of patients with aGvHD will have a complete response to steroids, underscoring the need for developing alternative treatment strategies for steroid-refractory aGvHD(MacMillan et al. 2002;Martin et al. 1990;Van Lint et al. 2006). Concomitant tacrolimus and steroid treatment was reported to be beneficial, mainly, in patients with mild to moderate GvHD (Dignan et al. 2012). Tacrolimus was also shown to be effective for the treatment of steroid-refractory GvHD(Kanamaru et al. 1995;Koehler et al. 1995).

The mTOR inhibitor, rapamycin, also known as sirolimus, is an immunosuppressant that is used as a second line treatment for aGvHD if steroids are unsuccessful(Benito et al. 2001;Ghez et al. 2009;Hoda et al. 2010). Studies on sirolimus alone as a first line intervention for aGvHD have shown comparable responses to that achieved with high doses of steroids(Pidala et al. 2011).

A number of studies have also explored the combination of these drugs for GvHD prophylaxis. A recently published study showed the feasibility of combining prednisolone with tacrolimus and methotrexate, as graft-versus-host disease prophylaxis in non-T cell-depleted haplo-identical transplant recipients(Mochizuki et al. 2011). In this study, no patient experienced grade IV GvHD, while all patients who developed aGvHD responded to steroid treatment. Recently, the combination of sirolimus and tacrolimus with or without ATG showed promising results for GvHD prophylaxis(Khaled et al. 2012;Ringden et al. 2011).

These studies support a role for combining these drugs for the prevention or treatment of aGvHD *in vivo.*

The aim of this chapter was to investigate the *in vitro* effect of combining steroids (dexamethasone), calcineurin inhibitors (tacrolimus) and mTOR inhibitors (rapamycin) on proliferation and cytokine profile of $CD4^+CD161^+$ and frequencies of regulatory T cells ($CD4^+Foxp3^+$).

5.2 Results

5.2.1 Combinations of dexamethasone, tacrolimus and rapamycin induce variable effects on CD161^{+}CD4^{+} T cell proliferation *in vitro*

Using a CFSE assay, I assessed the proliferation and frequencies of CD161^{+}CD4^{+} T cells co-cultured *in vitro* in the presence of a combination of dexamethasone, tacrolimus or rapamycin. The percentage increase of CD161^{+}CD4^{+} T cells was calculated using the following formula:

$$\boldsymbol{x = \left[\left(\frac{\%\ of\ CD4 + CD161 + at\ Day5}{\%\ of\ CD4 + CD161 + at\ \ Day\ 0}\right) x100\right] - 100\%}$$

Firstly, I found that the combination of dexamethasone and tacrolimus inhibited the expansion of CD161^{+}CD4^{+} T cells significantly more than dexamethasone (percentage increase 7.4% ± 4.4% vs. 31.3% ± 17.7%., ***p*=0.05**) or tacrolimus alone (percentage increase 7.4% ± 4.4% vs. 23.6% ± 3%, ***p*= 0.009**) **(Figure 5.1A)**. This effect is largely due to the ability of this combination to control the proliferation of both CD161^{+} and CD161^{-} CD4^{+} T cells **(Figure 5.1B).**

Next, the combination of dexamethasone with rapamycin was assessed. This combination did not induce any significant drop in the frequencies of CD161^{+}CD4^{+} T cells compared to dexamethasone alone (percentage increase 20.5% ± 6.2% vs. 31.3% ± 17.7%, ***p*= 0.7**) although there were more proliferating CD161^{+}CD4^{+} T cells compared to cultures with rapamycin alone (percentage increase20.5% ± 6.2%vs. -15.6% ± 6.8%, ***p*= 0.015**) **(Figure 5.1 A&B)**.

Finally, I assessed the effect of combining tacrolimus and rapamycin on the frequencies and proliferation of $CD4^{+}CD161^{+}$ and $CD161^{-}$ T cells. This resulted in a significant drop in the frequencies of $CD161^{+}CD4^{+}$ T cells compared to tacrolimus alone (percentage increase -1.35% ± 7.7% vs. 23.6% ± 3%, ***p*=0.004**) **(Figure 5.1A)**. However, the impact of this combination on the proliferation of $CD161^{+}$ and $CD161^{-}$ $CD4^{+}$ T cells was not significant **(Figure 5.1B)**, suggesting that the drop in the frequencies resulted from both $CD161^{+}$ and $CD161^{-}$ proliferating at the same rate.

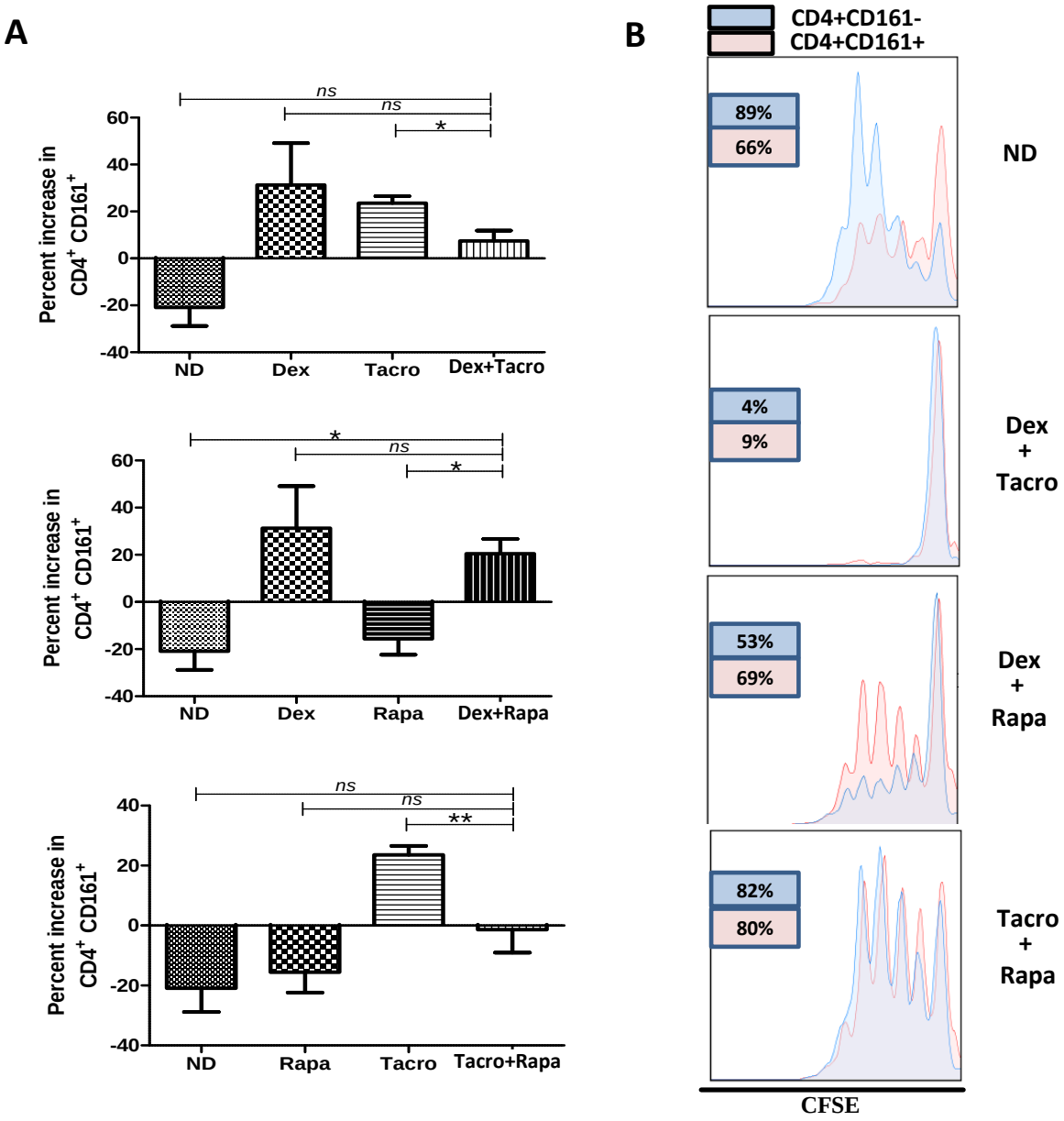


Figure 5.1:The effect of drug combinations on the viability and proliferation of CD161^{+}CD4^{+} T cells(n=3). A, Bar chart showing the percentage increase in CD161^{+}CD4^{+} T cells in response to drugs combinations. **B,** Histograms showing the difference in proliferation between CD4^{+}CD161^{+} (Pink) and CD161^{-} (Blue) T cells. Results are presented as mean ± SEM. One way ANOVA was used.

5.2.2 Combinations of dexamethasone, tacrolimus and rapamycin prevent IL-17 production by $CD4^+CD161^+$ T cells

In the previous chapter, I reported that tacrolimus successfully inhibits the production of IL-17 by $CD161^+CD4^+$ T cells, whereas rapamycin and dexamethasone fail to suppress IL-17 production by $CD161^+CD4^+$ T cells. Here, I evaluated the effect of combining dexamethasone, tacrolimus or rapamycin on $CD4^+CD161^+$IL-17-producing T cells. I calculated the fold increase in IL-17-producing $CD161^+CD4^+$ T cells by dividing the percentage of IL-$17^+CD161^+CD4^+$ T cells at day 5 by IL-$17^+CD161^+CD4^+$ T cells at baseline. I found that the combination of tacrolimus with dexamethasone resulted in a significant reduction in the percentage of IL-17-producing $CD161^+CD4^+$ T cells compared to dexamethasone alone (fold increase 0.05 ± 0.02 vs. 5.2 ± 0.7, ***p*=0.0013**). Similarly, the combination of rapamycin and dexamethasone resulted in a significant decrease in IL-17-producing $CD161^+CD4^+$ T cells(fold increase 2.75 ± 0.5%) compared to dexamethasone alone (fold increase 5.2 ± 0.7%; ***p*=0.03**) and rapamycin alone (fold increase 4.4 ± 0.9, ***p*= 0.14**). The frequency of IL-17-producing $CD161^+CD4^+$ T cells in response to this combination was comparable to the no drug control (fold increase 2.75 ± 0.5 vs. 1.9 ± 0.3%, ***p*=0.18**), (**Figure 5.2 B)**. Lastly, the combination of tacrolimus with rapamycin synergized to significantly lower the percentage of IL-17-producing $CD161^+CD4^+$ T cells compared to rapamycin alone or no drug control (fold increase 0.26 ± 0.07 vs. 4.4 ± 0.9; ***p*= 0.018** and 1.9 ± 0.3, ***p*=0.006,** respectively)**(Figure 5.2 C)**.

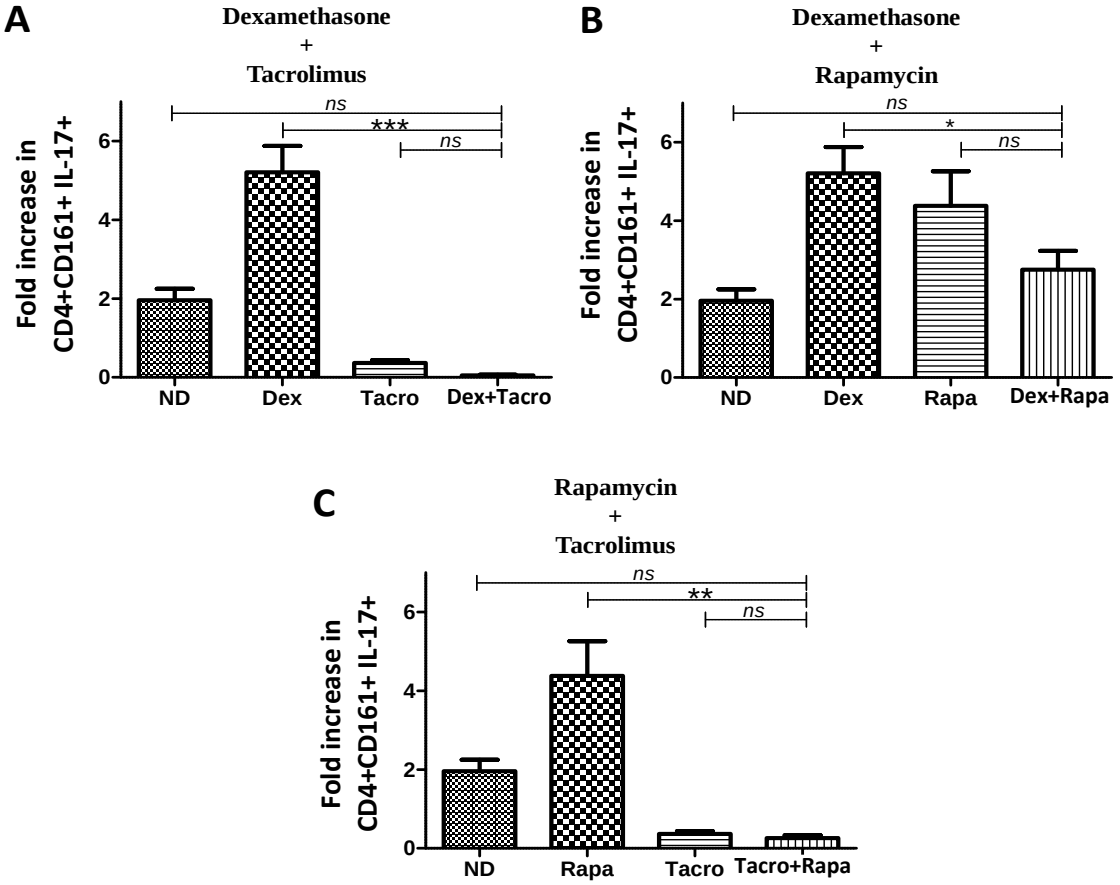


Figure 5.2:Percentages of IL-17-producing CD161$^+$CD4$^+$ T cells following co-culture with combinations of dexamethasone, tacrolimus or rapamycin (n=3). **A**, Bar chart showing the fold increase in IL-17-producing CD161$^+$CD4$^+$ T cells in response to Dex/Tacro **(A)**, Dex/Rapa **(B)** and Rapa/Tacro **(C)**. Fold increase was calculated by dividing the percentage of IL-17-producing CD161$^+$CD4$^+$ T cells at day 5 on theirs at the beginning of the experiment. Results were shown as mean ± SEM. One way ANOVA was used to test significance

5.2.3 Combinations of dexamethasone, tacrolimus and rapamycin prevent IFNγ production by $CD4^+CD161^+$ T cells

I demonstrated in the previous chapter that the frequencies of IFNγ-producing$CD161^+CD4^+$ T cells increase when co-cultured with rapamycin *in vitro*, but not with tacrolimus or dexamethasone **(section 4.2.3)**. Therefore, I evaluated the effect of combinations of dexamethasone, tacrolimus or rapamycin on $CD4^+CD161^+$IFNγ-producing T cells. I calculated the fold increase in IFNγ-producing$CD161^+CD4^+$ T cell by dividing the percentage of $IFN\gamma^+CD161^+CD4^+$ T cells at day 5 by $IFN\gamma^+CD161^+CD4^+$ T cell at baseline. In keeping with the findings outlined above, I found that the combination of tacrolimus with dexamethasone resulted in significant reductions in the percentage of IFNγ-producing$CD161^+CD4^+$ T cells compared to dexamethasone or no drug control (fold increase 0.06 ± 0.02 vs. 0.93 ± 0.09; ***p*= 0.0005**, and 1.24 ± 0.16 ***p*=0.002,** respectively)**.**

Next, I combined dexamethasone with rapamycin and found that, in contrast to co-culture with rapamycin alone where I reported a selective increase in IFNγ-producing$CD161^+CD4^+$ T cells(**section 4.2.3)**, the drug combination reduced IFNγ production in $CD161^+CD4^+$ T cells to a level similar to the no drug control (fold increase 1.27 ±0.2 vs.1.24 ± 0.16**, *p*=0.9**) **(Figure 5.3 B).** Similarly, combining tacrolimus with rapamycin reduced IFNγ production in $CD161^+CD4^+$ T cells compared to rapamycin alone (fold increase 0.59 ± 0.38 vs. 1.8 ± 0.17, ***p*=0.007**). **(Figure 5.3 C).**

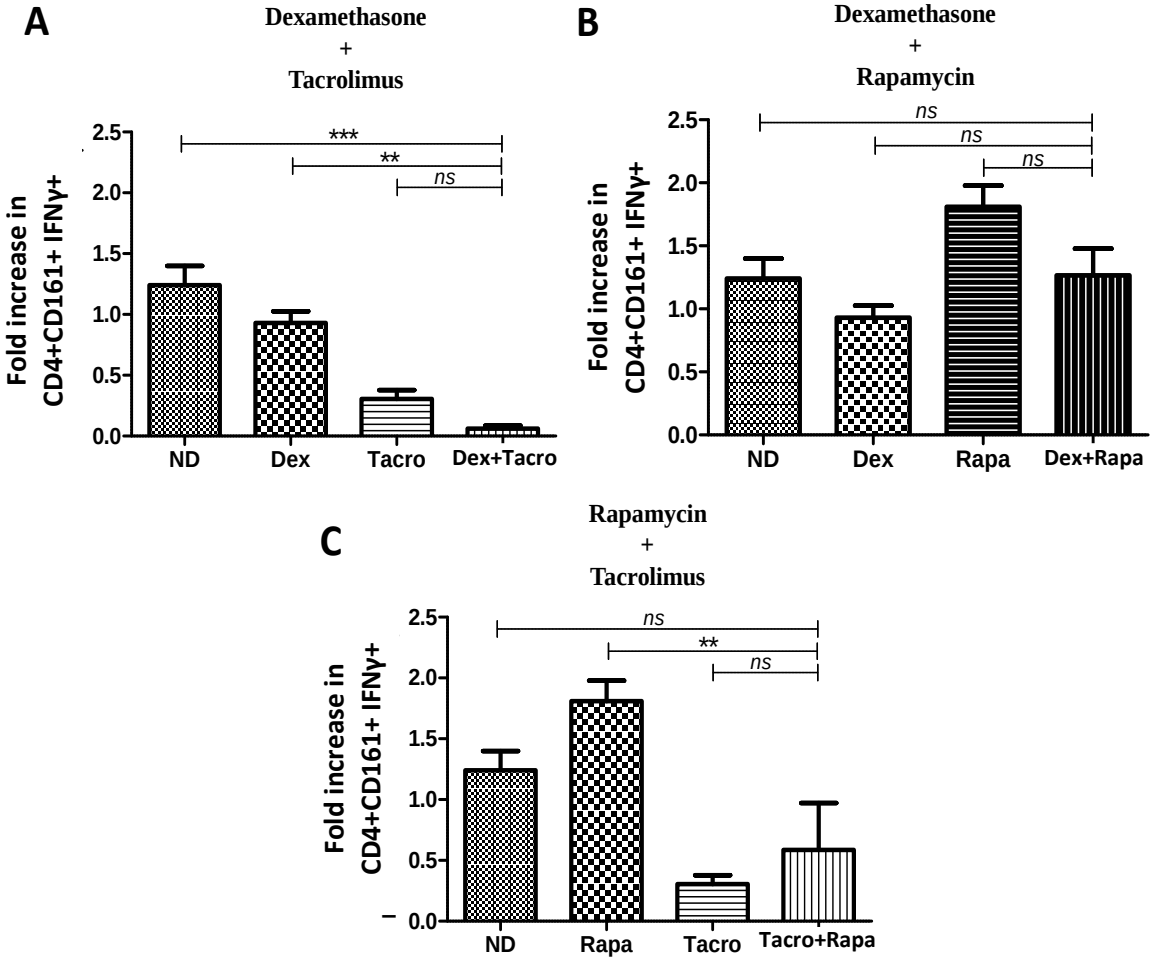


Figure 5.3: Frequencies of IFNγ-producingCD161$^+$CD4$^+$ T cellsfollowing co-culture with dexamethasone, tacrolimus or rapamycin (n=3). Bar charts showing the fold increase in IFNγ-producingCD161$^+$CD4$^+$ T cells in response to Dex/Tacro **(A),** Dex/Rapa **(B)** and Rapa/Tacro **(C).** Fold increase was calculated by dividing the percentage of IFNγ-producing CD4$^+$CD161$^+$ at day 5 by IFNγ-producing CD4$^+$CD161$^+$ at baseline. Results were shown as mean ± SEM. One way ANOVA was used.

5.2.4 The addition of tacrolimus or rapamycin to dexamethasone does not prevent dexamethasone-induced decrease in $CD4^+Foxp3^+$ regulatory T cell

I reported in section **4.2.6** that dexamethasone might induce apoptosis in regulatory ($Foxp3^+CD4^+$) T cells **(Figure 5.3)**. Here, I evaluated whether the addition of tacrolimus or rapamycin to dexamethasone will impact on $CD4^+Foxp3^+$ T cell frequencies *in vitro.* The fold increase in $CD4^+Foxp3$ T cells was calculated by dividing $CD4^+Foxp3^+$ T cells frequencies at day 5 by their frequencies at baseline.

The addition of tacrolimus or rapamycin to dexamethasone did not prevent dexamethasone-induced reduction in Tregs frequencies (fold increase in Treg frequencies following culture 0.07 ± 0.01 vs. 0.09 ± 0.1, ***p*=0.2)** and (0.1 ± 0.05 vs. 0.09 ± 0.1, ***p*=0.66**) respectively. Moreover, the combination of rapamycin and tacrolimus was also highly suppressive to Tregs compared to no drug control (fold increase 0.34 ± 0.12vs. 0.72 ± 0.06, ***p*=0.018)**.

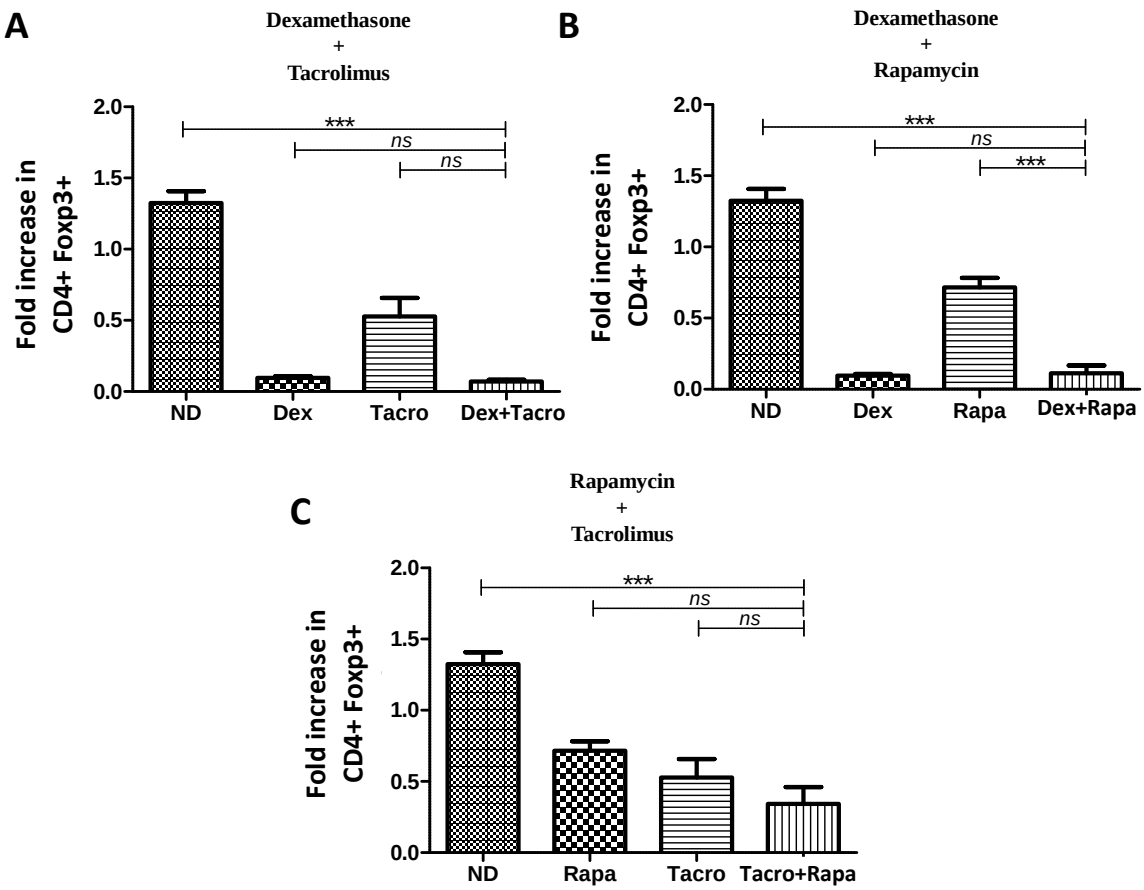


Figure 5.4:Frequencies of Foxp3$^+$ CD4$^+$ T cells in the presence of drugs alone or in combination (n=3). Bar charts show the fold increase in CD4$^+$Foxp3$^+$ T cells in response to dexamethasone/tacrolimus **(A)**, dexamethasone/rapamycin **(B)** and rapamycin/ tacrolimus **(C)**. Fold increase was calculated by dividing the percentage of CD4$^+$ Foxp3$^+$ at day 5 by frequencies at baseline. Results were shown as mean ± SEM. One way ANOVA was used.

5.3 Discussion

A number of different prophylactic regimens are used to prevent GvHD following allogeneic stem cell transplantation. However, in a significant proportion of patients, these regimens might fail to protect against GvHD(Deeg 2007). I demonstrated in **chapter 4** that $CD161^{+}CD4^{+}$ T cells might play a role in the pathogenesis of steroid-refractory aGvHD and also showed that the 3 different groups of immunosuppressive drugs (steroids, calcineurin inhibitors and mTOR inhibitors) have differential effects on $CD4^{+}CD161^{+}$ T cells. Therefore, I hypothesized that through their different mechanisms of action, combinations of these drugs may have a synergistic effect to suppress $CD4^{+}CD161^{+}$ T cells. Overall, I demonstrated in this study the advantage of combining dexamethasone with tacrolimus or rapamycin in suppressing $CD161^{+}CD4^{+}$ T cell proliferation and IL-17 production.

Clinical data supports the use of steroids in combination with calcineurin inhibitors(Dignan et al. 2012). In addition, Mochizuki et al recently showed that the addition of steroids to tacrolimus and methotrexate in the haplo-identical stem cell transplantation setting reduces the severity of GvHD and increases the response to steroids treatment(Mochizuki et al. 2011). In accordance with these studies, I showed that the combination of dexamethasone and tacrolimus effectively prevents the expansion of $CD4^{+}CD161^{+}$and $CD161^{-}CD4^{+}$T cells. Moreover, I also demonstrated that the addition of tacrolimus to dexamethasone significantly reduces the production of pro-inflammatory cytokines such as IL-17 and IFNγ by $CD4^{+}CD161^{+}$ T cells. Interestingly, the suppression of $CD161^{+}CD4^{+}$ T cell proliferation and cytokine production were not associated with increased apoptosis**.**

Interestingly, the combination of these drugs had deleterious effect on $Foxp3^+$ regulatory T cells. A number of studies have shown that Tregs appear to be highly sensitive to CD95L-induced apoptosis, in contrast to their more resistant Teff counterparts.(Fritzschinget al. 2005;Krammeret al. 2000). In vivo, the apparent selective sensitivity of Treg to CD95L might be a mechanism to eliminate Treg during the acute effector phase of an immune response at a time when Teff are resistant to CD95-mediated apoptosis. However, this same phenomenon may also contribute to, and exacerbate, GVHD.

Rapamycin has been reported to induce durable responses, similar to responses achieved with steroids when used as a “first-line” treatment for aGvHD(Pidala et al. 2011). Rapamycin has also been used as second line treatment for steroid-refractory aGvHD. However, the efficacy of rapamycin alone compared to rapamycin plus dexamethasone has not been formally addressed. In addition, the combination of dexamethasone and rapamycin has been shown to induce apoptosis in leukemic cells both *in vitro* and *in vivo*(Gu et al. 2010). In this study, I showed that the combination of dexamethasone and rapamycin induced apoptosis within the $CD161^+CD4^+$ T cell population. Although the drug combination did not significantly suppress the proliferation of $CD4^+CD161^+$ T cells, it inhibited the production of IFNγ and IL-17 by $CD4^+CD161^+$ T cells.

In these experiments, I also demonstrated that the combination of rapamycin and tacrolimus is highly effective at reducing the production of IFNγ and IL-17 by $CD4^+CD161^+$ T cells. However, this combination did not suppress the proliferation of $CD161^+$ and $CD161^-$ $CD4^+$ T cells.

These data support the use of combinations of immunosuppressive drugs with different modes of action for prophylaxis and the treatment of aGvHD.

CHAPTER 6. ASSESSMENT OF $CD161^{+}CD4^{+}$ T CELL FREQUENCIES AND FUNCTION IN PATIENTS WITH STEROID-REFRACTORY AND STEROID-RESPONSIVE ACUTE GvHD

6.1 Introduction

Acute GvHD is the most frequent complication after stem cell transplantation. Donor allo-reactive T lymphocytes play a significant role in its initiation and propagation(Shlomchik 2007). The occurrence of acute GvHD varies from10% to 80% according to risk factors and prophylaxis regimens used prior to SCT(Deeg 2007). High doses of systemic corticosteroids are the first-line therapy of choice for aGvHD (Dignan et al. 2012). However, steroid therapy fails to resolve GvHD in a significant proportion of patients(MacMillan et al. 2002;Martin et al. 1990;Van Lint et al. 2006). So far, the pathophysiology of steroid-refractory GvHD is not well defined.

The role of T cells in the refractoriness and resistance to steroids has been demonstrated in a number of autoimmune diseases. In keeping with the findings presented in **Chapter 4**, Lee and colleagues reported the presence of a subset of steroid-resistant $CD4^{+}$ T cells in uveitis patients.(Lee et al. 2009a)The same group in a separate publication showed that these cells have intermediate expression of CD25 ($CD25^{int}$)(Lee et al. 2007). Interestingly, mAbs against the IL-2 receptor (anti-CD25) have been used as second-line therapy in patients with refractory aGvHD, suggesting

that $CD4^{+}CD25^{int}$ T cells may play a role in the pathogenesis of steroid-refractory aGvHD (reviewed in (Dignan et al. 2012)).

It has been shown that steroids promote the switch from Th1 (IFNγ and IL-2) to Th2 (IL-4 and IL-10) phenotype through inhibition of IL-12 production by macrophages and dendritic cells(Blotta et al. 1997;Franchimont et al. 1998).Reports on the effect of steroids on Th17 cells have been conflicting ;a recent murine study of airway inflammation, reported that steroid resistance is mediated by Th17 cells(McKinley et al. 2008).

In this chapter I aim to examine the role of $CD161^{+}CD4^{+}$ T cells in steroid-refractory aGvHD by analysing the frequency and function of these cells in patients with steroid-refractory and steroid-responsive aGvHD. The hypothesis is that, in keeping with the *in vitro* experiments presented in **chapter 4,** $CD4^{+}CD161^{+}$ $IL\text{-}17^{+}$ T cells will be expanded in the peripheral blood of patients with steroid-refractory aGvHD compared to patients with steroid-responsive or no GvHD.

6.2 Results

6.2.1 Patients characteristics and timing of peripheral blood sampling

Twenty one patients who underwent haematopoietic stem cells transplantation at the Hammersmith hospital between 2009 and 2011 were studied. PBMC samples were collected pre- and post-transplantation at days 30, 45, 60, 90 and 120 days following stem cell infusion. PBMC were processed as outlined in section 2.2. PBMC samples collected just before or at the time of GvHD diagnosis and after or during steroid treatment were examined. Patients were divided into three groups; No acute GvHD (n=8), steroid-responsive (SRs) (n=6) and steroid-refractory acute GvHD (SRf) (n=7)**(Table 6.1)**. Acute GvHD was graded in the transplant clinic following the modified Seattle Glucksberg criteria.

	All (N=21)	No acute GvHD (N=8)	Steroid-responsive acute GvHD (N=6)	Steroic refracto acute GvHD (N=7)
	number (percent)			
Median age in years (range)	**49 (25-66)**	**50 (25-64)**	**60 (26-64)**	**47 (41-66**
Sex				
Male	15 (71.4)	6 (75)	4 (66.7)	5 (71.4
Female	6 (28.6)	2 (25)	2 (33.3)	2 (28.6
Diagnosis				
Acute leukaemia	5 (23.8)	3 (37.5)	1 (16.7)	1(14.3
Chronic myelogenous leukaemia	4 (19)	2 (25)	0 (0)	2 (28.6
Malignant lymphoma	9 (42.9)	2 (25)	4 (66.6)	3 (42.8
Others	3 (14.3)	1 (12.5)	1 (16.7)	1 (14.3
Conditioning regimen				
Myeloablative conditioning	7 (33.3)	1(12.5)	4 (66.7)	2 (28.6
Reduced intensity conditioning	14 (66.7)	7 (87.5)	2 (33.3)	5(71.4
Stem cell donors				
Related	7 (33.3)	2 (25)	2 (33.3)	3 (42.9
Unrelated	14 (66.7)	6 (75)	4 (66.7)	4 (57.1
HLA Match				
HLA-identical	20 (95.2)	7 (87.5)	6 (100)	7 (100
HLA-mismatched	1 (4.8)	1 (22.5)	0 (0)	0 (0)
Stem cell source				
Bone Marrow	1 (4.8)	1 (22.5)	0 (0)	0 (0)
Peripheral Blood Stem Cells	20(95.2)	7 (87.5)	6 (100)	7 (100
Post-transplant immunosuppressive prophylaxis				
Cyclosporine-Methotrexate	11(52.4)	4 (50)	0 (0)	6 (85.7
Cyclosporine-Methotrexate-ATG	7 (33.3)	2 (25)	5 (83.3)	1 (14.3
Cyclosporine-Methotrexate-Campath	3 (14.3)	2(25)	1 (16.7)	0 (0)
Acute GVHD				
Grades I-II	5(23.8)	0 (0)	4 (66.7)	1 (29)
Grades III-IV	7(33.3)	0 (0)	2 (33.3)	5 (24)

Table 6.1: Characteristics of stem cell transplantation patients. Patients were sub-divided according to GvHD occurrence and response to steroids**.**

6.2.2 $CD161^{+}CD4^{+}$ T cells are increased in the peripheral blood of patients with steroid-refractory aGvHD

To assess if$CD161^{+}CD4^{+}$ T cells are associated with steroid-refractory aGvHD, I measured the expression of CD161 on $CD4^{+}$T cells in PBMC samples from patients with steroid-refractory and steroid-responsive aGvHD and in those without GvHD directly *ex vivo*. Because of inter-individual variability in $CD4^{+}CD161^{+}$ percentage, the fold increase in $CD161^{+}CD4^{+}$ T cells was measured before and after initiation of aGvHD therapy with corticosteroids. This was calculated by dividing the frequencies of $CD161^{+}CD4^{+}$ T cells in PBMC samples after treatment with steroids by the their frequencies before treatment.

Patients with SRf aGvHD had significantly higher frequencies of $CD161^{+}CD4^{+}$ T cells after steroid treatment (30.4% ± 2.2% vs. 17.2% ± 2%before steroid therapy, ***p*= 0.004**)**(Figure 6.1A)**. In contrast, the frequency of $CD161^{+}CD4^{+}$ T cells was not significantly different before and after steroid therapy in patients with steroid-responsive aGvHD (17.9% ± 1.3 vs.17.5% ± 0.4 **p= 0.8)**.Following steroid therapy, patients with SRf aGvHD had significantly higher frequencies of $CD4^{+}CD161^{+}$T cells, presented as fold increase, compared to no GvHD patients (1.8 ± 0.23 vs. 1.1 ± 0.07, ***p*= 0.002**)and SRs aGvHD patients (1.8 ± 0.23 vs. 0.55 ± 0.38, ***p*= 0.02**)**.** Moreover, the frequencies of $CD161^{+}CD4^{+}$ T cells was not significantly different in GvHD patients who responded to steroid therapy compared to patients with no GvHD (fold increase 0.55 ± 0.38 vs. 1.1 ± 0.07, ***p*=ns**)**(Figure 6.1B)**.

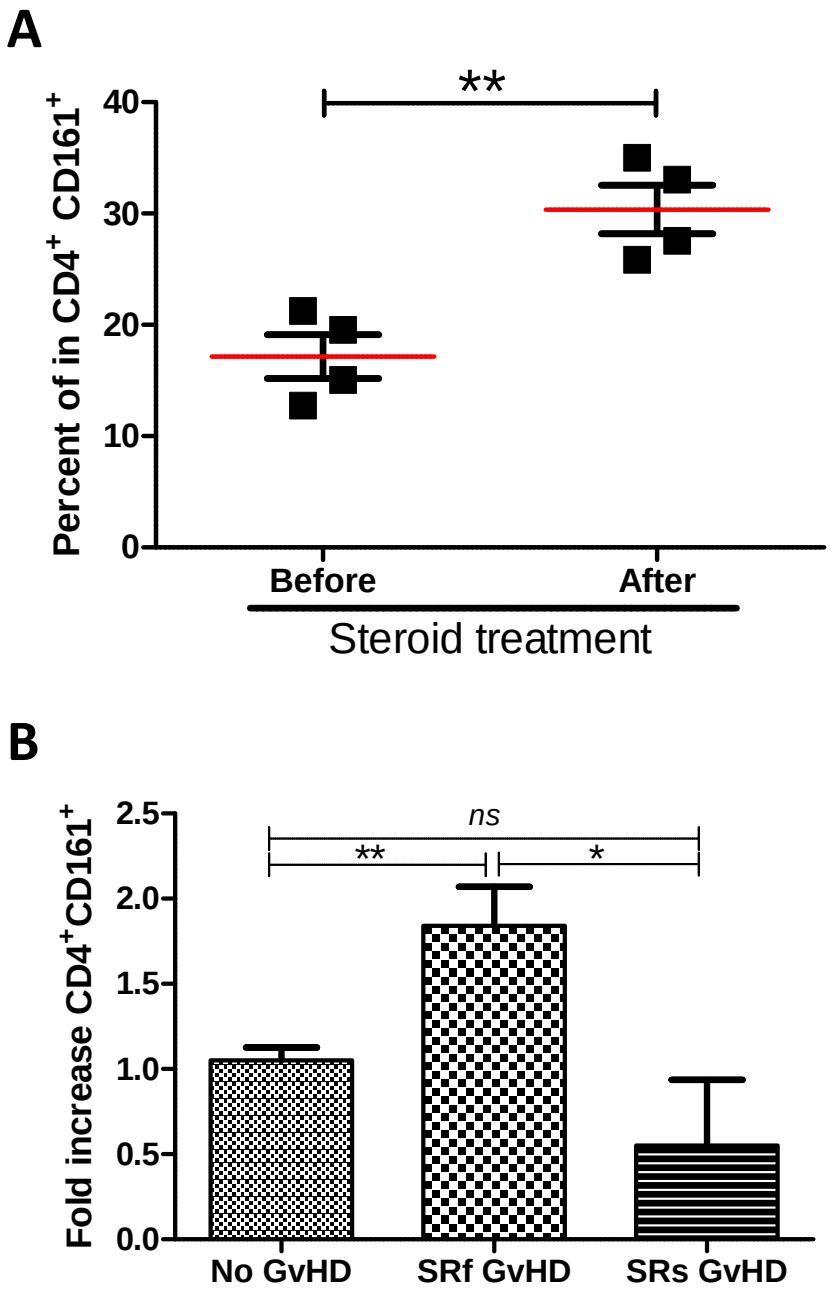


Figure 6.1: Change in the frequency of $CD161^{+}CD4^{+}$ T cells post-transplant. A, Scatter plot showing the increase in the frequencies of $CD4^{+}$ $CD161^{+}$ T cell in steroid-refractory aGvHD patients after steroid treatment**(n=4)B,** Bar chart comparing the fold increase in $CD161^{+}CD4^{+}$ T cells in patients with steroid-responsive**(n=3)**, steroid-refractory**(n=4)** and no GvHD **(n=8)**. Fold increase was calculated by dividing $CD4^{+}CD161^{+}$ before and after GvHD occurrence (steroid-treatment). Results presented as mean ± SEM. Student *t*-test and one way ANOVA were used.

6.2.3 The expanded$CD161^+CD4^+$ T cells in patients with aGvHD have a Th17 phenotype

In **chapter 4**I showed that $CD161^+CD4^+$ T cells that resist the *in vitro* lymphocytotoxic effect of dexamethasone have a Th17 phenotype. Therefore, I sought to examine the frequencies of IL-17-producing $CD4^+CD161^+$cells in patients with aGvHD.

In keeping with the *in vitro* data, I found that $CD4^+CD161^+$IL-17^+T cell frequencies increased in patients with SRf aGvHD (as assessed by fold increase in $CD4^+CD161^+$IL-17^+ T cells following treatment) compared to patients with no GvHD and SRs GvHD patients (fold increase 1.7 ± 0.9 vs. 1.15 ± 0.15 and 0.4 ± 0.3, **p= 0.4 and 0.4,** respectively), although the difference did not reach statistical significance (**Figure 6.2**).

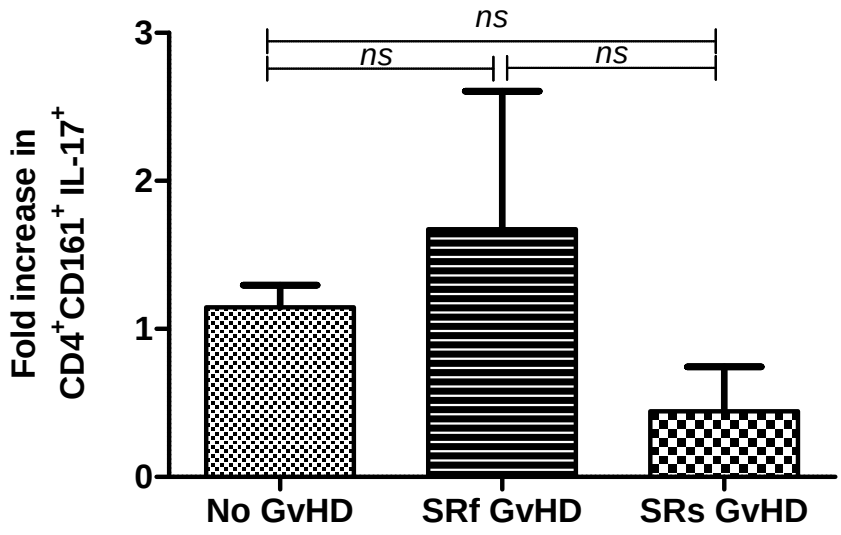


Figure 6.2:Percentage of $CD4^+CD161^+IL\text{-}17^+$ T cells post-transplant. Bar chart comparing the fold increase in $CD4^+CD161^+IL\text{-}17^+$ T cells in patients with steroid-responsive**(n=3)**, steroid-refractory**(n=4)** and no GvHD patients **(n=8)**. Fold increase was calculated by dividing the frequencies of $CD4^+CD161^+IL\text{-}17^+$ after GvHD occurrence (steroid-treated) by theirs before. Results presented as mean ± SEM.

6.2.4 IFNγ-producingCD161^{+}CD4^{+} T cells are not increased in patients with steroid-responsive or steroid-refractory GvHD

Previous data (**section 4.2.3**) demonstrated that *in vitro* exposure to steroid (dexamethasone) has minimal effect on IFNγ production by CD4^{+}CD161^{+} T cells. For this reason, I evaluated the IFNγ production before and after GvHD onset and steroid therapy.

As seen previously in the in-vitro experiments, exposure to steroids in both SRs as well as SRf aGvHD patients did not have a significant impact onIFNγ-producingCD4^{+}CD161^{+}cellswhen compared with patients with no GvHD (fold increase 0.87 ± 0.11 and 1.02 ± 0.59 vs. 1.5 ± 0.46 , ***p*=0.4**) . Furthermore, unlike IL-17, there was no difference in IFNγ production between SRf and SRs GvHD patients(***p*= 0.9**) **(Figure 6.3)**.

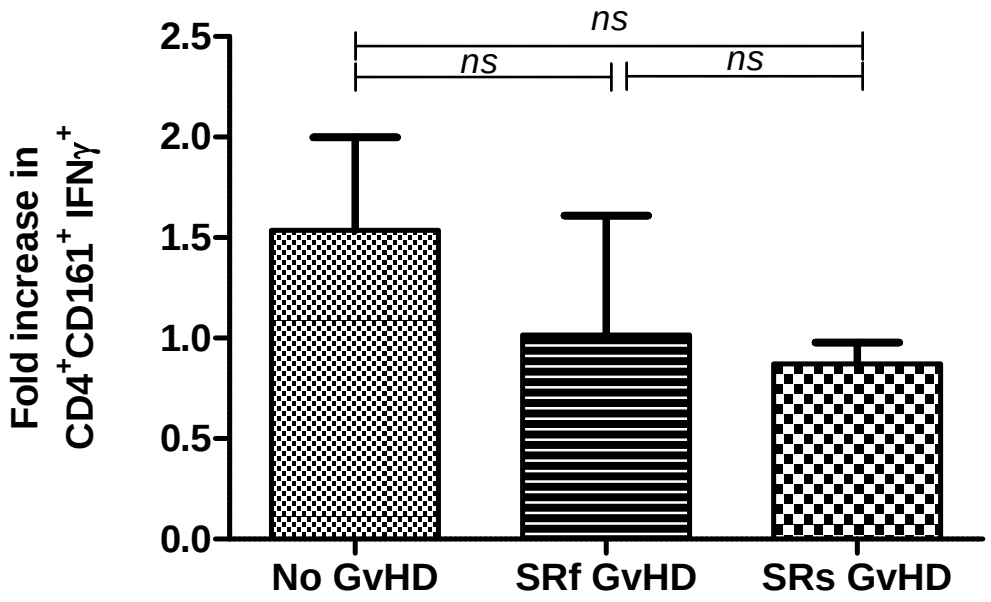


Figure 6.3:Percentage of $CD4^+CD161^+IFN\gamma^+$ T cells post-transplant. Bar chart comparing the fold increase in $CD4^+CD161^+IFN\gamma^+$ T cells in patients with steroid-responsive**(n=3)**, steroid-refractory**(n=4)** and no GvHD patients **(n=8)**. Fold increase was calculated by dividing $CD4^+CD161^+IFN\gamma^+$ before and after GvHD occurrence (steroid-treated). Results presented as mean ± SEM.

6.2.5 Corticosteroid therapy is associated with a significant reduction in $CD4^+Foxp3^+$T cells *in vivo*

Next, I sought to examine if treatment with corticosteroids could disturb the balance between T cell subsets, especially by suppressing regulatory T cells ($Foxp3^+$). Thus, I compared the frequencies of $CD4^+Foxp3^+$ T cells pre- and post- steroid intervention.

I calculated the ratio of $CD4^+Foxp3^+$ T cells in patients with aGvHD by dividing their frequencies after treatment with corticosteroids by frequencies before treatment. I observed a significant reduction in $CD4^+Foxp3^+$ T cells frequencies, presented as fold increase, following treatment with steroids both in patients with SRs and SRf GvHD compared to pre-GvHD therapy (fold increase 0.58 ± 0.16 and 0.67 ± 0.11, respectively) **(Figure 6.4 A)**. This suggests that corticosteroids induce apoptosis in $CD4^+Foxp3^+$ T cells. Of note, $CD4^+Foxp3^+$ T cells were almost two fold higher in patients with no GvHD compared to patients with SRs and SRf aGvHD(fold increase 1.8 ± 0.43 vs. 0.6 ± 0.08,***p*= 0.026**) **(Figure 6.4 B)**.

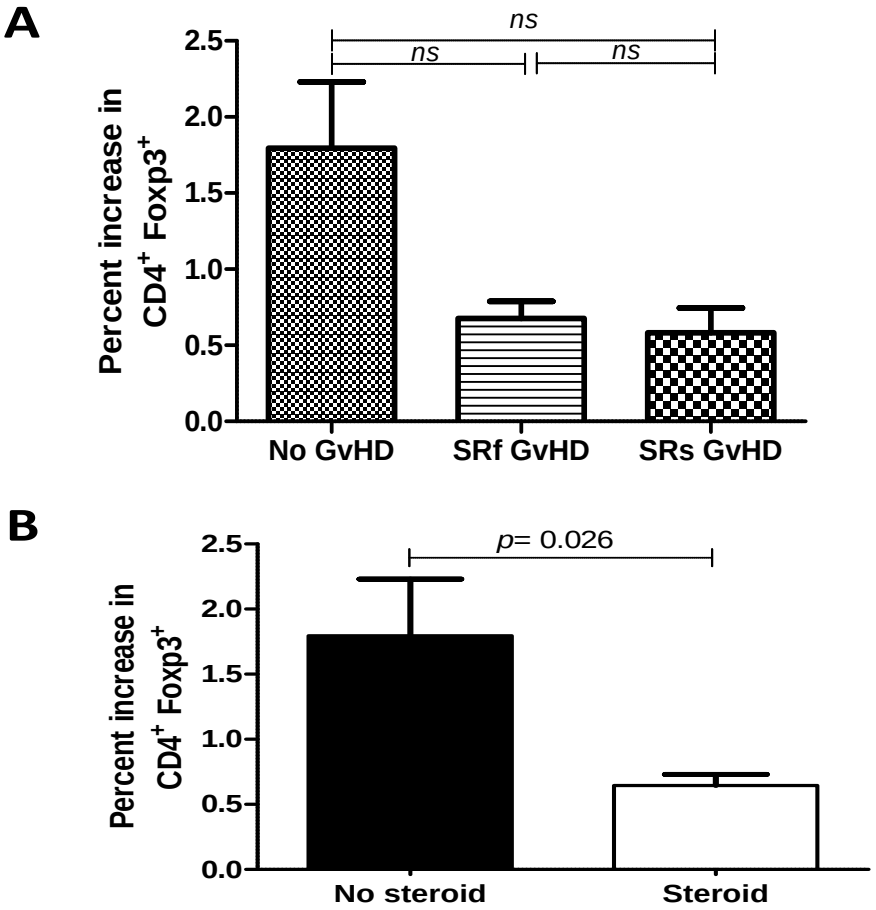


Figure 6.6: :**Percentage of regulatory T cells post-transplant. A,** Bar chart comparing the fold increase in $CD4^+Foxp3^+$ T cells in patients with steroid-responsive**(n=3),**steroid-refractory**(n=4)**and patients with no GvHD **(n=8). B,** bar chart showing the effect of steroid treatment on $CD4^+Foxp3^+$ in presence of no steroid **(n=8)** and steroid-treated **(n=7).** Fold increase was calculated by dividing $CD4^+$ $Foxp3^+$ before and after GvHD occurrence (steroid-treated). Results presented as mean ± SEM. One way ANOVA were used.

6.3 Discussion

In this chapter, I assessed the change in the frequencies of $CD161^{+}CD4^{+}$, IL-17 producing and $Foxp3^{+}$regulatory T cells in acute GvHD patients following steroid therapy, and patients with no sign of aGvHD exploring their role in steroid-refractory GvHD *in vivo*. As shown previously with the *in vitro* data in the previous **chapters**, I here showed that after treatment with corticosteroids, the frequencies of $CD161^{+}CD4^{+}$ T cells significantly increases in patients with steroid-refractory GvHD, suggesting that a subset of $CD161^{+}CD4^{+}$ T cells have the ability to survive corticosteroid therapy *in vivo.* In addition, I have previously**(chapter 3)** shown that the vast majority of $CD161^{+}CD4^{+}$ T cells fall within the$CD25^{int}$. Therefore, this finding indirectly correlates with data by Lee and colleagues demonstrating the role of $CD25^{int}CD4^{+}$ T cells in steroid-refractory uveitis (Lee et al. 2009a). These data propose that CD161 could be a marker for steroid-resistant T cells.

I have also shown here that the increase in the frequencies of $CD161^{+}CD4^{+}$ T cells in patients with steroid-resistant aGvHD was concomitant with an increase in IL-17 producing T cells. The frequencies of IL-17-producing $CD4^{+}$ T cells only decreased in patients who responded to steroid treatment. This findings further support the *in vitro* experiments carried out in **chapter 4** and are in keeping with previously published reports, demonstrating the resistance of Th17 cells to steroid treatment(McKinley et al. 2008). Collectively, these data suggest that the IL-17-producing $CD161^{+}CD4^{+}$ T cells might contribute to the pathogenesis of steroid-refractory aGvHD.

The fundamental role of regulatory T cells in immune tolerance has been well established. In addition, these cells have been shown to play a role in preventing or at least ameliorating GvHD symptoms**(section 1.5**). Their role, however, in steroid-

refractory GvHD is not well defined. Previous studies in autoimmune models suggest that steroids may induce Tregs, although with impaired suppressive activity, suggesting that Foxp3 upregulation in these studies might have actually been a sign of T cell activation(Prado et al. 2011b). In contrast, in the previous chapters, I demonstrated that corticosteroids induce reduction in$CD4^+Foxp3^+$ T cell frequencies. In keeping with previous data and in contrast to the *in vitro* experiments in chapter 4, I observed in this study that there was no difference in the frequencies of Foxp3+CD4+ T cells following steroid treatment between steroid responsive and non-responsive aGvHD patients *ex-vivo*. Although the percentage of regulatory T cells was comparable in aGvHD patients, irrespective of their responsiveness to corticosteroids treatment, the reduction in the frequencies of Tregs following steroid therapy was associated with a concomitant increase in IL-17-producing $CD161^+CD4^+$ T cells only in patients with steroid-refractory aGvHD. However, the suppressive properties of Treg from both patient groups could not be compared in view of the paucity of the available samples. Altogether, these data suggest that the imbalance between the effector and regulatory T cells; in favour of the effector phenotype, might play a significant role in the pathogenesis of steroid-refractory acute GvHD (**section 1.4.3**) supporting the notion that steroids can disturb the balance between Th17 and Tregs.

In summary, the data in this chapter are consistent with the *in vitro* experiments presented in **chapter 4**.This study is however limited by the small number of samples, which preclude a definitive conclusion. Also, the timing for the sample collection, especially before GvHD onset, was another obstacle that needs to be considered. A larger prospective study is required to establish the role of $CD4^+CD161^+IL\text{-}17^+$ T cells as well as their balance with regulatory T cells, in respect of frequencies and functions, in steroid-refractory aGvHD.

CHAPTER 7. GRAFT TH17 AND REGULATORY T CELL CONTENT AND RISK OF ACUTE GvHD POST-TRANSPLANT

7.1 Introduction

Allogeneic stem cell transplantation remains the treatment of choice for a variety of haematological disorders. The source of the donor graft and its content are likely to play an important role in determining the success of this therapy. Historically, bone marrow was the main source of haematopoietic stem cells (HSC). More recently, peripheral blood stem cells (PBSC) mobilized with granulocyte-colony stimulating factor (G-CSF) has been increasingly used as the source of stem cells for AHSCT, due to its ease of procurement and more rapid engraftment. In patients who continue to have residual disease following AHSCT or those with persistent mixed chimerism, donor lymphocyte infusion (DLI) is used to prevent disease relapse. However, mature T cells within the graft as well as DLI might induce undesirable reactions such as acute GvHD.

The effect of G-CSF mobilization on immune cells has been extensively studied(Abbi et al. 2012;Dhedin et al. 2006;Joo, Lee, Won, Lee, Kim, Park, Park, Choi, Choi, & Seo 2012;Toh et al. 2009;Vela-Ojeda et al. 2005;Zhao, Xu, Lu, & Huang 2011).Interestingly, in spite of harbouring ten times more T cells, the incidence of acute GvHD after PBSC is similar to BM transplantation(Bensinger et al. 2001), although more recent data suggest an increase in the rates of chronic GvHD following PBSC transplants(Anasetti et al. 2012). In addition, although it has been determined that the regulatory T cell content is lower in G-CSF

mobilized graft compared to conventional bone marrow graft, the effect of G-CSF mobilization is believed to be immunomodulatory, thereby reducing the risk and severity of acute GvHD(Abbi et al. 2012).

Graft versus host disease post-transplantation hampers the success of AHSCT. A number of studies have examined the relationship between the cellular content of the graft or the DLI product and the risk of aGvHD following AHSCT. T cells have been shown to play an indispensable role in the pathogenesis of GvHD. Several studies have focused on the importance ofTh17/Treg balance and risk of aGvHD**(section 1.5)**.

Data presented in the chapters4 and 5 of this thesis suggest a potential role for Th17 precursors, $CD4^+CD161^+$ T cells, in refractory aGvHD. I showed that $CD161^+CD4^+$ T cells may play a role in the pathogenesis of steroid-refractory aGvHD through their ability to efflux dexamethasone, an ABCB1 transporter substrate.

The objective of this chapter is to examine the T cell content of the donor graft, specifically the frequencies of $CD4^+CD161^+$ T cells, Th17 and $Foxp3^+$ regulatory T cells, the Th17/Treg ratio, and their role in the development of acute GvHD. In addition, by comparing T cell content of G-CSF mobilised PBSC graft and DLI (not G-CSF mobilised), I will study the effect of G-CSF on the phenotype and function of these different T cell subsets.

7.2 Results

7.2.1 Patients characteristics

In recipients of allogeneic PBSC, GCSF-mobilised donor grafts**(n=24)**and donor lymphocytes (DLI)**(n=20)** were cryopreserved and stored in the John Goldman centre for cellular therapy at Hammersmith Hospital. All donors and patients gave written informed consent for the use of their material for the research study.

To assess the association between the content of GCSF-mobilised donor graft and the risk of aGvHD, patients were divided into two groups according to the onset of acute GvHD; No acute GvHD **(n=17)** and acute GvHD **(n=7)(Table 7.1).** GvHD diagnosis and grading was done routinely in transplant clinic using the modified Seattle Glucksberg criteria. Patient characteristics are summarised in **Table 7.1**.

	All (N=24)	No acute GvHD (N=17)	acute GvHD (N=7)
	number (percent)		
Median age in years (range)	**44.7 (18-63.4)**	**47.1 (18-56.1)**	**45.3 (28.3-63.4)**
Sex			
Male	15 (62.5)	10 (58.8)	5 (71.4)
Female	9 (38.5)	7 (41.2)	2 (28.6)
Diagnosis			
Acute leukemia	8 (33.3)	7 (41.2)	1 (14.2)
Chronic myelogenous leukemia	6 (25)	5 (29.4)	1 (14.2)
Malignant lymphoma	4 (16.7)	1 (5.9)	3 (42.8)
Others	6 (25)	4 (23.5)	2 (28.6)
Conditioning regimen			
Myeloablative conditioning	8 (33.3)	2 (11.8)	6 (85.7)
Reduced intensity conditioning	16 (66.7)	15 (80.2)	1(14.3)
Stem cell donors			
Related	23(95.8)	16 (94.1)	7 (100)
Unrelated	1 (4.2)	1 (5.9)	0 (0)
HLA Match			
HLA-identical	17 (70.8)	11 (64.7)	6 (85.7)
HLA-mismatched	7 (29.2)	6 (35.3)	1 (14.3)
Stem cell source			
Bone Marrow	0 (0)	0 (0)	0 (0)
Peripheral Blood Stem Cells	24 (100)	17 (100)	7 (100)
GvHD grading			
Grades I-II	2 (8.3)	0 (0)	2 (28.6)
Grades III-IV	5 (20.8)	0 (0)	5 (71.4)
CMV serology status			
CMV postive	18 (75)	13 (76.4)	5 (71.4)
CMV negative	6 (25)	4 (23.6)	2 (38.6)
Transplant outcome			
Relapse	9 (37.5)	8 (47.1)	1 (14.3)
No relapse	15 (62.5)	9 (52.9)	6 (85.7)

Table 7.1: Characteristics of stem cell transplantations patients. Patients were sub-divided into two groups according to acute GvHD occurrence.

7.2.2 G-CSF effect on the frequencies of $CD4^+CD161^+$ T cells, Th17 and $Foxp3^+$ T cells.

I first sought to assess the effect of G-CSF on the frequencies of T cell subsets by comparing percentages of $CD4^+CD161^+$ T cells, Th17 and Tregs in G-CSF-mobilized grafts and the DLI samples. There was no significant difference in the frequencies of Th17 cells in G-CSF mobilised grafts compared to DLI samples (0.68% ± 0.1 vs. 0.69% ± 0.09, ***p*=0.96**) **(Figure 7.1A)**. Similarly, G-CSF mobilization did not have an impact on the frequencies of $CD4^+Foxp3^+$ T cells(4.3% ± 0.25 vs. 3.9% ± 0.28, ***p*=0.4**) **(Figure 7.1C),** although naïve Tregs ($CD45RA^+CCR7^+$) were more abundant in G-CSF mobilised grafts compared to DLI (33.4% ± 3.5 vs. 23.1%± 3, ***p*=0.035**) **(Figure 7.1D)**. On the other hand, $CD161^+CD4^+$ T cells were significantly lower in G-CSF mobilised grafts compared to DLI samples (4.4% ± 0.56 vs. 6.5% ± 1.1, ***p*= 0.04**) **(Figure 7.1B)** .These data suggest that G-CSF skews the T cell composition of the graft to a more immunomodulatory phenotype by increasing the proportion of naïve Tregs and reducing the frequencies of $CD161^+$ $CD4^+$T cells.

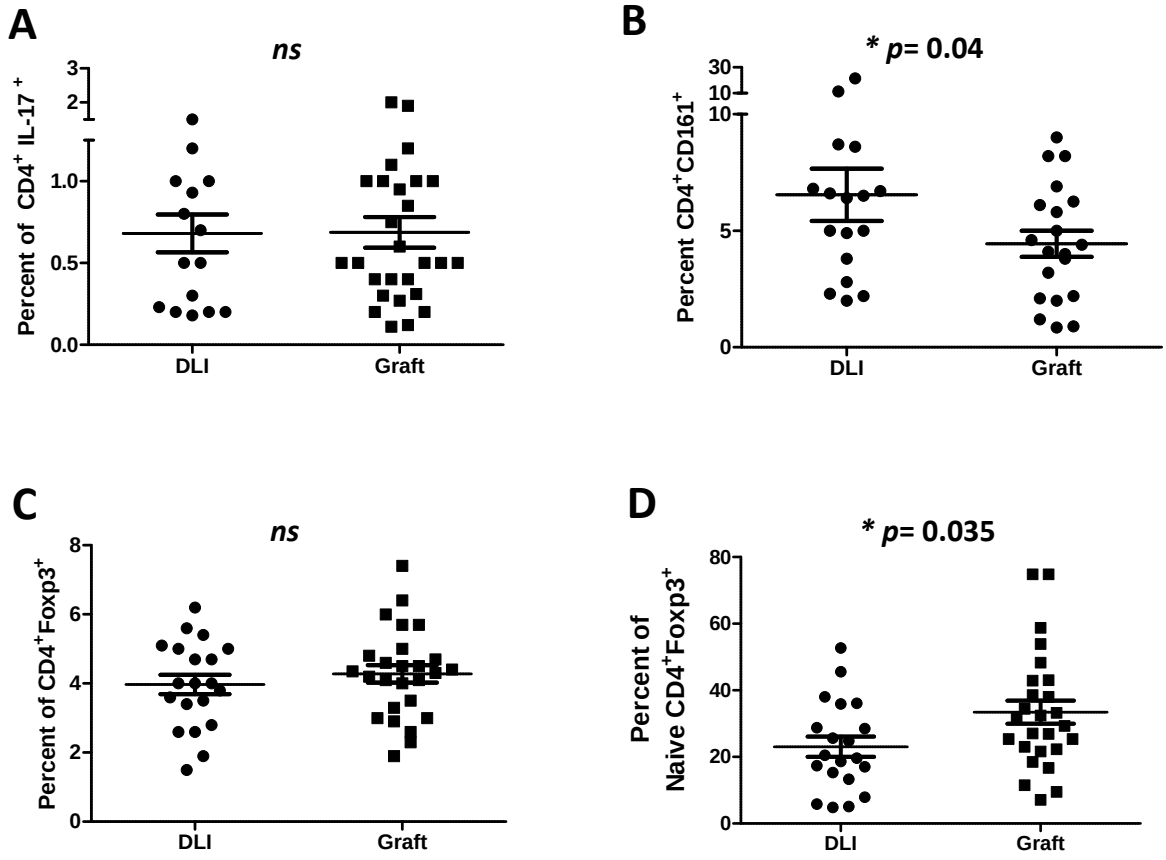


Figure 7.1:Scattered plots showing the differences in the proportions of T cell subsets in G-CSF mobilised grafts compared to DLI (without G-CSF). A, proportion of$CD4^+$ $IL\text{-}17^+$ T cells **B,** frequency of $CD4^+161^+$T cells. **C,** Frequency $CD4^+Foxp3^+$regulatory T cells. and **D,** Frequency of naïve $Foxp3^+$ Treg. Data represent the mean ± SEM. Student *t*-test was used.

7.2.3 Frequencies of $CD4^+CD161^+$ T cells, Th17 and regulatory T cells in the donor grafts do not predict for risk of acute GvHD post-transplant

A number of studies suggest a role for Th17 cells in the pathogenesis of acute GvHD; $CD4^+CD161^+CCR6^+$ T cells, the precursor for Th17 cells, are enriched in the gastrointestinal tract biopsy of aGvHD patients(Bossard et al. 2012). In contrast, regulatory T cells have been shown to protect against GvHD**(section 1.5)**. Therefore, I sought to evaluate the frequencies of these subsets in the donor graft and correlate their frequencies with the risk of aGvHD.

There was no significant difference in the frequencies of IL-17-producing $CD4^+$ T cells (Th17) (0.55% ± 0.12% vs. 0.74% ± 0.12%, ***p*= 0.39**)**(Figure 7.2A)**or $CD161^+CD4^+$ T cells(4.9% ± 1.0% vs. 4.2% ± 0.7%, ***p*=0.5**)in grafts transfused into patients with or without aGvHD **(Figure 7.2B)**. Similarly, there was no significant difference in the frequencies of $CD4^+Foxp3^+$ regulatory T cells, both total and naïve, in patients with or without aGvHD; ***p*= 0.3 and *p*=0.8,** respectively **(Figure 7.2C and D)**.

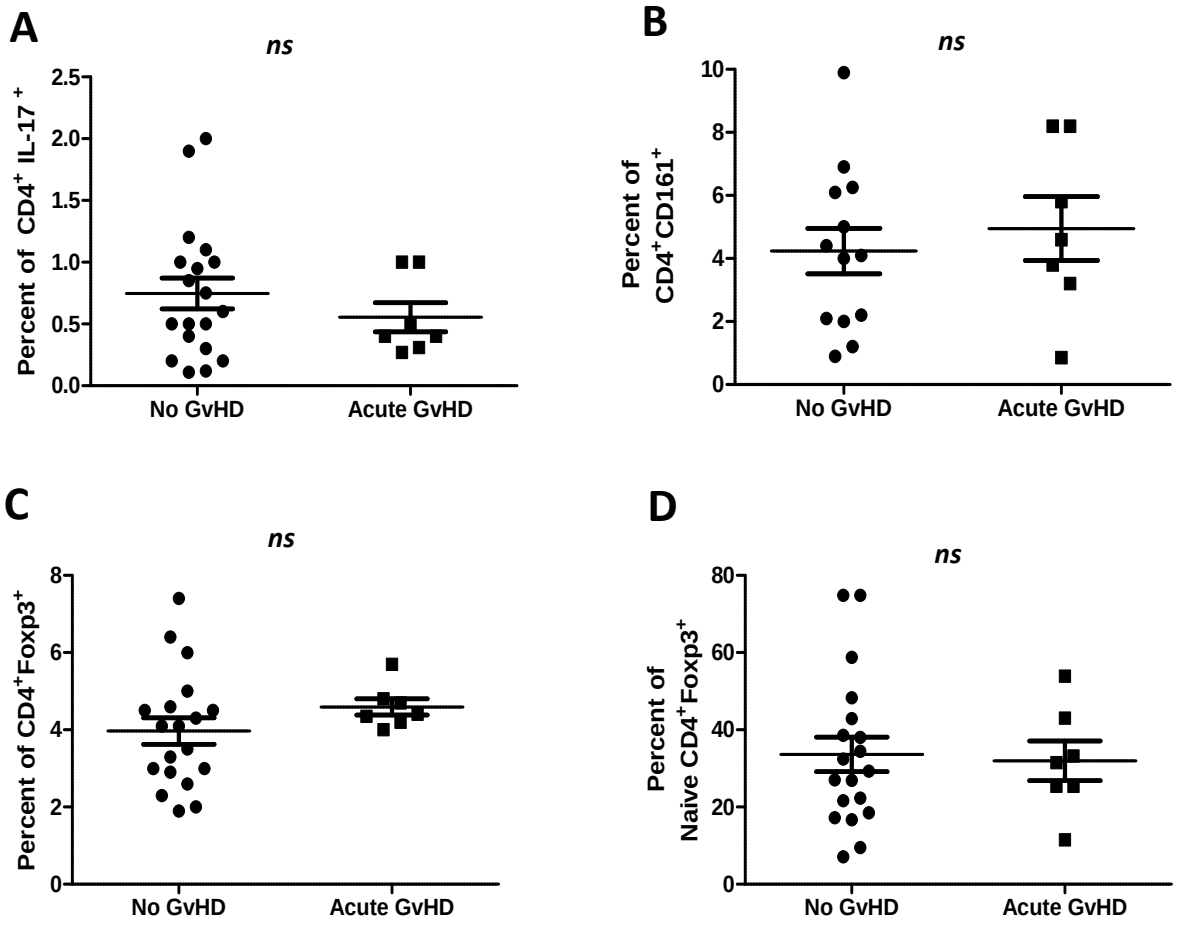


Figure 7.2:Scatter plots comparing frequencies of T cell subsets in donor grafts of patient with and without acute GvHD. A, Frequencies of$CD4^+$ $IL\text{-}17^+$ T cells **B,** Frequencies of $CD4^+161^+$T cells. **C,** Frequencies of $CD4^+Foxp3^+$regulatory T cells and **D,** Frequencies of naïve $CD4^+Foxp3^+$ Treg. Data represent the mean ± SEM.

7.2.4 Ratios of $CD4^+CD161^+$ T cells/Tregs and Th17/Tregs do not predict for risk of aGvHD.

A number of studies have suggested that the ratio of Treg/Th17 cells, rather than the frequencies of these T cell subsets, is predictive for risk of aGvHD(Dander et al. 2009;Ratajczak et al. 2010). Thus, the ratios of Th17/Tregs and $CD4^+CD161^+$ T cells/Tregs in the donor graft and the risk of aGvHD were assessed. I found no significant difference in the Treg: Th17 ratio (11.3 ± 2.5 vs. 10.3 ± 1.7, ***p*=0.8**)or Treg/$CD161^+CD4^+$ T cell ratio in patients with or without aGvHD (1.6 ± 0.41 vs.1.5 ± 0.62), ***p*=0.9**; **(Figure 7.3)**

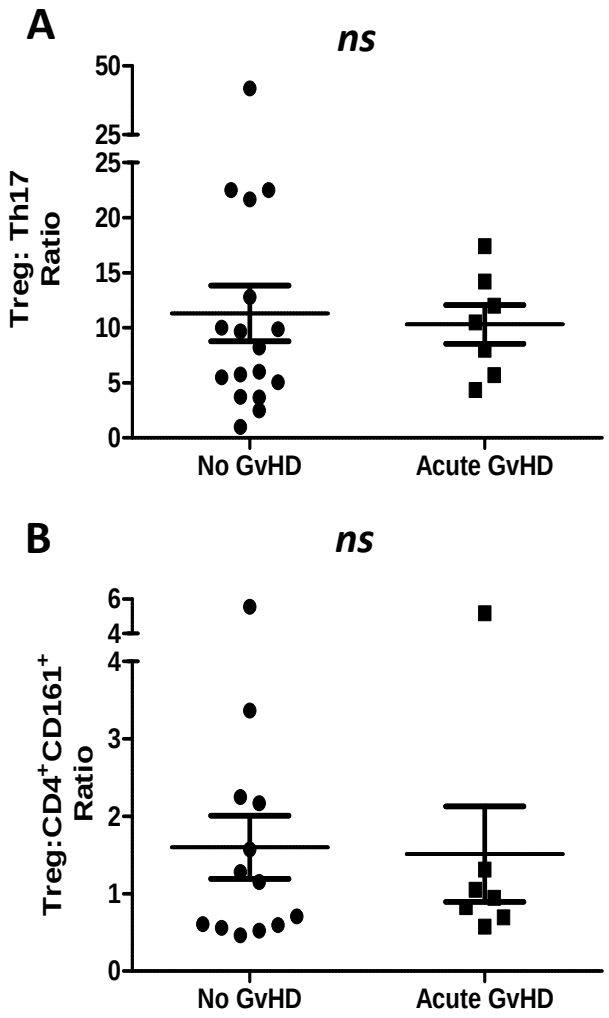


Figure 7.3:Scatter plots examining Th17/Tregs and $CD4^+CD161^+$ T cells/Tregs ratios in the donor graft and the risk of aGvHD. Ratios were calculated by dividing the frequencies of Th17 cells by Tregs and $CD161^+CD4^+$ T cells by Tregs. **A,** Treg:Th17 ratio in patients with or without aGvHD. **B,** Ratio of Treg/$CD4^+161^+$ T cells in patients with or without aGvHD. Data represent the mean ± SEM. Student *t*-test was used.

7.3 Discussion

In this study, the association between Th17, regulatory T cells and $CD4^+CD161^+$ T cells, and the risk of aGvHD was investigated. I used cryopreserved pilot samples of donor grafts that were infused into patients undergoing allogeneic G-CSF mobilized grafts. I also assessed the impact of G-CSF on T cell subsets, by comparingTh17, Treg and $CD161^+CD4^+$ T cell frequencies in G-CSF mobilised donor grafts and DLI samples.

A number of studies suggest that G-CSF may exert an immunomodulatory effect on T cell subsets in the graft(Toh et al. 2009;Vela-Ojeda et al. 2005;Vela-Ojeda et al. 2010;Zhao, Xu, Lu, & Huang 2011). In support of these data, Toh et al recently demonstrated that whereas G-CSF induces Treg-related genes such as TGF-β, it reduces the expression of the Th17 transcriptional factor gene, RORγt. The observed changes in the molecular profile of T cells in response to G-CSF were associated with a reduction in Th17 and increase in regulatory T cells. The observation that G-CSF can ameliorate the symptoms of acute GvHD is thought to be at least partly mediated through inhibition of Th17 differentiation(Joo et al. 2012).However, in this study I did not observe an impact of G-CSF mobilisation on the frequencies of IL-17-producing $CD4^+$ T cells. Furthermore, I did not observe an association between the Th17 content in the graft and the risk of aGvHD. However, our observation that G-CSF mobilisation reduces the$CD161^+CD4^+$ T cell content in the donor graft further supports an immunomodulatory effect of G-CSF on T cell subsets.

Several studies have shown a crucial role for regulatory T cells in immune tolerance and protection from GvHD in mice and human **(section 1.5)**. Indeed, in the HSCT setting, Rezvani et al. showed that the higher $Foxp3^+$ T cell content in the donor graft is associated with a lower risk of aGvHD(Rezvani et al. 2006). Moreover, the balance of Treg and Th17 cells has been shown to play a role in the pathogenesis of aGvHD(Ratajczak et al. 2010). I found that naïve $Foxp3^+$ T cells are significantly higher in G-CSF mobilised donor graft; and this data is in agreement with previous reports that $CD62L^+$naïve regulatory T cells are more protective than $CD62L^-$ counterparts(Ermann et al. 2005;Vela-Ojeda et al. 2010).

Under normal circumstances the ratio between T cell subsets are tightly controlled. However, disruption of this balance may result in autoimmune disorders or acceleration of inflammatory reactions such as GvHD. Disturbances in the balance of regulatory T cells and Th17 cells has been suggested to a play a role in the pathogenesis of GvHD(Ratajczak et al. 2010). In the present study however, I found no significant differences in the ratios of Treg/Th17 and Treg/$CD161^+CD4^+$ T cells in the donor graft of patients with or without aGvHD.

Collectively, our findings that G-CSF mobilisation reduces the$CD161^+CD4^+$ T cell content in the donor graft and increases naïve Tregs support an immunomodulatory effect of G-CSF on the donor graft. However, the low number of available samples and the retrospective nature of the analysis significantly limit this study. I plan to validate these data in a larger prospective study.

CHAPTER 8. GENERAL DISCUSSION AND FUTURE PLANS

8.1 General discussion

Allogeneic stem cell transplantation (AHSCT) is the only successful therapeutic option for a number of haematological malignancies. Eradicating the residual leukemic cells and replenishing the recipient's haematopoietic stem cell compartment and cellular immunity are the main actions of this modality. However, the ultimate success of AHSCT is hindered by a number of complications including GvHD, initiated by the interaction between the graft cellular components, mainly T lymphocytes, and the recipient's tissue antigens and cytokine milieu. Nonetheless, the cellular content of the graft in AHSCT controls to a great extent the ultimate outcome of the procedure. Indeed, the presence of T cells in the graft determines GVL and immune reconstitution as well as the risk of GvHD. Compelling evidence suggests that the interplay between T cell subsets infused within the graft, including Th1, Th17 and regulatory T cells, is a major factor influencing the outcome of AHSCT. Therefore, maintaining the balance between these subsets is likely to be paramount for the success of this treatment.

The specific aims of this thesis were:

1. To evaluate the existence of stem-like memory T cells with drug effluxing ability in the peripheral blood of healthy controls and patients with AML.
2. To examine the functional phenotype of drug effluxing $CD4^+$ and $CD8^+$ T cells, including their Th1 and Th17 profile.
3. To examine mechanisms of steroid-refractory GvHD by assessing the *in vitro* and *in vivo* resistance of T cell subsets (Th1, Th17 and Tregs) to corticosteroids as well as calcineurin and mTOR inhibitors.

4. To evaluate the impact of donor T cell subsets (Th1, Th2, Th17 and Tregs) infused with the graft on risk of GvHD.

The achievements of this thesis can be summarised as follows: In chapter 3, I demonstrated the existence of a sub-population of $CD4^+$ T lymphocytes with stem cell-like properties. I demonstrated that this subset of $CD4^+$ T cells can be defined phenotypically by co-expression of CD161, and have the ability to rapidly efflux rhodamine through the MDR pump. Interfering with the ABC transporters using specific chemical inhibitors such as CsA abrogated the effluxing ability of these cells. Furthermore, I showed that drug-effluxing $CD161^+CD4^+$ T cells are enriched within the viral-specific Th1 repertoire and survive lymphocytotoxic chemotherapy, such as daunorubicin, through their ABCB1 pump effluxing ability. These data are in keeping with work by Turtle and colleagues (Turtle et al. 2009)which showed the existence of a similar subset of stem-like memory T cells in CD8 compartment, capable of rapidly effluxing cytotoxic chemotherapeutic agents through the ABCB1 transporter complex. These cells were found to possess stem-like characteristics, including expression of the anti-apoptotic molecules; Bcl-2 and Bcl-xl, in addition to their quiescence under physiological conditions.

In chapter 4, I explored whether drug-effluxing $CD161^+CD4^+$ T cells may also play a role in the pathogenesis of GvHD and whether they exhibit *in vitro* resistance to the lymphocytotoxic and immunosuppressive effects of corticosteroids and calcineurin and mTOR inhibitors. Immunosuppressive drugs such as tacrolimus and in some centres, rapamycin, are used as prophylaxis to minimize the risk of acute and chronic GvHD. In addition, upon the occurrence of GvHD other interventions (primarily corticosteroids) are used to treat the disease. Interestingly, some of the drugs used in

GvHD prophylaxis and treatment, including corticosteroids, tacrolimus and rapamycin, are substrates of the ABCB1 transporter complex(Schinkel & Jonker 2003). In chapter 4, I indirectly evaluated the effect of dexamethasone, tacrolimus and rapamycinon the survival, proliferation and effector function of the drug-effluxing $CD4^+CD161^+$ T cells. I showed that$CD161^+CD4^+$T cells stimulated *in vitro* with anti-CD3/CD28 beads have the ability to resist the immunosuppressive and lymphocytotoxic effects of tacrolimus and dexamethasone. Additionally, using a CFSE proliferation assay, I demonstrated that $CD161^+CD4^+$ T cells proliferate preferentially, suggesting that this subset of $CD4^+$ T cells may have an inherent resistance to the immune suppressive effect of these drugs. These results are in keeping with a study by Lee and colleagues, reporting the existence of subset of $CD4^+$T cells with resistance to steroids. These cells were characterized by the intermediate expression of IL-2Rα (CD25)(Lee et al. 2007). Similarly, I showed that the vast majority of $CD161^+CD4^+$ T cells also displayed intermediate expression of CD25. The promising reports of clinical response to treatment with Daclizumab, a mAb against alpha subunit of the IL-2R (anti-CD25) in steroid-refractory GvHD (reviewed in (Dignan et al. 2012)), may be partially attributable to the extermination of $CD4^+CD25^{int}$ $CD161^+$ T cells. However, the fact that regulatory T cells share CD25 expression with these cells might hinder the usefulness of this approach. Therefore, specific targeting of these cells using a monoclonal antibody to CD161,for instance, may potentially facilitate eradication of steroid-resistant subsets while sparing regulatory T cells. I showed that the preferential survival and expansion of$CD161^+CD4^+$ T cells in the presence of dexamethasone is concomitant with a dramatic induction in IL-17, but not IFNγ production. The role of Th17 cells in the pathogenesis of GvHD is controversial although Th17 cells have been shown to mediate resistance to steroids in airway inflammation(McKinley et al. 2008).

Interestingly, I found that $CD4^+Foxp3^+$ T cells are highly susceptible to the lymphocytotoxic effect of dexamethasone *in vitro*. Collectively, these data suggest that $CD161^+CD4^+$ T cells may play a role in steroid-refractory GvHD through their ability to preferentially survive the cytotoxic effect of steroids, accompanied with an induction in their pro-inflammatory functions (Th17). In addition, the increased susceptibility of $CD4^+Foxp3^+$ T cells to treatment with steroids may further skew the Th17/Treg ratio and contribute to the pathogenesis of GvHD.

I then explored the combination of ABCB transporter inhibitors and immunosuppressive drugs and their effect on $CD4^+CD161^+$ Th17 cells. Expression of multi-drug transporters e.g. ABCB1, is a mechanism through which $CD161^+CD4^+$ T cells efflux cytotoxic drugs that are ABCB1 substrates. Inhibitors of the ABCB1 transporter, which prevent the efflux of its substrate, have been widely used in combination with cytotoxic drugs to enhance their activity. This approach is already exploited in cancer therapy(Walter et al. 2004). Here, I showed that the addition of inhibitors for both ABCB1 and ABCC1 (vinblastine) to dexamethasone increased the lymphocytotoxic effect of dexamethasone on $CD4^+CD161^+$ Th17 cells. These data support a role for the use of ABCB1 transporter inhibitors in combination with dexamethasone to overcome the resistance of $CD161^+CD4^+$ T cells to steroids.

In chapter 5, I explored the impact of combinations of dexamethasone with tacrolimus or rapamycin on $CD161^+CD4^+$ T cells *in vitro*. I showed that combining tacrolimus with dexamethasone or with rapamycin significantly reduces IL-17 production by $CD4^+CD161^+$ T cells. Prospective and randomised clinical studies are now needed to evaluate the *in vivo* safety and efficacy of these drug combinations in the prevention and treatment of GvHD.

In Chapter 6, I explored the role of $CD161^{+}CD4^{+}$ T cells in steroid-refractory GvHD *in vivo.* I studied the frequency and functionality of $CD161^{+}CD4^{+}$ T cells in patients with aGvHD before and after steroid therapy. Interestingly, I found that the frequency of $CD161^{+}CD4^{+}$ T cells increased significantly in patients with steroid-refractory GvHD compared to patients with steroid-responsive GvHD or those without evidence of GvHD patients. In keeping with my *in vitro* data **(chapter 4)**, steroid treatment resulted in a significant reduction in the number of regulatory T cells, irrespective of treatment outcome. Altogether, these results suggest that steroid-refractory GvHD might be partly caused by induction of $CD161^{+}CD4^{+}$ T cells and the concurrent reduction in regulatory T cells in response to steroid treatment. However, these data will need further validation using a confirmatory and larger cohort of patients.

Finally, I explored the impact of donor T cell content on risk of GvHD; specifically asking the following questions: 1) what is the impact of G-CSF on the phenotype and function of T cell subsets in the graft and 2) Does the frequency of Th17 and Tregs in the donor graft predict for risk of aGvHD post AHSCT? Interestingly, I observed a shift to a more immunomodulatory phenotype, with higher naïve regulatory T cells and lower $CD161^{+}CD4^{+}$ T cell percentages in G-CSF-mobilised grafts compared to DLI samples (non-G-CSF mobilised). However, I did not observe a correlation between $CD4^{+}CD161^{+}$, Th17 and Treg content in the graft and the risk of GvHD occurrence. However, these data are limited due to the small number of samples analysed and the retrospective nature of the study.

Collectively, the results presented in this thesis show the existence of a memory subset of $CD161^{+}CD4^{+}$ T cells with stem cell-like properties. The enrichment of antigen specific memory T cells within this subset might have significant implications for the development of the next generation of T cell based immunotherapies against pathogens and tumours. Moreover, $CD161^{+}CD4^{+}$ T cells showed a selective survival and proliferation advantage in the presence of steroids with skewing towards a Th17 phenotype and concomitant reduction in $CD4^{+}Foxp3^{+}$ regulatory T cells. These changes were partially reversed by combining steroids with other immunosuppressive drugs such as calcineurin and mTOR inhibitors and selective inhibitors of ABC transporters. Altogether, these data might help in developing a graft engineering approach aimed at controlling the expansion of these cells and maintaining the balance between the effector and regulatory arms of the cellular immune response.

8.2 Future plans

I have shown the existence of stem cell-like memory $CD4^+CD161^+$ Th1 and Th17 cells with rapid drug-effluxing capacity. I propose to characterize these cells more comprehensively. Specifically, I propose to sort purify the rapidly-effluxing $CD4^+CD161^+$ $Rh123^-$ and the non-effluxing $CD4^+CD161^+$ $Rh123^+$ T cells to compare their molecular signature by gene expression profiling. I would like to specifically study the differential expression of ATP-binding cassette B1 (ABCB1), anti-apoptotic molecules (Bcl-2 and Bcl-xl) and transcription factors.

To further investigate the preferential enrichment of antigen specific $CD4^+$ T cells including CMV, EBV and influenza within the sorted Rh123-effluxing population, I propose to optimise assays of HLA Class II tetramer staining, to allow sort-purification of viral-specific $CD4^+$ T cells within the $CD161^+$ T cell fraction. Using a humanized mouse model of CMV infection(Berges and Rowan 2011;Smith et al. 2010), I will compare the ability of the adoptively transferred $CD161^+$ and $CD161^-$ $CD4^+$T cells to protect against viral infection and their role in providing long-term immunity against secondary viral challenge. This model will also allow me to examine the resistance of the adoptively transferred sort-purified viral-specific $CD161^+$ $CD4^+$ T cells to cytotoxic drugs including chemotherapeutic agents and steroids. These data will be especially important for the development of clinical protocols of adoptive therapy in patients with viral reactivation (e.g. CMV) during corticosteroid therapy for aGvHD, where resistance of the adoptively transferred viral-specific T cells to the lymphocytotoxic effect of the steroids will be highly desirable.

To further investigate the role of "drug-effluxing" $CD161^{+}CD4^{+}$ T cells in the pathogenesis of steroid-refractory GvHD, I propose a prospective study to compare the frequencies of $CD4^{+}CD161^{+}$ Th17 cells in patients with steroid-refractory and steroid-responsive GvHD and those without GvHD. The aim will be to validate the *ex vivo* findings presented in **chapter 6,** confirming a role of $CD161^{+}CD4^{+}$ T cells in the pathogenesis of steroid-refractory GvHD. I would also like to assess the effect of second line interventions employed for the treatment of steroid-refractory GvHD on $CD161^{+}CD4^{+}$ T cell frequencies, and the reciprocal balance between Th17 and regulatory T cells.

Using a Human/Mouse xenogeneic SCID mouse model I will study the contribution of $CD161^{+}CD4^{+}$ T cells to the pathogenesis of GvHD by infusing sort-purified $CD161^{+}CD4^{+}$or $CD161^{-}CD4^{+}$T cells along with the graft and I will then evaluate the severity of ensuing GvHD in the two animal groups and their response to steroid treatment. These data will have important implications in the development of novel strategies to prevent and manage steroid-refractory GvHD, including approaches to graft engineering and selective depletion of CD161 expressing T cells, as well as development of monoclonal antibodies to target and deplete CD161 expressing T cells in patients with steroid-refractory GvHD.

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List of Publications

Publications:

Alsuliman A, Garland P and Rezvani K. The Role of Regulatory T Cells in GVL and GVHD**: Book Chapter,** Nova Science Publishers, and Inc. **(2010)** ISBN: 978-1-61761-554-2

Alsuliman A, Khoder A, Stringaris K1, Cooper N, Sekine T, de Lavallade H, Sarvaria A, Mielke S, Marin D, Rezvani K. Stem-like characteristics in a subset of CD161 expressing human memory $CD4^+$ T cells may facilitate their survival after chemotherapy: **Original article submitted to Blood (2012).**

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