The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation

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Candidate Declaration

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Abstract

Graft versus host disease (GvHD) is a complication of allogeneic haematopoietic stem cell transplantation (HSCT) which can result in significant patient morbidity and mortality. Recent advances in the understanding of the different types of immune cells, especially those that play a role in tolerance and immune regulation, has led to investigation of their role in transplant tolerance. These include: plasmacytoid dendritic cells (pDC), DC-10 cells and Type 1 regulatory T cells (Tr1).

This study aims to determine any associations between tolerogenic cell populations including pDCs, DC-10 cells and Tr1 cells, along with other T cells, with the development of GvHD, and to analyse other allogeneic transplant outcomes including engraftment. It was postulated that there would be an inverse relationship between the number of tolerogenic cells and GvHD. In doing so, the study will determine if any or all of the analysed tolerogenic cells have the potential to be used as a biomarker for GvHD.

24 patients receiving transplants and five donors were consented and recruited onto the study between November 2018 and November 2019. The donors had their peripheral blood stem cell collection(s) and the recipients had their peripheral blood tested for: pDC, mDC1, DC-10, Tr1 and T cells at 2, 4, 8 weeks and 3 and 6 months post-transplant. Six patients were diagnosed with acute GvHD (aGvHD) and one patient was diagnosed with chronic GvHD within the 6-month follow up time period.

There was no statistically significant difference between the patients diagnosed with GvHD versus those that were not with regards to graft $CD34^+$ dose received ($P = 0.68$). There was no statistically significant difference between the patients who received a 'high' graft $CD34^+$ dose versus those that received a 'low' $CD34^+$ graft dose with respect to neutrophil ($P = 0.7938$) or platelet ($P = 0.4197$) engraftment.

Chi-square analysis found no connection between GvHD diagnosis and the known risk factors for GvHD (*P* values ranging between 0.404 - >0.999). Patients diagnosed with GvHD had a similar mean age to those that were not, 54.83 years and 54.71 years respectively.

Peripheral blood cell count data at the five post-transplant time points was split into GvHD and no GvHD groups and analysed using the Mann-Whitney test. No statistically significant difference was found between these groups, and the pDC, mDC1, DC-10, Tr1 and T cell counts at any time point (*P* values ranging between 0.077 - >0.999). The sample size in this study was small, and if the study was performed with a larger sample size then statistical significance may have been achieved. However,

there was an overlap in cell counts between patients diagnosed with GvHD and those that were not, and without a separation of the cell counts between the GvHD and no GvHD groups, it would be difficult to determine a cut off value.

An observation from the study was unusually high DC-10 cell counts prior to the diagnosis of GvHD in the one patient who died of GvHD. Further work is required to see if this finding is repeated.

Abbreviations

7-AAD – 7-Amino-Actinomycin D

AIM – Absent-in-melanoma

AML – Acute myeloid leukaemia

AN – Anthony Nolan

APC – antigen presenting cell

BM – Bone marrow

BDCA – Blood dendritic cell antigen (cell marker)

CD – cluster of differentiation (cell markers)

CLR – C-type lectin receptors

DAMPs – damage associated molecular patterns

DC – Dendritic cell

DFS – Disease free survival

EFS – Event free survival

cDC – Conventional dendritic cell

mDC – Myeloid dendritic cell (currently referred to as a conventional dendritic cell)

pDC – Plasmacytoid dendritic cell

EFS – Event free survival

G-CSF – granulocyte stimulating factor

GM-CSF – granulocyte-macrophage stimulating factor

aGvHD – acute graft versus host disease

cGvHD – chronic graft versus host disease

GvHD – graft versus host disease (Grades ≤ I - clinically insignificant. Grades II-IV - clinically significant)

GvT – graft versus tumour

GvL – graft versus leukaemia

HPC – Haematopoietic progenitor cell

HSC – Haematopoietic stem cell

HSCT – Haematopoietic stem cell transplant

IL – Interleukin

LTHT – Leeds Teaching Hospital Trust

MHC – Major histocompatibility complex

MMF – Mycophenolate mofetil

MoDC – Monocyte derived dendritic cell

MPP – Multipotent progenitors

MRD – Matched related donor

MTA – Material transfer agreement

MUD – Matched unrelated donor

NHSBT – NHS Blood and Transplant

NOD – Nucleotide oligomerization domain

NRM – Non relapse mortality

OS – Overall survival

PAMPs – Pathogen-associated molecular patterns

PBSC – peripheral blood stem cells (stem cells that have been mobilised into the peripheral blood)

PFS – Progression free survival

PRR – Pattern recognition receptor

RFS – Relapse-free survival

SHU – Sheffield Hallam University

SJUH – St James's University Hospital

TGF-β – Transforming growth factor-β

TLR – Toll-like receptors

Tr1 – Type 1 regulatory T cells

T reg – T regulatory cells

WBC – White blood cell

Glossary of Medical Terms

Disease-free survival (DFS) / Relapse-free survival (RFS). Time to relapse or death from any cause, whichever comes first. Patients evaluable for DFS are patients in remission pre or post-transplant (at time of inclusion). So DFS also means survival without evidence of disease (Labopin *et al.* no date).

Event free survival (EFS) is an alternative term for Disease-free survival (DFS) (Labopin *et al.* no date).

Neutrophil engraftment is the first of 3 consecutive days where neutrophil count is >0.5 x 10^9/L. Platelet engraftment is defined as the first of 3 consecutive days where the platelet count is >20 x10^9/L without transfusion (Sohn *et al.* 2003).

Overall survival (OS). Time to death, irrespective of the cause. There is no need to specify whether the death was due to the disease or not (Labopin *et al.* no date).

Progression free survival (PFS). For patients transplanted in an active phase of the disease (not disease-free at time of inclusion), the term PFS is appropriate. The term “progression” refers to any stage advanced of that at the beginning of the study (Labopin *et al.* no date).

Relapse applies to a patient transplanted after achieving a remission phase and for whom relapse of disease can be recorded (Labopin *et al.* no date).

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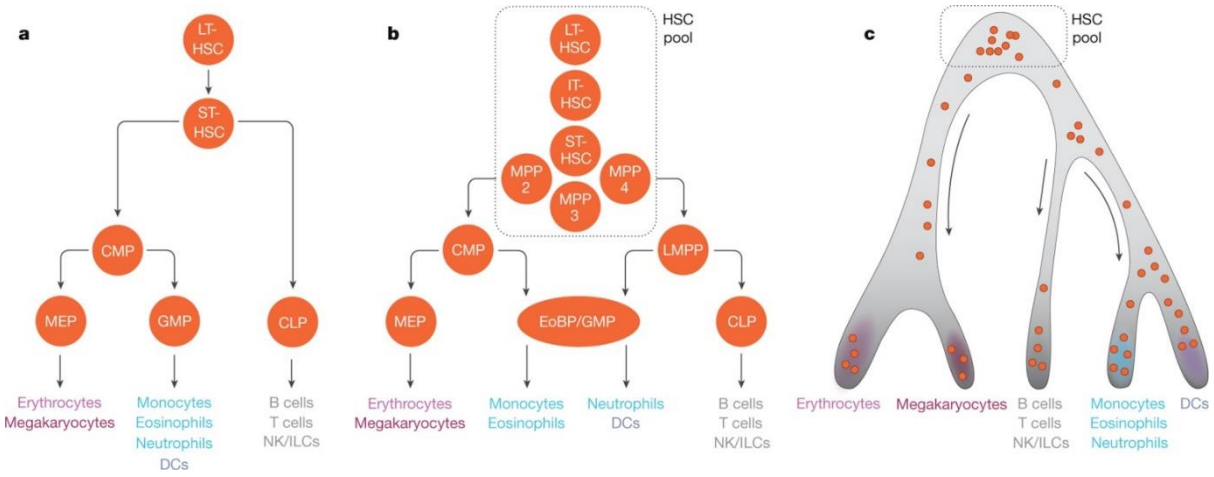
Chapter 1 – Review of literature

1.1 Haematopoiesis

Haematopoietic stem cells (HSCs) originate in foetal tissues and reside primarily in the bone marrow (BM) (Punt *et al.* 2019). HSCs give rise to the entire haematopoietic cell system (Dong *et al.* 2020). However, HSCs are a rare subset of cells within the BM, with less than 1 HSC present per 5 x 10^4 cells (Punt *et al.* 2019). Since the start of the 21st century our understanding of haematopoiesis has evolved, as illustrated in Figure 1.1.

Circa 2000: HSCs are split between long term (LT) - and short term (ST) -HSCs which then divide into the myeloid and lymphoid lineages via the common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) populations (Fig 1.1a). Between 2005–2015, a dynamic HSC pool emerges above the myeloid and lymphoid lineages (Fig1.1b). From 2016 onwards, single-cell transcriptome analysis updates the differentiation pathways (Fig 1.1c). Computational predictions of single-cell transcriptome analysis have suggested an early lineage restriction and that lineage specific fates are a continuous process, and that unilineage-restricted cells emerge from a continuum of undifferentiated HSCs (Laurenti and Göttgens 2018)**.**

Figure 1.1 Models of haematopoiesis since the late 1990s



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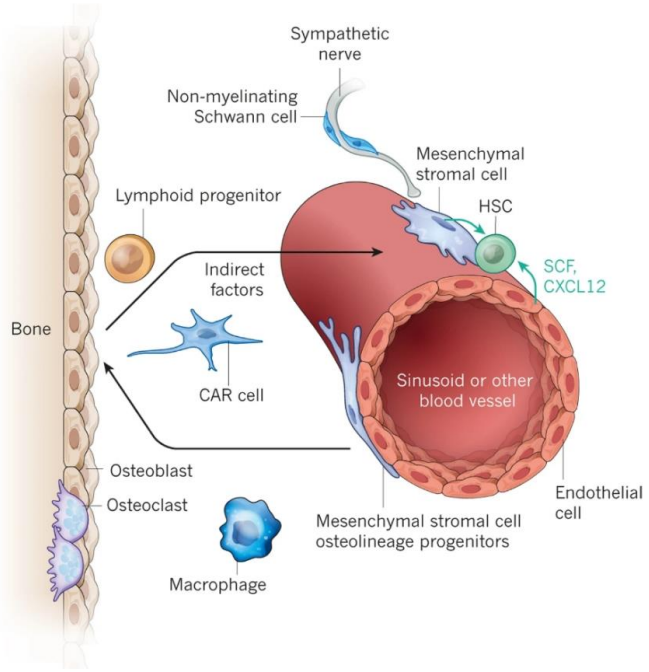
Haematopoiesis models since the late 1990s, the cell groups coloured according to (c) 2016 onwards are applied to the earlier models. (a) Crica 2000: HSCs are split between long term (LT) - and short term (ST) -HSCs which then divide into the myeloid and lymphoid lineages via the CMP and CLP populations (b) 2005-2015, a dynamic HSC pool emerges above the myeloid and lymphoid lineages (c) 2016 onwards, single-cell transcriptome analysis updated the differentiation pathways. LT-HSC = Long term HSC, ST-HSC = Short term HSC, CMP = common myeloid progenitor, CLP = common lymphoid progenitor, MEP = megakaryocyte–erythrocyte progenitors, GMP = granulocyte–monocyte progenitors, MPPs = Multipotent progenitors, LMPP = lymphoid-primed multi potential progenitor, EoBP = eosinophil–basophil progenitor, DCs = dendritic cells, NK = natural killer cells, ILCs = innate lymphoid cells (Laurenti and Göttgens 2018)**.**

The BM is the paradigmatic adult stem cell niche (Figure 1.2) and is divided into two niches, the endosteal and perivascular niches. The osteoblasts are located in the area of the endosteal niche and the perivascular niche is the area around the blood vessels. The quiescent HSCs are in the perivascular niche (Punt *et al.* 2019). The differentiated blood cells exit the BM from the perivascular niche via the blood vessels.

Figure 1.2 The haematopoietic niche



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HSCs are usually found adjacent to sinusoids that can be found throughout the BM. Mesenchymal stromal cells (MSCs) and endothelial cells in the perivascular niche produce stem cell factor (SCF) that promote HSC maintenance. It is likely cells around the bone surface in the endosteal niche also contribute to HSC maintenance, via the secretion of cytokines and expression of cell surface markers. In this way the perivascular and endosteal niches interact with each other (arrows). SCF = Stem cell factor. CAR = CXCL12-abundant reticular (Morrison and Scadden 2014).

HSCs can be used in regenerative medicine (transplantation) for patients with various malignant and non-malignant diseases such as leukaemia, lymphoma, myeloma, aplastic anaemia and beta thalassaemia. HSC transplantation (HSCT) was the original stem cell therapy. Allogeneic HSCT can be an effective treatment for patients with genetic conditions (Sung and Chao 2013).

1.2 Allogeneic Haematopoietic Stem Cell Transplantation

The first successful bone marrow transplant took place in 1959 when E. Donnall Thomas performed a syngeneic (identical twin) transplant for a patient with leukaemia (Thomas *et al.* 1959). It is a potentially curative therapy for haematological cancers, in which a patient receives a cytotoxic conditioning regimen to kill cancer cells, followed by an infusion of haematopoietic precursor cells

from a donor matched for major histocompatibility complex (MHC) antigens (Peled *et al.* 2020). Several studies have provided evidence that the HSC compartment consists of long-term repopulating (LTR) and short-term repopulating (STR) cells, and these are illustrated in table 1.1. In murine studies a long-term repopulating cell has been defined as a cell population that can repopulate lethally irradiated mice for over 6-months (Sitnicka *et al.* 2016).

Table 1.1 Haematopoietic stem/progenitor cell descriptions

Cell	Description
LT - HSCs	The most quiescent HSC which retains pluripotency throughout the life of the organism.
ST - HSCs	Predominantly quiescent HSCs but divide more frequently than LT HSCs and have a more limited self-renewal capacity compared to LT HSC.
MPPs	Limited ability to self-renew but can proliferate rapidly and give rise to both lymphoid and myeloid cell lineages.

Adapted from Punt *et al.* (2019). LT-HSC = Long term Haematopoietic Stem cell, ST-HSC = Short term Haematopoietic Stem Cell, MPPs = Multipotent progenitors

However, allogeneic HSCT has three major limitations (1) the pre-transplantation conditioning regime procedure's toxicity, and infection as a result of depletion of the body's immune cells, (2) graft versus host disease (GvHD) and (3) a potential lack of histocompatible donors – a human leukocyte antigen (HLA) matched sibling or unrelated donor is essential (Luznik *et al.* 2012). 70% of patients do not have an HLA matched donor in their family, and the chance of finding a matched unrelated donor depends on the patient's ethnicity and is between 23%-77% (National Marrow Donor Program – Be The Match (no date)).

The European Society for Blood and Marrow Transplantation (EBMT) annual activity survey found that its members had performed 18,483 first allogeneic HSCT and a total of 19,630 allogeneic HSCTs in 2018 (The European Society for Blood and Marrow Transplantation (no date)). During the 60 years of allogeneic HSCT treatments there have been many methodological changes designed to expand eligibility to older patients and/or those with comorbidities. Reduced intensity conditioning (RIC) regimens have been introduced and rely on the beneficial graft versus tumour (GvT) effects of the transplant to eliminate residual malignant cells (Blazar *et al.* 2012).

There are three different sources of HSCs: bone marrow, mobilised peripheral blood and umbilical cord blood, and each has its advantages and disadvantages for both the donor and the patient.

Advantages for the donor for mobilised peripheral blood stem cell collections (PBSC) are faster haematological recovery, and an avoidance of anaesthesia and potential exposure to blood products. Several studies have shown in the HLA matched sibling donor setting a survival advantage for PBSC over BM for patients with advanced leukaemia, but an increased risk of chronic GvHD (Eapen *et al.* 2007). The therapeutic efficacy of allogeneic HSCT for haematological malignancies relies largely on the graft versus leukaemia (GvL) effects exerted by the donor $CD3^+$ T cells, but there is a risk of uncontrolled GvHD (Delia *et al.* 2013) as the $CD3^+$ T cells may cause immunologic damage to the recipient's organs and tissues (Torelli *et al.* 2011).

Consensus discussions reported from the Center for International Blood and Marrow Transplant Research (CIBMTR, USA) have defined myeloablative or high-dose regimens, as most often including single or multiple alkylators and sometimes including total body irradiation (TBI) (Weisdorf 2017). A myeloablative regimen is a conditioning regimen that cannot be administered without stem cell support. The definition of myeloablative conditioning regimens allow for everything else to be considered an RIC regimen by default (Giralt *et al*. 2009).

Mobilised PBSC donations have an increased percentage of plasmacytoid dendritic cells (pDCs) without altering the number of myeloid dendritic cells (mDCs) (Morelli and Thomson 2007). T helper (T_H) cells can be divided into subgroups depending on their function. Following maturation pDCs favour allogeneic T-helper 2 (T_H2)-cell responses, while mDCs favour T_H1-cell responses. T_H2 cell responses are associated with immune responses to larger parasites and trigger IgE and eosinophils, while T_H1-cell responses are associated with immune responses to viral and bacterial infections and trigger cytotoxic responses (Punt *et al.* 2019). It may therefore be the case that the increased numbers of pDCs in the graft promotes a T_H2-cell response, which could favour engraftment. Although there is a 10-fold higher dose of transplanted T cells in PBSC recipients compared to BM cell recipients, acute GvHD (aGvHD) does not occur in a significantly higher proportion among PBSC recipients as might be expected based on the T cell dose (Rajasekar *et al.* 2010). Because these cells within the graft may be low in number compared to those present within the recipient, and may have limited potential for survival post infusion, it has been questioned if they have a significant impact on clinical outcomes.

A key element of the success of allogeneic HSCT is post-transplant immune reconstitution (Torelli *et al.* 2011). In allogeneic HSCT the transplanted HSCs commonly encounter diseased or damaged recipient environments (Dong *et al*. 2020). The important phases following transplantation of the stem cells are:

(1) Homing

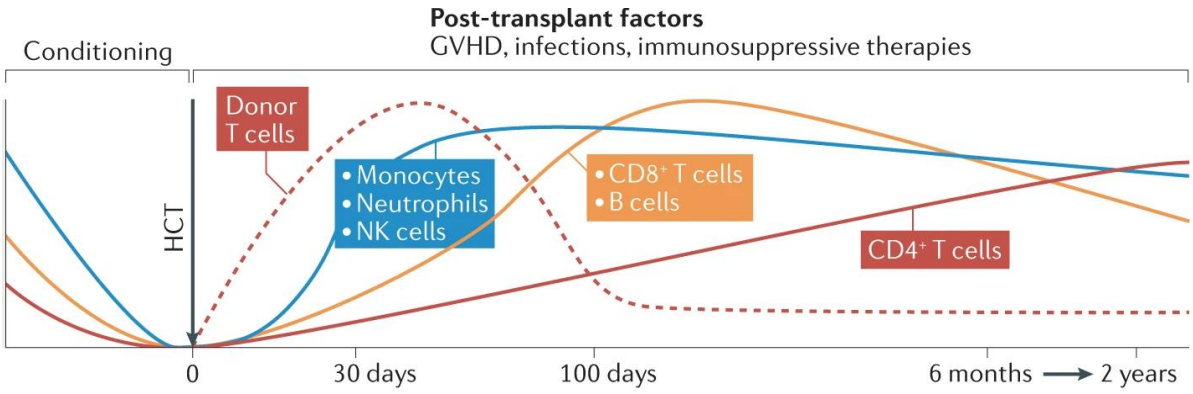
(2) Lodgement

(3) Localisation

(4) Niche interactions and proliferation

Figure 1.3 illustrates immune reconstitution following HSC transplantation. HSCs are thought to reach homeostatic levels during long term engraftment. Restoration of normal immune homeostasis requires engraftment and expansion of donor T cells contained in the graft and the differentiation of immune cells from donor haematopoietic progenitor cells (Lonial *et al*. 2013). Donor T cells not only lower the risk of tumour recurrence by inducing the GvL effect, but they also reduce the incidence of infections. Host T cells in the PB are depleted by the conditioning regimen, however memory T cells can reside in human tissues and Divito *et al.* (2020) report that host T cell chimerism in the gut and skin can be markedly different to the T cell chimerism in PB.

Figure 1.3 Time frame of immune cell reconstitution up to two years following haematopoietic stem cell transplantation



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The dynamics of cell reconstitution post HSC transplantation. Innate immune cells recover relatively early, while T and B cells can take up to 2+ years to recover. The 'first wave' of T cells post allograft are donor T cells undergoing alloactivation and proliferation. HCT = Haematopoietic cell transplantation, NK cells = Natural killer cells. Each line represents repopulation of recipient blood by specific cell types (Velardi *et al.* 2020).

Once the transplanted cells are in the recipient, they must ensure myeloid repopulation, immunological reconstitution and the acquisition of tolerance to host HLA molecules via central or peripheral mechanisms. Peripheral tolerance after allogeneic transplantation depends on multiple regulatory mechanisms aimed at blocking allo-immune activity while maintaining immune responses

to pathogens and tumour antigens (Roncarolo *et al.* 2011). The first six months after allogeneic HSCT is the most sensitive time window for tolerance induction (Ukena *et al.* 2011).

The following key events occur at varying times post allogeneic HSCT (Apperley *et al.* 2012):

(1) Neutrophil and platelet engraftment – usually within the first 28 days.
(2) Acute graft versus host disease (aGvHD) – usually within the first 100 days.
(3) Relapse/Progression – can occur at any time.
(4) Death – can occur at any time.
(5) Chronic graft versus host disease (cGvHD) – usually after the first 100 days.

Dong *et al.* (2020) studied HSC transplantation in murine models using transcriptomics and immunophenotyping of the transplanted cells. They were able to group the haematopoietic cells into 21 populations based on transcriptome analysis. They found that as expected most HSCs were quiescent, but they also found that the multipotent progenitors (MPPs) and committed progenitor cells (CPs) were remarkably variable in terms of their cell cycle status. The cellular dynamics one week following transplantation showed the percentage of HSCs gradually decreased, while the MPPs emerged as a major population. The data was consistent with the model that long-term reconstitution in the early post-transplant phase is mostly derived from a reserved, small sub-set of HSCs that maintain both their molecular and functional status. The data also suggested there is an early erythroid and myeloid cell biased differentiation from HSCs and/or MPPs after transplantation, and that the physiological roles and significance of these cells warrant further study in human transplantation.

The major adverse outcomes following allogeneic HSCT are relapse, GvHD, infection and toxic effects on organs, these can be due to natural processes or as a consequence of treatments (Peled *et al.* 2020).

Most deaths after allogeneic HSCT occur within the first 2 years post-transplant and may be due to: relapse, acute or chronic GvHD, infection, or other acute or subacute toxicities of HSCT. Death beyond 2 years is infrequent (Wingard *et al.* 2011). Disease relapse is currently the major cause of death following allogeneic HSCT for haematological malignancies (Blazar *et al.* 2020).

1.3 Donor selection and laboratory testing to prevent Graft versus Host Disease

Donor selection is an important factor in the success of allogeneic HST. In general, an HLA matched related donor (MRD) and an HLA matched unrelated donor (MUD) are the first and second preference for allogeneic HST (Kawamura *et al.* 2019).

The British Society for Histocompatibility & Immunogenetics (BSHI) published recommendations in 2016 on HLA matching and donor selection (Little *et al.* 2016). They recommend HLA high-resolution typing should be performed on potential matching; mismatching and haploidentical related donors, when familial haplotypes cannot be fully assigned. That a 10/10 high-resolution HLA-A, -B, -C, -DRB1 and -DQB1-matched unrelated PBSC or bone marrow donor should be used where possible. If no such donor exists, a single mismatch at HLA-A, -B, -C, -DRB1 or -DQB1 is acceptable. Cytomegalovirus status matched donors should be selected if possible. They recommend ABO matched donors and male donors be selected if the patient has multiple HLA matched donors, and that young donors should be preferentially selected.

While ever closer matching in the future may reduce the risk of GvHD, some mismatched antigens may be important for beneficial GvL responses (Zeiser and Blazar 2017).

1.4 The aetiology of Graft versus Host Disease

GvHD is the most recognised complication post allogeneic HSCT and was first observed in 1956 in a murine model (Barnes and Loutit 1957). Allogeneic GvHD and GvL are driven by the interaction of host and donor APCs that encounter mature T cells from the donor graft (Magenau *et al.* 2016). A proportion of the APCs present express host MHC or minor histocompatibility antigen peptides, which interact with a T cell expressing a suitable T cell receptor (Zeiser and Blazar 2017), and initiate an immune response against them (Hippen *et al.* 2011). Examples of minor histocompatibility antigens are HY and HA-3 which are found on all tissues (Ferrara *et al.* 2009). Historically acute and chronic GvHD were separated by the 100-day marker, but it is now acknowledged that aGvHD can occur after 100 days, as late aGvHD, for example after donor lymphocyte infusion (DLI) (Ghimire *et al.* 2017). A DLI is an infusion of lymphocytes from the original donor that can be used for mixed chimerism and/or relapse treatment. Acute GvHD typically targets the skin, gut and liver. Chronic GvHD most commonly affects: skin, nails, mouth, eyes, female genitalia, gastrointestinal (GI) tract, liver, lungs, muscles, fascia and joints (Apperley *et al.* 2012).

GvHD classically develops over five steps (Sung and Chao 2013):

(1) The conditioning regimen (radiation or chemotherapy) given to the recipient causes tissue damage and releases pro-inflammatory cytokines.
(2) Donor T cell activation is triggered by recipient antigens presented by host APCs and sustained by donor APCs.
(3) Donor T cells proliferate and differentiate into naïve, effector, memory, regulatory and other subsets.

(4) Activated self-reactive T cells migrate from the secondary lymphoid organs to target tissues such as the skin, liver or gut.
(5) Once these T cells arrive at the target organ(s), they cause tissue destruction.

Skin is the principal target organ of aGvHD, and the initial manifestation is a maculopapular rash, which can spread throughout the body. Martin *et al.* (1990) studied 740 allogeneic transplants in Seattle, USA and found 81% of patients with aGvHD had skin involvement. Damage to the skin was defined by vacuolar degeneration of the basal cell layer, dyskeratotic keratinocytes and mononuclear cell infiltrates.

The GI tract can be the most severely affected organ. It is manifested by secretory and voluminous diarrhoea, severe abdominal pain, vomiting and anorexia (Ferrara *et al.* 2009). Apoptosis of epithelial cells has been observed with patchy ulcerations and apoptotic bodies in the base of crypts, with loss of surface epithelium.

Hepatic GvHD is characterised by abnormal liver function and a rise in bilirubin and alkaline phosphatase levels in the peripheral blood (Ghimire *et al.* 2017). Donor lymphocytes target the bile duct epithelial cells and cause endothelialitis, pericholangitis and apoptotic bile duct destruction.

1.5 Immunological response in graft versus host disease

GvHD and relapse are the two most important causes of post transplantation recipient mortality (Podgorny *et al.* 2014). Donor $CD4^+$ and $CD8^+$ T cells have crucial roles in the pathogenesis of GvHD (Blazar *et al.* 2012). Transplants carried out in the HLA matched sibling can still produce GvHD due to differences in minor HLA antigens (Ghimire *et al.* 2017), and these have been associated with both GvHD and GvL. Other major complications include viral and fungal infections, which can occur due to the immunosuppressive nature of allogeneic HSCT. The treatment of GvHD can also risk infectious complications, and unfortunately patients suffering from severe GvHD may die from these (Ghimire *et al.* 2017). Non-relapse-related mortality (NRM) (for example infections) can occur independently from the occurrence of GvHD.

GvHD occurs because of immunologic damage to host tissue by conventional T and NK cells in the transplanted donor graft. GvHD is classically $CD8^+$ T cell and MHC class I dependant. Molecules known as damage associated molecular patterns (DAMPs), which are released from cells following the conditioning regimen, can induce tissue damage and therefore may also play a role in GvHD. In murine models, dying cells in the gut release ATP, which can bind to its receptor P2X7 on host APCs and activate the inflammasome (Robb *et al.* 2012). An inflammasome is an intracellular multiprotein

complex that contains caspases, for example caspase-1 and caspase-11 (Jankovic *et al*. 2013). This leads to upregulation of the expression of co-stimulatory molecules on APCs, leading to increased antigen presentation and activation of T cells (Blazar *et al.* 2012).

There is a lack of conclusive and reproducible evidence supporting roles for $CD4^+$ T cell subsets, T_H1 cells (associated cytokines include: interleukin (IL) IL-1, IL-2, IL-6, IL-12 and interferon [IFN]-γ) or T_H2 cells (associated cytokines include: IL-4, IL-5, IL-9, IL-10 and IL-13) in GvHD (Blazar *et al.* 2012). T_H17 cells (associated cytokines include: IL-17A, IL-17F, IL-21 and IL-22) have been shown to have a role in GvHD pathobiology, as studies have shown that T_H17 cells are sufficient, but not necessary to induce GvHD. Biopsy samples from patients with gut aGvHD contain IL-17 producing cells, though interestingly not from patients with skin GvHD (Blazar *et al.* 2012).

A deficiency in immune tolerance mechanisms may allow newly transplanted T cells to react with host antigens and cause tissue damage that may manifest as GvHD (McIver *et al.* 2008). Allogeneic HSC transplantation can foster the development of tolerance as host APCs are replaced with donor APCs and donor T cells (Hippen *et al.* 2011). Following transplantation, in the direct pathway, donor DCs from the graft present intact donor MHC molecules to allospecific T cells. In the semi direct pathway, recipient T cells interact with donor MHC molecules that have been transferred from the surface of donor cells to recipient DCs. In the indirect pathway, recipient DCs that have processed donor alloantigen, present the allo-peptides on the recipient MHC molecules to donor reactive T cells. The type of graft and the time after transplantation influences the participation of each of these pathways (Morelli and Thomson 2007). The risk period for donor anti-host alloresponses that result in GvHD is typically highest in the first 1-3 months post HSC transplant.

The success of allogeneic HSCT is limited by the occurrence of GvHD, which is fatal in approximately 15% of transplant recipients. Steroids are the first line of treatment, but patients with steroid refractory aGvHD have a dismal outcome (Blazar *et al.* 2012) with the 1-year mortality rate as high as 80% (Yu *et al.* 2019).

1.6 Acute Graft versus Host Disease

Acute GvHD involves alloreactive donor T cell mediated cytotoxic responses against the tissues of the recipient, mediated by cell surface and secreted factors. Immune cell activation initiates intracellular biochemical cascades that induce the transcription of genes for many proteins including cytokines and cytokine receptors (Ferrara *et al.* 2009) such as interferon γ, IL-2 and tumour necrosis factor-alpha (TNF-α), which are released during acute GvHD. The immune system insufficiently controls the alloreactive effector T cells, which expand and are recruited to the site of inflammation

(Ukena *et al.* 2011). Tissue damage caused by the cytotoxic T cells leads to the recruitment of other effector cells (including NK cells and neutrophils) which further augment tissue injury and can result in a self-perpetuating state of GvHD that can be difficult to control once it is initiated (Blazar *et al.* 2012).

Moderate to severe aGvHD occurs in approximately 40% of all recipients of allogeneic HSCT (Apperley *et al.* 2012). The risk factors for aGvHD are shown in table 1.2.

Table 1.2 Risk factors for GvHD

Donor	Recipient
HLA compatibility (related/unrelated)	Age (older)
Sex mismatched (F→M)	Conditioning regimen
Alloimmunisation (parity, transfusions)	Prevention of GvHD
Stem cell source (PBSC>BM>CB)	

F = Female, M = Male, PBSC = Peripheral blood stem cell, BM = Bone marrow, CB = Cord blood. Stem cell source - PBSC has the highest risk of GvHD and CB the lowest (Apperley *et al.* 2012).

A three-phase model has been created for aGvHD (Ghimire *et al.* 2017):

Phase 1. Tissue damage due to conditioning that activates host antigen presenting cells

Conditioning is used to eradicate disease and prepare the recipient's BM to receive the donor HSCs, to enable engraftment without rejection. Damage to the recipient tissue is caused by the disease, treatment(s), any infections, and the conditioning. The damaged recipient tissue releases danger signals such as TNF-α and IL-1. This activates the recipient APCs.

Phase 2. Donor T cell activation

The donor T cells recognise alloantigen on recipient APCs in a process known as direct antigen presentation. They can also recognise alloantigen on donor APCs in a process known as indirect antigen presentation. $CD8^+$ T cells recognise variations in the MHC class I antigens, while $CD4^+$ T cells recognise variations in the MHC class II antigens. Peyer's patches in the GI tract, are probably the location of the initial interaction between activated APCs and donor T cells (Ferrara *et al.* 2009).

Phase 3. Target cell apoptosis

In this phase both innate and adaptive immune cells work synergistically to exacerbate the T cell induced inflammation. Inflammatory cytokines synergize with the $CD8^+$ T cells resulting in further

tissue injury and possible target organ dysfunction. Monocytes/macrophages are stimulated to secrete inflammatory cytokines resulting in amplification and propagation of a cytokine storm.

In 1974 Glucksberg (Glucksberg *et al.* 1974) published the first aGvHD classification, which is still in use today. The Glucksberg grade is obtained using two tables. The first gives each affected organ a stage, and is shown in table 1.3. The second table then uses these stages to assign the grade, and is shown in table 1.4.

Table 1.3 Glucksberg table one used for staging GvHD

Stage	Skin/Maculo-papular rash	Liver / Bilirubin (µmol/L)	GI tract / Diarrhoea (ml)
+	<25% of body surface	34-50	>500
++	25-50% of body surface	51-102	>1000
+++	Generalised erythroderma	103-255	>1500
++++	Generalised erythroderma with bullae formation and desquamation	>255	Severe abdominal pain with or without ileus

Adapted from (Apperley *et al.* 2012)

Table 1.4 Glucksberg table two used for staging GvHD

Grade of aGvHD	Degree of organ involvement
I	Skin: + to ++
II	Skin: + to +++ Gut and/or liver: + Mild decrease in clinical performance
III	Skin: ++ to +++ Gut and/or liver: ++ to +++ Marked decrease in clinical performance
IV	Skin: ++ to ++++ Gut and/or liver: ++ to ++++ Extreme decrease in clinical performance

Adapted from (Apperley *et al.* 2012)

It is routine practice to split aGvHD into two groups: clinically insignificant (grades 0-I) and clinically significant (grades II-IV).

1.7 Chronic Graft versus Host Disease

Chronic GvHD occurs in 40% of HLA identical sibling unmanipulated HSC transplants, more than 50% of HLA- non-identical related HSC transplants, and in 70% of matched unrelated HSC transplants (Apperley *et al.* 2012). The risk factors for cGvHD are the same risk factors as for aGvHD and/or prior aGvHD. Chronic GvHD may manifest simultaneously from aGvHD, develop after treatment for aGvHD, or may occur *de novo*. Classical cGvHD occurs after 100 days post-transplantation and may overlap with aGvHD (Ghimire *et al.* 2017). The development of sclerotic lesions is a hallmark of cGvHD, and they can occur in almost every organ (Ghimire *et al.* 2017). The National Institute of Health (NIH) (USA) consensus development project graded cGvHD in the following levels: mild, moderate or severe (Filipovich *et al.*2005).

1.8 Prevention and Treatment of Graft versus Host Disease

Methotrexate has been used since the 1950s to shut down T cells through inhibition of the enzyme dihydrofolate reductase, which is essential to produce purines for DNA synthesis, in several clinical settings, including HSCT (Sung and Chao 2013). GvHD prophylaxis can include calcineurin inhibitors despite their incomplete efficacy, and their impairment of GvL responses (Shrestha *et al.* 2020). Calcineurin inhibitors exert their immunosuppressive effects by reducing IL-2 production and IL-2 receptor expression, leading to a reduction in T cell activation (Ghimire *et al.* 2017). Examples of calcineurin inhibitors are tacrolimus or ciclosporin A (Sung and Chao 2013). *Ex vivo* T cell depletion is no longer routinely used in HLA matched transplantation as it largely abolishes GvL effects (Ghimire *et al.* 2017).

Anti-thymocyte globulin (ATG), anti-T lymphocyte globulin (ATLG) are frequently used for GvHD prevention in Europe (Bonifazi *et al.* 2020). ATG causes a rapid decrease in naïve $CD4^+$ and $CD8^+$ T cells in both adults and children that usually lasts for at least 2 months (Safinia *et al.* 2012). In contrast memory $CD4^+$ and $CD8^+$ T cells are resistant to depletion by ATG and these cell subsets increase over the first 6 months post transplantation. To limit this memory T cell expansion, transplant recipients are placed on other immunosuppressive drugs, most commonly tacrolimus or ciclosporin A, (Sung and Chao 2013), and mycophenolate mofetil which is an anti-proliferative agent. Patients may also be given the monoclonal antibody alemtuzumab, which lyses lymphocytes. These drugs can markedly deplete most of the leukocyte populations in peripheral blood (Safinia *et al.* 2012).

Because ATG/ATLG will react against both recipient and donor lymphocytes, the expected effects are a reduction in the risk of both graft failure and GvHD. However, their use is associated with delayed

immune reconstitution, which may result in an increased risk of infections and relapse (Bonifazi *et al.* 2020).

Afferent phase treatments include targeting donor T cell activation by various methods: calcineurin inhibitors and mycophenolate which interfere with T cell activation signals. Post-transplant cytotoxic approaches that target activated T cells include cyclophosphamide (Ghimire *et al.* 2017).

Efferent phase treatments include corticosteroids. The broad activity of these includes induction of T cell apoptosis, suppression of macrophage activation and cytokine release. Corticosteroids are the first line treatment for both acute and chronic GvHD. GvHD may also be treated with drugs such as sirolimus, tacrolimus and methotrexate. Extracorporeal photopheresis is also a treatment option for acute and chronic GvHD, and it has been postulated that this may induce T regs (Ghimire *et al.* 2017).

Cellular therapy is a promising strategy for steroid refractory aGvHD, and this group includes T regulatory cells and mesenchymal stromal cells (Ghimire *et al.* 2017). Since acute GvHD requires T cell migration into the target organ(s), which is influenced by chemokine gradients, targeting the relevant chemokine receptor (e.g. CCR5 for liver GvHD) could be a potential treatment strategy (Zeiser and Blazar 2017). Potential future treatments for GvHD include chimeric antigen receptor T (CAR-T) cells. Using a xenogeneic GvHD murine model Shrestha *et al.* (2020) developed $CD83^+$ targeted CAR-T cells for GvHD prevention. CD83 is expressed on allo-activated conventional $CD4^+$ T cells and proinflammatory DCs, both of which are implicated in GvHD. The authors demonstrated the CD83 CAR-T cells provided lasting GvHD prophylaxis in a mouse model.

While the ability to prevent and treat GvHD improves, further research is required to improve the treatment of GvHD while maintaining or maximising GvT effects. It is also believed that many treatments are initiated too late, after major changes have already damaged the recipient's tissue(s). Any biomarkers allowing early identification of patients who are at risk of GvHD would therefore be extremely useful to prevent early detrimental changes to recipient tissue(s).

1.9 Predictive factors of Graft versus Host Disease

HLA mismatch is the strongest determinant of GvHD (Sung and Chao 2013). Other risk factors include use of female donors for male recipients, multiparity in donors, and total body irradiation versus reduced intensity conditioning (as the latter causes less damage and less GvHD).

1.10 Biomarkers

The word “biomarker” is a portmanteau of “biological marker” and refers to medical signs (not symptoms) that can be measured objectively, accurately, and reproducibly. Because there may be multiple pathways involved in a particular disease, a particular biomarker may only correlate with clinical endpoints under limited circumstances. The NIH has defined a biomarker as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses, to a therapeutic intervention” (Biomarkers Definitions Working Group 2001). The relevance of a biomarker refers to the ability of a biomarker to provide clinically relevant information (Strimbu and Tavel 2010).

The following characteristics are important for biomarkers (Edelstein 2010):

1. Non-invasive, easily measured, inexpensive and produce quick results
2. Be from readily available sources e.g. blood or urine
3. Have high sensitivity allowing early detection, with no overlap in results between diseased patients and healthy controls
4. Have high specificity, being highly up or down regulated specifically in diseased patients and unaffected by other conditions
5. Biomarker levels should change in response to treatment
6. Biomarker levels should enable risk stratification and have prognostic value in the real world
7. The biomarker should be biologically plausible and may provide insight into the disease mechanism.

While invasive biopsies are usually a safe procedure, they are not risk free. Stec *et al.* (2010) found major and minor complication rates of 6.1% and 27% respectively in a group of 115 open biopsy renal patients. In addition to these health risks, biopsies can cause anxiety for the patient. Blood and urine are collected during the normal care of a patient and have low health risks for the patient.

Peripheral blood (PB) is the most studied material for immune monitoring, due to ease of sampling and the application of well-developed techniques such as flow cytometry. However, trials of T reg transfusions have shown homing of the T reg cells to ‘sites of action’ (Stark *et al.* 2021) and therefore data obtained from tissue analysis could be important and have biomarker value. However, trials on potential biopsy biomarkers may require repeated invasive sampling which would be difficult to gain ethical approval for as well as possibly detrimental to the patient. A biomarker from an invasive

source (for example spleen biopsy for Tr1 cells) may therefore be moot, and would likely have to be a very good biomarker to be considered in the clinic.

Receiver operating characteristic (ROC) curves can be used to determine the clinical diagnostic value of a biomarker, and the area under the ROC curve (AUC) is a statistic used to evaluate the value of a biomarker. An AUC of 1.0 would be a perfect biomarker, while an AUC of 0.5 is a result no better than chance. A good biomarker would be expected to score at least 0.75, an excellent biomarker would be expected to score at least 0.9 (Edelstein 2010).

Biomarkers can be used for assessment of the risk of disease progression, treatment decisions and medical interventions. Biomarkers may be molecules (for example cytokines) and/or cells (for example DCs). Biomarkers can be categorised into the following groups:

1. Diagnostic (an example in GvHD would be to identify GvHD patients at the onset of the disease to differentiate their disease from other conditions).
2. Prognostic (an example for GvHD would be to identify patients with different likelihood of GvHD before the onset of disease).
3. Predictive (an example for GvHD would be to identify patients' likelihood to respond to therapy before therapy has been initiated.
4. Response to treatment (an example for GvHD would monitor response to treatment which would require a pre-treatment sample).

This study is studying peripheral blood tolerogenic cells as a prognostic biomarker.

A $CD4^+$ T cell count of > 50/µl within 100 days of transplant has been proposed as a reliable predictor of outcomes including relapse, survival and non-relapse mortality (Boelens *et al.* 2020). Acute GvHD predictive biomarkers include IL-6 and Stimulation-2. Adom *et al.* (2020) reported that regulatory T cells as defined by the $CD4^+CD25^{hi}FoxP3^+$ phenotype can be a diagnostic and predictive biomarker of GvHD. $CD4^+CD25^{hi}FoxP3^+$ lymphocytes have also been shown to be a potential response to treatment biomarker (Magenau *et al.* 2010). However, no single biomarker has established itself in routine care of transplant patients, and biomarkers remain an active area of research (Magenau *et al.* 2016).

1.11 Immune cell migration

Immune cells, including the dendritic and regulatory cells analysed in this study, are produced from haematopoietic stem cells. Following production in the primary lymphoid organs the cells are released into the bloodstream. Immune cells circulate between the peripheral blood, lymphatic system, secondary lymphoid organs and tissues, and most white blood cells function in locations

other than blood, blood is just the transport system allowing cells to migrate to their site of action (Punt *et al.* 2019). This study is only obtaining samples from PB, PB being a non-invasive source suitable for a potential biomarker. (Peripheral blood could also be used for molecular biomarkers, for example cytokines, but these were not analysed in this study). The other locations immune cells are present in would require an invasive procedure, which is less suitable for a potential biomarker. However, peripheral blood is not a 'site of action' in GvHD, which in its acute setting is usually the skin, gut and liver, and it is in these 'sites of action' and the secondary lymphoid organs, where the circulating tolerogenic cells may exert their influence.

Using a murine model of pancreatic islet transplantation, Gagliani *et al.* (2013) studied transfused FoxP3 T regs and Tr1 cells and transplant tolerance. They found these different regulatory T cells acted differently *in vivo*, the FoxP3 T regs accumulated into the graft while the Tr1 cells were found in the spleen and were maintained there long term. The group postulated that the graft FoxP3 T regs "hand tolerance over" to the Tr1 cells within the spleen. The spleen is a large secondary lymphoid organ which responds to bloodstream antigens and is therefore important in systemic infections (Punt *et al.* 2019). Panoskaltsis-Mortari *et al.* (2004) found that donor T cells located to the lymphoid tissues within hours of transplantation in an allograft murine model, and between the third and seventh day the allogeneic T cells increased in numbers in the GvHD target organs. Donor T cells migrate to peripheral lymphoid tissues and the spleen within hours of transplantation, where they can interact with host APCs that have been matured by the effects the patient's conditioning regimen (Wysocki *et al.* 2005).

The cell counts obtained in this study may or may not offer insight into the pathophysiology of GvHD, but that was not the study's purpose. The study aims to discover if certain PB tolerogenic cell counts can be used as a biomarker for patients at risk of GvHD.

1.12 Tolerance and Tolerogenic cells

The immune system can attack pathogens, while acquiring and maintaining a state of tolerance to the body's own tissues, commensal microorganisms, and food antigens that it encounters every day (Manicassamy and Pulendran 2011). The study of clinical transplant tolerance has resulted in enhanced understanding of the mechanisms and cells involved in immune regulation, including APCs and regulatory T cells, which play key roles in promoting tolerance (Ezzelarab and Thomson 2011). Transplantation tolerance can be defined as a state of specific unresponsiveness to host and donor allo-antigens, with preserved responsiveness towards pathogens (Roncarolo *et al.* 2011). While there has been much progress in understanding the role of innate immunity in inducing protective responses against pathogens, little is known about its ability in promoting tolerogenic responses and

suppressing immune responses (Manicassamy and Pulendran 2011). Immunological tolerance is often split into two processes: central tolerance (operating in the primary lymphoid organs, BM and thymus) and peripheral tolerance (believed to operate in the secondary lymphoid organs or tissue site (Punt *et al.* 2019)). Because the thymus has undergone involution in adults, post HSCT immunity in adults lacks central tolerance mechanisms. This implies a greater role for peripheral tolerance mechanisms in allogeneic responses (Magenau *et al.* 2016). Tolerogenic DCs present antigen to antigen specific T cells, but do not give adequate co-stimulatory signals for effector T cell activation and proliferation. This then manifests as T cell death, T cell anergy or T reg generation and/or expansion (Morelli and Thomson 2007). Mechanisms of peripheral tolerance consist of peripheral clonal deletion or active suppression mediated by regulatory cells. Within the $CD4^+$ regulatory T cell subsets, the best characterised are the $CD4^+CD25^+FoxP3^+$ T reg and the Type 1 regulatory T (Tr1) cells (Roncarolo *et al.* 2011).

1.13 Dendritic cells

DCs are a heterogeneous group of professional APCs that are derived from the BM (Morelli and Thomson 2007) and can be isolated fresh from blood, lymphoid or non-lymphoid organs (Rogers *et al.* 2013). DCs are potent APCs with dual functions, they can be either immunogenic or tolerogenic in nature (Moreau *et al.* 2012). DCs also have a role in both central and peripheral tolerance. DCs bridge the innate and adaptive immune system; as well as being able to recognise and eliminate 'foreign' antigen, they circulate through tissues and lymphoid organs, taking up and presenting peptides from apoptotic cells and other 'self' antigens to T cells. Through this mechanism, they play a critical role in peripheral tolerance (Volchenkov *et al.* 2013). DCs promote peripheral tolerance by generating regulatory T cells and by altering the T-helper 1 (T_H1)/T_H2/T_H17 balance (Manicassamy and Pulendran 2011).

There are several subtypes of human DCs including mDC, pDC and Langerhans cells *in vivo*. Both mDC and pDC originate from $CD34^+$ haematopoietic progenitor cells in the BM and enter the circulation as precursor DCs, before reaching their final tissue locations, facilitated by the expression of L-selectin (in non-inflamed states) or E-selectin (in inflamed states) (Rogers *et al.* 2013). In the steady state, DCs in blood and non-lymphoid tissues are phenotypically immature (Morelli and Thompson 2007). And they exist as conventional DC (cDC) or precursor DC (pre-DC). In tissue culture experiments DCs are typically 2 orders of magnitude more effective as APCs than B cells or macrophages (Hawiger *et al.* 2001, Moreau *et al.* 2012). Circulating DCs are important in two different arms of immune tolerance induction (1) initiating central tolerance through negative

selection, by migrating from the periphery to the thymus, bringing with them peripheral self-antigens and (2) Induction of T regs in the periphery (Proietto *et al.* 2008).

Conventional/myeloid DC (cDC/mDC) and plasmacytoid DC (pDC) play distinct roles in the innate and adaptive immune responses by the expression of their specialised cytokines and molecules. Maturing pDCs, unlike mDCs, increase their levels of inducible co-stimulator ligand (ICOS-L) which endows those APCs with the ability to promote *de novo* differentiation of T regs (Ogata *et al.* 2012).

Another subgroup of DCs are the more recently defined DC-10 cells. DC-10 cells are APCs that have been found to efficiently promote the induction of IL-10 producing Tr1 cells *in vitro* (Amodio and Gregori 2012). Tolerogenic DCs are characterised by low production of pro-inflammatory and high production of anti-inflammatory cytokines, reduced expression of co-stimulatory molecules, and high levels of inhibitory molecules and can induce T reg cells (Volchenkov *et al.* 2013). IL-10 is a cytokine that plays a central role in controlling inflammatory responses, suppressing T cell responses and maintaining immunological tolerance (Battaglia *et al.* 2006). IL-10 also inhibits cytokine production by T cells and monocytes/macrophages and can induce long lasting antigen specific anergy in both $CD4^+$ and $CD8^+$ T cells. IL-10 also plays a fundamental role in the reciprocal effects of tolerogenic DC and T reg cells (Ezzelarab and Thomson 2011).

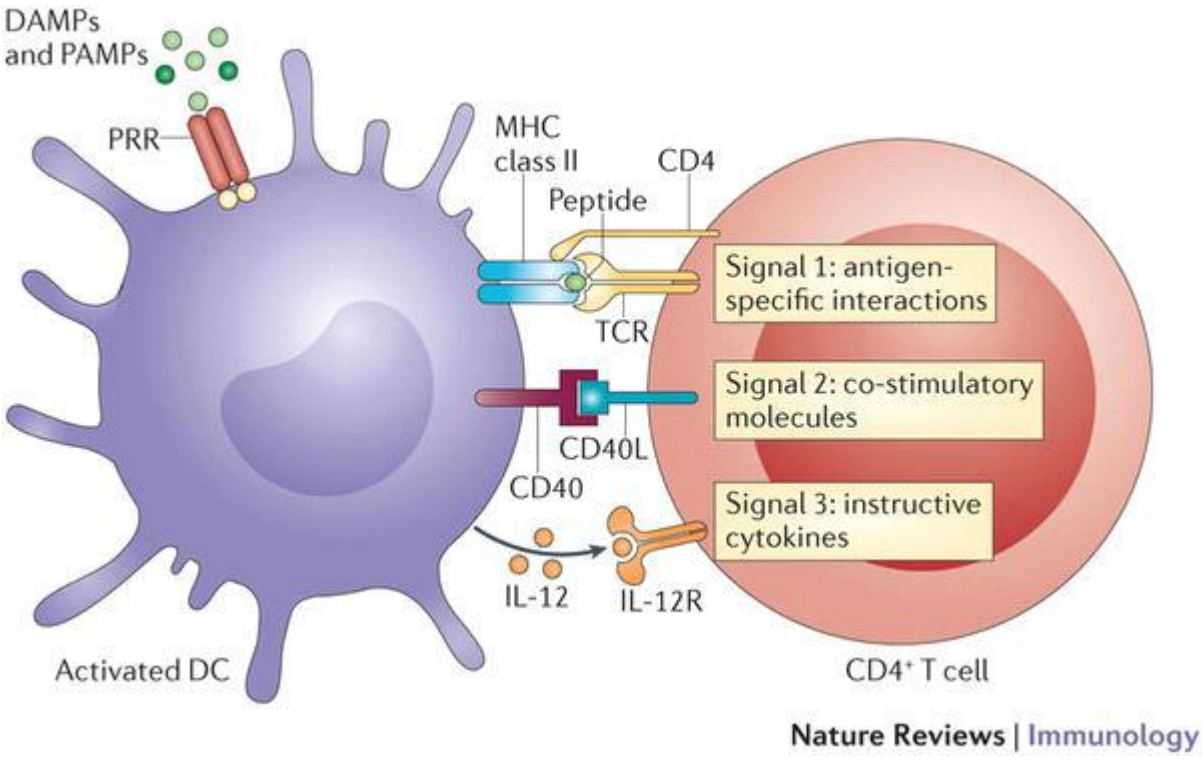
1.14 Dendritic cells are the bridge between innate and adaptive immunity

The innate immune system relies on a variety of receptors to sense pathogens and these pattern recognition receptors (PRR) include: Toll-like receptors (TLR), C-type lectin receptors (CLR), Retinoic acid-inducible gene I (RIG-I) like receptor, Nucleotide oligomerization domain (NOD) like receptors and Absent-in-Melanoma (AIM) like receptor (Punt *et al.* 2019). Activation of most TLRs promotes T_H1 responses (Iwasaki and Medzhitov 2010). Studies have shown that TLR2 signalling promotes IL-10 and tolerogenic responses (Manicassamy and Pulendran 2011).

APCs, activated by PRR signals, engulf and process antigen at the site of infection. Activated APCs in barrier tissues up-regulate chemokine receptor CCR7, which interacts with chemokine CCL21, which is present on the endothelial cells of the lymphatic vessels. The activated APCs can migrate to local (draining) lymph nodes and travel via the afferent lymphatic vessels to the lymph nodes. Once in the lymph node, each APC can be scanned by up to 5000 naïve T cells per hour (Punt *et al.* 2019). The DCs may induce effector responses, however if there is an absence of inflammatory or infectious signals, DCs present self-antigens for the induction and maintenance of self-tolerance (Hadeiba *et al.* 2008). The responses initiated are a combination of direct cell-cell interactions and indirect cell-cytokine interactions (Proietto *et al.* 2008).

DCs are the only APCs that can activate naïve T cells. Three distinct signals are required to induce naïve T cell activation, proliferation, and differentiation (Punt *et al.* 2019). Signal 1 is the T cell receptor (TCR)/MHC-peptide interaction, signal 2 is the costimulatory action of molecules such as CD28-CD80/86 or CD40L-CD40. Signal 3 is initiated by the polarising cytokines, IL-2 and transforming growth factor (TGF)-β which leads to T reg differentiation, as shown in figure 1.4.

Figure 1.4 Three signals are required for activation of a naïve T cell by activated DCs



Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Immunology. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? Taku Kambayashi *et al*. Macmillan Publishers Limited. 2014.

DAMPS = Damage-associated molecular patterns, PAMPS = Pathogen-associated molecular patterns, PRR = Pattern recognition receptor, TCR = T cell receptor. The three-signal hypothesis is as follows. Signal 1 – TCR and MHCII-peptide interactions with co-receptor CD4. Signal 2 – A separate set of molecules interact and co-stimulate, such as CD28-CD80/86 or CD40L-CD40. Signal 3 – Cytokines are released from DCs that direct T cell proliferation (IL-2) and differentiation (e.g. IL-12) via the corresponding receptor (e.g. IL-12R) (Kambayashi and Laufer 2014).

In the HLA matched allogeneic HSCT setting, DCs arising from the donor graft, and DCs persisting from recipients are expected to present endogenous donor minor histocompatibility antigen

peptides that will influence T cell activation and/or induction of tolerance. Recipient DCs are radioresistant and can survive pre transplant conditioning regimens that target cycling or proliferating cells (Rajasekar *et al.* 2010).

A murine model has shown that recipient DCs generated *in vitro* and activated by lipopolysaccharide, have regulatory properties that could be used to prevent lethal GvHD following allogeneic BM transplantation in mice. The tolerogenic DCs downregulated the anti-host reactivity of donor T cells and prevented GvHD. This correlated with an increased incidence of donor derived natural T regs (nT regs), this did not affect GvL, which gives the technique a potential advantage (Sato *et al.* 2003). Using a mouse model Larsen *et al.* (1990) found that donor DCs migrated out of mouse cardiac allografts into the recipient's spleen where they stimulated T cells. This provides evidence that DCs migrate from peripheral tissues to lymphoid organs post transplantation in mice. Clinical trials have been performed where immature DCs pulsed with antigen have been injected into volunteers, and this injection resulted in the specific inhibition of antigen specific $CD8^+$ T cell effector function and the emergence of antigen specific IL-10 producing cells (Dhodapkar *et al.* 2001).

To avoid graft rejection, recipients receive lifelong immunosuppressive drugs such as tacrolimus, mycophenolate mofetil (MMF) and prednisolone (Moreau *et al.* 2012). Tolerogenic DC therapy could be an alternative and improved approach to immunosuppression post-transplantation. Due to their dual nature (immunogenicity v tolerogenicity), DCs can be considered as potential therapeutic agents (for organ transplantation or autoimmune diseases) or cellular carriers (anti-cancer vaccines) (Ezzelarab and Thomson 2011). DCs may have a potential role to ameliorate or prevent graft rejection, GvHD and autoimmune disorders. Moreau *et al.* (2012) used tolerogenic DCs that expressed both self and donor MHC molecules, the self MHC were loaded with donor allopeptides, and this resulted in the indefinite survival of kidney grafts in murine models of kidney transplantation.

1.15 Plasmacytoid Dendritic Cells

The ability of pDCs to induce immunity or tolerance may reflect that they exist at different stages of maturation, or that this group might include more than one population (Morelli and Thomson 2007).

The role of pDCs in clinical allogeneic HSC transplantation and their role in clinical outcomes such as survival, event free survival (EFS), incidence of acute and/or cGvHD is not clearly defined in the current literature (Rogers *et al.* 2013). pDCs in 'steady state' conditions (in the absence of any detectable infection or overt inflammation) are immature, they express low levels of MHC class II and costimulatory molecules. It has been reported that pDCs have less capacity for foreign antigen

uptake than mDC, and that this may mean that pDC present more self-antigen–MHC complexes than mDC (Hadeiba *et al.* 2008). As pDC are normally found in the thymus and peripheral lymphoid tissues they may be involved in tolerance to a greater extent than other DCs.

In innate immunity, pDCs behave as type-I IFN producers due to their ability to produce robust amounts of type-I IFNs, which are essential cytokines in anti-viral immunity, via their expression of TLRs 7 and 9 (Ogata *et al.* 2012). pDCs can switch their functional properties following the innate response phase from cytokine producers to mature DCs and act as important initiators and/or controllers of adaptive immunity by priming naïve $CD4^+$ T cells. Signalling through TLR7/9 by viruses can trigger immature DCs to rapidly develop into mature APCs which instruct human naïve $CD4^+$ allogeneic T cells to differentiate into T regulatory type 1 (Tr1) cells that secrete high levels of IL-10 and IFN-γ. Thus, pDC have a cytokine producing capacity linking to their tolerogenic DC functions of adaptive immunity. pDCs have the potential to prime $CD4^+$ T cells to differentiate into IL-10 producing T regulatory cells through the preferential expression of ICOS-L (Ogata *et al.* 2012).

Using a murine model of GvHD Hadeiba *et al.* (2008) found the addition of chemokine receptor $CCR9^+$ pDCs to BM transplanted recipients 'rescued' the mice from death and ameliorated their clinical signs. The ligand for $CCR9^+$ is CCL25, a chemokine linked to the homing of DCs and T cells to the gut. They found that $CCR9^+$ pDCs were potent inducers of regulatory T cells that suppressed antigen specific immune responses, including inhibiting aGvHD in mice.

The limited immunostimulatory characteristics of pDC (that distinguish them from mDC) make them an attractive therapeutic target for promoting tolerance in solid organ and allogeneic HSC transplantation (Rogers *et al.* 2013). While the clinical significance of the DC content of mobilised PBSC grafts has been studied, there are conflicting results. Lonial *et al.* (2013) compared granulocyte-macrophage colony-stimulating factor (GM-CSF) with granulocyte colony-stimulating factor (G-CSF) mobilised grafts and found that there was no significant difference between either the $CD34^+$ dose, engraftment or survival when using GM-CSF or G-CSF. However, there was a significant difference in pDC content, with G-CSF mobilized collections containing significantly more pDCs. The authors conclude that mobilisation with GM-CSF resulted in a shift to an mDC phenotype and T_H1 polarization of T cells, whereas mobilisation with G-CSF resulted in a shift to the pDC phenotype that is associated with T_H2 polarization. This contrasts with Rajasekar *et al.* (2010) who found that a group with 'high' pDC content had a significantly higher risk of relapse and lower overall survival (OS) and event-free survival (EFS). While both Lonial *et al.* (2013) and Rajasekar *et al.* (2010) used mobilised PBSCs, the contrasting results may be explained by differences in conditioning regimens and/or pDC identification method by flow cytometry.

Plasmacytoid DCs are distributed in the PB and lymphoid organs. Their migration from the PB to the lymph nodes is regulated by expression of the C-C chemokine receptor, CCR7, mDCs also utilise CCR7 to migrate to the lymph nodes. The ligands for CCR7 are CCL19 and CCL21 (Liu *et al.* 2021). CCL19 is expressed by stromal cells within secondary lymphoid organs while CCL21 is expressed by lymphatic endothelial cells.

1.16 DC-10 dendritic Cells

Gregori *et al.* (2010) identified and characterised a subset of DCs which they termed DC-10 cells. The authors found that DC-10 cells were present *in vivo,* can secrete large amounts of IL-10, and are potent inducers of antigen specific IL-10 producing Tr1 cells. They proposed that DC-10 cells are a novel subset of tolerogenic DCs that have the function to induce Tr1 cells. Tr1 cells are known to promote and maintain peripheral tolerance (Amodio and Gregori 2012). The secretion of high levels of IL-10 by DC-10 cells induces hyporesponsiveness in allogeneic T cells (Moreau *et al.* 2012). DC-10 cells express tolerogenic molecules including immunoglobulin-like transcript (ILT)2, ILT3, ILT4, and HLA-G (Comi *et al.* 2018). However, DC-10 cells have a low capability to stimulate naïve $CD4^+$ T cells (Hippen *et al.* 2011).

DC-10 cells are present in peripheral blood and in the secondary lymphoid organs of healthy subjects and accumulate in human decidua in the first trimester of pregnancy. The frequency of DC-10 cells in peripheral blood of pregnant and non-pregnant women is comparable (Comi *et al.* 2018). DC-10 cells were identified in the peripheral blood and spleen of healthy donors and in the decidua of pregnant women as $CD11c^+CD14^+CD83^+$ cells (Comi *et al.* 2019). Generally, DC-10 cells express CD11c, CD14, and CD16, and have a mature phenotype as they express costimulatory molecules CD83 and CD86 (Comi *et al.* 2019).

DC-10 cells can be differentiated from peripheral blood monocytes, (MoDC) *in vitro* and they express CD14, CD16, CD11c and CD11b (Amodio and Gregori 2012) and although not activated, express CD83, CD86 and HLA-DR. Despite the expression of CD14 and CD16, these moDC-10 cells differ from type 2 macrophages (M2) because while both produce IL-10 and low amounts of IL-2, DC-10 cells produce IL-6 and M2 cells do not.

1.17 T regulatory cells

T reg cells that express the transcription factor FoxP3 are critical for limiting immune responses and suppressing tissue inflammation (Vasanthakumar *et al.* 2020). T reg cells are just 5% of the total $CD4^+$ T cell population in PB (Ukena *et al.* 2011), however they are critical regulators in the induction and maintenance of peripheral tolerance, and loss of immune tolerance can contribute to

autoimmune diseases (Hand *et al.* 2020). This percentage varies by tissue site, T regs are approximately 20% of skin resident $CD4^+$ T cells, and 5-10% of tissue resident adult colon $CD4^+$ T cells (Ali and Rosenblum 2017). T reg cell activation is antigen specific, as for effector T cells. However, the mechanism of action of T regs is different to that of effector T cells, as the latter function mainly in a cell contact dependant manner directed against the antigen bearing cells, whereas T regs function via secreted factors and expression of negative regulatory cell surface receptors (Raffin *et al.* 2020).

T regs are predominant in the PB and in the lymph nodes (Shevyrev and Tereschchenko 2020). T reg function is believed to operate in two principal locations, secondary lymphoid tissues and peripheral tissues. In the secondary lymphoid tissues, T regs suppress induction of immunity by regulating T cell priming and expansion, which require T reg homing to lymphoid tissues and interaction with APCs and T cells. In the periphery, the T regs suppress effector immune cells (Chauhan *et al.* 2014). Cytotoxic T lymphocyte antigen 4 (CTLA-4/CD152) expression reflects suppressive activity of Tregs (Shevyrev and Tereschchenko 2020).

Peters *et al.* (2013) found activated FoxP3 T regs were widely distributed in human lymph nodes and spleen. However, they were not located in proximity to APCs which reduced the likelihood that APCs were responsible for the activation. The group found that unlike T regs in the secondary lymphoid organs, T regs in PB and BM expressed CCR7, a homing receptor for migration towards secondary lymphoid organs. T regs in draining lymph nodes and spleen were found to express CCR9, a homing receptor for the gut and CCR4, a homing receptor for the skin.

Chemokine receptors, CXCR3, CCR4 and CCR6 allow T regs to home to the same sites as T_H1, T_H2 and T_H17 cells respectively. Peters *et al.* (2013) found PB and BM T regs expressed CCR4 and CCR6, while lymph node T regs expressed CXCR3. The ligands for CXCR3 are CXCL4, CXCL10, CXCL9 and CXCL11, the ligands for CCR4 are CCL17 and CCL22, while the ligand for CCR6 is CCL20 (Punt *et al.* 2019).

It is believed that the pathway of inhibition by T reg cells is initially highly antigen specific (Punt *et al.* 2019). Once activated, T regs exert bystander suppression which results in suppression regardless of their antigen specificity (Raffin *et al.* 2020). T regs can suppress the functional activity of both $CD4^+$ and $CD8^+$ cells directly by preventing their differentiation, activation, and proliferation via either cell to cell contact or a contact independent route (Safinia *et al.* 2012). T reg cells have been shown to kill APCs or effector T cells directly, and they have been shown to modulate the function of other cells responding to antigen via surface receptor engagement.

T reg cells detect antigen presented in association with MHC class II molecules and CD4 molecules (Raffin *et al.* 2020). They can be subdivided into naïve, effector and memory cell compartments. The expression of CD45RA is generally associated with naïve T reg cells (Tian *et al.* 2017). Several studies have suggested that naïve CD45RA$^+$ T reg cells have a higher suppressive capacity compared with effector or memory T reg cells. However, it is known the percentage of naïve T reg cells decreases with age and most T reg cells in adult blood are CD45RA$^-$. (Raffin *et al.* 2020).

T reg cells confer immune tolerance via multiple mechanisms: soluble mediators IL-10, TGF-β and IL-35, the consumption of IL-2 and the expression of negative regulatory cell surface molecules such as CTLA-4 (CD152). T reg binding to APCs can result in stripping off the cell surface molecules (trogocytosis) which can alter co-stimulation and antigen presentation (Raffin *et al.* 2020). IL-10 directly regulates T cells by inhibiting their ability to produce IL-2, TNF-α and IL-5, and to proliferate. IL-10 indirectly suppresses T cell responses by downregulating the expression of HLA class II and co-stimulatory molecules such as CD80/CD86 on APCs (Roncarolo *et al.* 2011). IL-10 upregulates the production of IL-10 itself on DC precursor cells, rendering them regulatory cells capable of dampening immune responses and inducing T regs. Anergy induced by IL-10 during priming of T cells is a profound state of antigen specific unresponsiveness without cell death (Amodio and Gregori 2012).

Data from human studies implicates reduced T reg numbers in the periphery and target organs contributes to GvHD. Therefore, insufficient reconstitution of T regs after transplantation might be part of the uncontrolled expansion of effector T cell clones (Ukena *et al.* 2011). The T reg subsets have receptors for inflammatory chemokines such as CCR2, CXCR3, CCR4, CCR5 and CCR8 which suggests T regs can enter inflamed tissues and could therefore play a role in GvHD. Ukena *et al.* (2011) studied 141 patients post allogeneic transplantation; they analysed the T reg transcriptomes of patients with and without GvHD. They found the T regs isolated from patients with severe aGvHD had downregulation of molecules related to migration/homing of T cells to inflamed tissue and secondary lymphoid organs for example CCR5, CXCR3, CCR3, CXCR6 and CCR1. Ukena *et al.* (2011) found overexpression of CDK6 in T regs in patients with GvHD and this provides evidence that T reg cell cycle progression from G_1 to S phase may be a critical step in the pathogenesis of cGvHD. The results of Ukena *et al.* (2011) suggest that homing of T regs to secondary lymphoid tissue and sites of inflammation play an important role in the control of GvHD.

There are now more than 50 active or completed clinical trials testing the safety and efficacy of T reg cell therapy for a variety of indications (Raffin *et al.* 2020). It is believed there are two advantages to using antigen specific T regs in therapy (1) their action would be limited to the site of the alloantigen

source and immune activation (2) this may avoid undesirable pan-suppression, which would have advantages for infection and cancer risk.

It has been demonstrated in murine models that T reg cells exert their suppressive function both at the tissue site of inflammation and in the local secondary lymphoid tissues (Zhang *et al.* 2009). Murine models also suggest the chemokine receptor, CCR5, is important for the recruitment of T regs to GvHD target tissues and is a prerequisite for their suppressive function on alloreactive T cells (Ukena *et al.* 2011).

Trafficking and migration to tissues and secondary lymphoid organs are required for T reg cell function *in vivo*. In murine models, Zhang *et al.* (2009) found T regs migrated from blood to the inflamed allograft where they supressed alloimmunity. This process was dependent on chemokine receptors CCR2, CCR4 and CCR5. Within the graft, the T regs were activated and they subsequently migrated to the draining lymph nodes, in a CCR2, CCR5 and CCR7 dependant manner, this movement was essential for optimal suppression. The T regs inhibited DC migration in a TGF-β and IL-10 dependant manner and suppressed antigen specific effector T cell migration and proliferation in the draining lymph nodes and allografts.

Antigen specific T regs have been found to be more potent than polyclonal T reg cells in models of autoimmune disease and transplantation. Antigen specific T reg cells predominately localise at the site of antigen presentation, which should decrease the risk of generalised immunosuppression (Raffin *et al.* 2020). They are therefore potentially of use in the GvHD setting.

Murine models have shown that the adoptive transfer of *ex-vivo* expanded donor T regs was highly effective in preventing acute or cGvHD (Cohen *et al.* 2002). Murine models have shown that immature DCs induce alloantigen specific T cell anergy *in vitro* and drive *de novo* differentiation of natural T (nT) regs and type 1 (Tr1) cells (Morelli and Thomson 2007).

T regs are split into two groups: tTreg cells which develop in the thymus (sometimes referred to as natural or nTregs) and pTreg cells which develop in the periphery (sometimes referred to as induced or iTregs) (Punt *et al.* 2019). T regs isolated from peripheral blood are likely to be a combination of tTreg and pTreg cells (Raffin *et al.* 2020). tTreg cells mainly recognise self-antigens, whereas the pTreg T cell receptor (TCR) repertoire also includes TCRs for non-self, infectious antigens, or innocuous commensal microbiota derived antigens (Raffin *et al.* 2020). If the TCR is engaged in the absence of a suitable co-stimulatory signal, that T cell clone becomes anergic (Punt *et al.* 2019). pTreg cell activation requires TCR engagement and cytokines (Safinia *et al.* 2012).

Investigations into the mechanisms of peripheral tolerance have led to the discovery of various subpopulations of T regs: FoxP3 T regs, NK T cells, γδ TCR T cells, $CD8^+CD28^-$ T cells, Tr1 cells and $CD3^+CD4/8/56^-\alpha/\beta TCR^+$ T regs (McIver *et al.* 2008).

1.17.1 Type 1 regulatory T cells

$CD4^+$ type 1 T regulatory (Tr1) cells are induced in the periphery and have an important role in promoting and maintaining tolerance (Gagliani *et al.* 2013). The main mechanism by which Tr1 cells achieve tolerance is by the secretion of large amounts of IL-10 and the killing of myeloid APCs by granzyme B. Tr1 cells can maintain peripheral tolerance and prevent allograft rejection and GvHD (Battaglia *et al.* 2006).

When Tr1 cells are activated via their TCR they produce large amounts of IL-10, but they are distinct from T_H2 cells since they do not produce IL-4, and only produce low levels of IL-2 (Battaglia *et al.* 2006). Tr1 cells have a low proliferative capacity upon TCR activation. Although Tr1 cells must encounter their antigen for activation, once activated they suppress in an antigen non-specific manner, presumably due to the release of IL-10 and TGF-β. Tr1 cells supress both T_H1 and T_H2 mediated immune responses.

1.18 Rare event analysis

Rare event analysis usually refers to the detection of events that occur at a frequency of 1 in 1000, (0.1%) or less (Donnenberg and Donnenberg 2007). Watanabe *et al.* (2011) state that DCs are <1% of circulating mononuclear cells. Mononuclear cells (defined by lymphocytes + monocytes) have a count range of 1,600/µl – 3,440/µl and are 22%-52% of leukocytes in PB. The PB T cell count range is 540-1790/µl (Punt *et al.* 2019), the $CD4^+$ T cell count range is 300 – 1500 (Stem Cell technologies, no date), T reg count range is 13.5-46/µl (Chevallier *et al.* 2013). The expected ranges for the tolerogenic cells in peripheral blood are: pDC 5-10/µl, mDC 4-30.5/µl, (Chevallier *et al.* 2013), DC-10 4-13/µl (Gregori *et al.* 2010) and Tr1 4-13/µl (Gagliani *et al.* 2013). These cell types are thus low frequency cells in peripheral blood. Because of these counts their enumeration within PB by flow cytometry is an example of rare event analysis.

The distribution obtained by rare event analysis is governed by Poisson statistics, and the measurement precision increases as higher numbers of cells are collected (Hedley and Keeney 2013). Rare cell detection can be enhanced by maximising the signal-to-noise ratio of the cells against the background. The flow cytometer to be used in the study has 488 nm and 638 nm lasers.

To obtain a desired CV of 10% in rare event analysis, 100 target events should be obtained; the total number of events to be obtained is determined by the ratio of target events:total events. If the

target event is 1 in 100 (1%), then 10,000 total events should be obtained, if the target even is 1 in 1000 (0.1%), then 100,000 total events should be obtained (Hedley and Keeney 2013).

1.19 Engraftment of transplanted cells

Engraftment is the process by which HSCs home to the BM niches. Once in the BM microenvironment, HSC proliferate and generate all haematopoietic cell subsets (Hutt 2017).

The NHSBT definition of engraftment defines engraftment to be achieved on the first of 3 consecutive days when unaided neutrophils are ≥0.5 x 10^6/ml and unaided platelets are ≥20 x 10^6/ml. Engraftment is an important staging post following allogeneic transplantation.

If the patient's neutrophil levels have not reached 0.5 x 10^6/kg by day 28, this is termed delayed engraftment. This does not automatically mean that graft failure has occurred, and it is a clinical decision as to when delayed engraftment is declared graft failure.

Graft failure is a serious complication of allogeneic HSCT defined as either lack of initial engraftment of donor cells - primary graft failure, or loss of donor cells after initial engraftment - secondary graft failure (Ozdemir & Civriz Bozdağ 2018).

1.20 Professional practice

My role at the Stem Cell and Immunotherapy laboratory at NHSBT Barnsley is Deputy Head of Bone Marrow Transplantation (BMT) Clinical Services. As a senior biomedical scientist, my role includes identifying areas to improve the service for patients and clinicians. GvHD, a complication of allogeneic HST, is an area of concern that together with my supervisors was identified as a topic that had unaddressed questions that were suitable for a DProf project. If, as the experimental hypothesis proposed, there was an association between the tolerogenic cell number(s) analysed in the graft and/or during host immune cell reconstitution and GvHD, then this could potentially serve as a biomarker for GvHD prior to any detrimental effects of this unwanted immune response, and would be of use to both clinician and patient, which could improve patient outcomes by enabling earlier intervention. If an association was found, further work could build on this finding and tolerogenic cell enumeration could potentially be introduced into routine practice going forwards.

This study created a much-increased dialogue between the staff in the clinic and the laboratory with regards to GvHD, with increased amounts of data flowing in both directions and has generated new knowledge that both clinical and laboratory staff were able to use.

1.21 Aims and objectives

The study of cells in the recipient's graft prior to transplant, and peripheral blood post-transplant may highlight peripheral mechanisms in countering alloreactivity and GvHD via peripheral tolerance following allogeneic HSCT. The aim of this research study was to determine any associations between specific tolerogenic cell types and allogeneic transplant outcomes. The transplant outcomes of interest are GvHD and engraftment. The cell types of interest are: pDCs, DC-10 cells and Tr1 cells. These cell types were enumerated in the graft prior to transplant and in the peripheral blood of recipients' post-transplant. The study assessed both DCs and Tr1 cells as DCs can induce immune tolerance by induction of T regs (Chen 2006).

Factors to consider in the study were that identification of these cells in peripheral blood would not necessarily mean cell anti-GvHD activity, further experiments would be required to provide evidence that the cells were actively inhibiting GvHD.

The study aims to answer the following two research questions:

(1) Is there an association between pDCs and/or DC-10 cells and/or Tr1 cells in the graft and GvHD and/or survival?
(2) Is there an association between pDCs and/or DC-10 cells and/or Tr1 cell levels during host immune reconstitution and GvHD and/or survival?

To answer these questions, the following null and alternative hypotheses are proposed:

H_0: There is no association between studied cell number(s) in the graft and/or during host immune reconstitution and GvHD or survival in the recipient.
H_1: There is an association between studied cell number(s) in the graft and/or during host immune reconstitution and GvHD or survival in the recipient.

Chapter 2 – Materials and methods

2.1 Overview

The study hypothesis is that GvHD in patients undergoing allogeneic HSCT is influenced by the number of tolerogenic cells in the mobilised PBSC grafts they receive, and/or by how soon after transplantation these cells emerge in the recipient's PB. The tolerogenic cells analysed were pDCs, DC-10 cells and Tr1 cells. Cell counts were performed on donor PBSC collection(s) and on the recipient's PB blood samples at the following post-transplant time points: 2-weeks, 4-weeks, 8-weeks, 3-months, and 6-months. These time points were selected because the first six months after allogeneic HSCT is the most sensitive time window for tolerance induction (Ukena *et al.* 2011) and because the timing of onset of GvHD with aGvHD typically occurring in the first 100-days post-transplant, and cGvHD typically occurs after this time point. Acute GvHD would typically be observed up to and including the 3-month sample point and cGvHD between the 3- and 6-month sample time points (Ghimire *et al.* 2017). Sampling was prioritised to time points within the first 2-months post-transplant to enable an assessment of whether the tolerogenic cell number could predict if the patient is at risk of acute and/or chronic GvHD. Moderate to severe aGvHD occurs in approximately 40% of all recipients of allogeneic HSCT (Apperley *et al.* 2012). Chronic GvHD occurs in 40% of HLA identical sibling unmanipulated HSC transplants, in more than 50% of HLA- non-identical related HSC transplants and in 70% of matched unrelated HSC transplants (Apperley *et al.* 2012). These percentages were used to estimate the number of patients within the study likely to be diagnosed with GvHD.

The first patient in the study received their transplant on 08/11/18 and the final patient in the study received their transplant on 01/11/19.

2.2 Research Ethics Committee approval

The on-line Health Research Authority (HRA) tools were used to determine if the study was classified as research and hence required review by an NHS Research Ethics Committee (REC):

http://www.hra-decisiontools.org.uk/research/

http://www.hra-decisiontools.org.uk/ethics/

The study was classified as research and therefore required review by an NHS REC.

An application for NHS REC review was made via the online Integrated Research Application System (IRAS):

https://www.myresearchproject.org.uk/

The study reference is IRAS ID 226012 and required the following additional documents: Sheffield Hallam University (SHU) study protocol, patient information sheet, patient consent form, donor information sheet, donor consent form, evidence of sponsor (SHU) insurance, SHU's: employers' liability, public liability, professional indemnity and directors' and officers' liability, CVs of student and supervisors and HRA statement of activities. The SHU study protocol, patient information sheet, patient consent form, donor information sheet and donor consent form are shown in appendices I-V.

The NHS REC application was reviewed on the 13th September 2017 by the Proportionate Review Sub-Committee of the Wales REC 7. REC reference: 17/WA/0288. This review was favourable with one condition. Final approval from Wales REC 7 was received 17th October 2017 and the final approval letter is shown in appendix VI.

An annual update was submitted to the NHS REC in 2019.

2.3 Health Research Authority approval

HRA statement of activities and schedule of events for both NHSBT and Leeds Teaching Hospital Trust (LTHT) were created for HRA approval. HRA approval (IRAS 226012) was received 16th October 2017. The HRA approval letter is shown in appendix VII.

2.4 NHS Blood and Transplant approval

An application was made to the NHSBT research and development committee on 23rd February 2017. Operational support for the study (reference Msc-17-03) was received on 20th March 2017 subject to REC and HRA approval. The NHSBT approval letter is shown in appendix VIII.

2.5 Sheffield Hallam University ethics approval

An application (SHUREC2B) was submitted on 5th July 2017, reference HWB-BIO-08. This application was approved 15th September 2017. The SHU ethics approval letter is shown in appendix IX.

2.6 Leeds Teaching Hospital Trust approval

The first meetings about the study with LTHT Research and Innovation were in October 2017. Operational support for the study (R&I reference number HM18/107461) was received on 6th August 2018. The LTHT approval letter is shown in appendix X.

2.7 Anthony Nolan Trust approval

To enable the study to test Antony Nolan (AN) PBSC collections a research application was made to The Anthony Nolan Trust on 21st September 2017 (AN reference RESDON062). The application was

approved on 4th October 2017. The AN Trust required a signed confidentiality agreement between it and NHSBT for the study. There was a pre-existing confidentiality agreement between the two organisations and the AN Trust confirmed it was acceptable for the study on 16th November 2017. The AN approval letter is shown in appendix XI.

The AN Trust performs collections and is involved in the import of donations collected overseas. During discussions with the AN Trust, the principal investigator was made aware that the study could only consent AN Trust donors. If the PI wished to include donations from international registries the PI would have to apply to each international registry they wished to use. It was deemed unfeasible to apply to the many international registries for this DProf research study so matched unrelated donors in the study were limited to AN Trust donors.

2.8 Collaboration agreement (Material Transfer Agreement)

The HRA required a material transfer agreement (MTA) to be created. This was included within a collaboration agreement between NHSBT, SHU, LTHT and the AN Trust. Each party signed the agreement, and this was completed on 7th August 2018. A copy of the MTA is provided appendix XII.

2.9 General Data Protection Regulation (GDPR) Transparency statement

During the study the Human Research Authority required that all donor information / consent forms contained a transparency statement. This was created in September 2019 and a copy is shown in appendix XIII.

Project files were created and stored securely in the SCI laboratory, NHSBT Leeds. NHSBT Leeds has controlled access and the files were kept in a locked room.

2.10 Patient selection

The study sought to recruit consecutive adult allograft patients at St. James University Hospital (SJUH) between November 2018 and November 2019. Patients had a six month follow up, so the study was scheduled to last 18 months, finishing in May 2020. After the 6-month post-transplant sample had been provided, the patient's involvement in the study ended. The following inclusion and exclusion criteria were applied when recruiting patients.

2.11 Inclusion and exclusion criteria

Inclusion criteria

1. Adult haematopoietic stem cell transplant patients undergoing mobilised PBSC transplantation at SJUH

2. Related donors
3. Anthony Nolan (AN), British Bone Marrow Registry (BBMR) and Delete Blood Cancer (DBC) matched donors

Exclusion criteria

1. Participants under the age of 18 years
2. Bone marrow and cord blood allogeneic stem cell source
3. Unrelated donors from non-AN, BBMR and DBC registries

Potential participants (donors and patients) were identified when the SJUH consultants were planning the allogeneic HSCT work. The principal investigator liaised with the research nurse at SJUH or the AN Trust contact, who would then approach the potential participants to ask whether they were interested in taking part in the study.

Not all potential patients could be recruited, one patient lacked capacity and a second patient was not appropriate to approach due to their disease status.

2.12 Obtaining informed consent

Patients and related donors were approached by a research nurse at SJUH. AN Trust donors were approached by the AN Trust staff. Patients and donors were informed about the study and were given patient/donor information sheets and consent forms. The patient/donor information sheet gave the principal investigator's contact details should they wish to contact them with further questions. Patients/donors were given at least 24 hours to decide if they wished to take part. Participants were informed that their decision about the study would not affect their care in any way and that they were free to withdraw from the study at any time. If the patient / donor (as appropriate), wished to participate in the study, informed voluntary consent was obtained by an appropriate member of staff.

2.13 Anonymisation of patients and donors

Patients and donors were anonymised by having a number assigned to them upon recruitment. The numbers were sequential. As patients were being followed at 5 separate time points post-transplant the principal investigator kept a record of the identity of the patients in password protected software in secure premises.

2.14 Patient and donor sample collection

No additional study samples were required from patients or donors for the study, as samples were taken from the cell collections for routine $CD34^+$ analysis at the Stem Cell and Immunotherapy (SCI) lab, NHSBT Leeds, and these sample were used for the tolerogenic cell count analysis.

Following transplant, patients are routinely sampled to assess cell counts weekly until day 100. The study used samples at the following routine sampling time points: 2-, 4-, 8-weeks, 3- and 6-months. The 2-, 4- and 8-week time points were selected for analysis because if tolerogenic cell results at these time points was significant for GvHD then it would enable the clinicians to take early action to prevent GvHD. The 3-month sample time point was near the day 100 time point, by which time aGvHD would typically have been expected to occur, and the 6-month sample was selected to give an insight into cGvHD, which typically occurs after 100 days (Ghimire *et al.* 2017). At the 2-week sample time point patients were in-patients, but after this time point, they may have been in-patients or out-patients depending on their clinical course. These routine peripheral blood samples were 4ml EDTA samples and were first sent to the SJUH haematology laboratory. Once the sample's full blood count (which includes white cell count, haemoglobin level, haematocrit and platelet count) had been analysed on the haematology analyser, the samples were transported to the SCI lab at NHSBT Leeds. This was on either the same day, or the following day. All samples were tested on either the same day or the following day after the samples were taken. When necessary, samples were stored overnight at 4^{o}C prior to analysis.

An initial study was performed using three healthy donor PB samples to establish the flow cytometry method. The donors had consented to research via the NHSBT 2B form. These samples were also used to assess any impact of storage of PB samples at 4^{o}C for up to 48 hours prior to testing by flow cytometry. Initially three healthy controls were recruited and as the data generated was consistent between these three individuals, no further healthy controls were recruited.

2.15 Key technology utilised in the study – Flow Cytometry

"The marriage between immunology and cytometry is one of the most stable and productive in the recent history of science" (Cossarizza *et al.* 2017). It is a technique for making rapid measurements on particles as they flow individually through a sensing point (Ormerod 2000). Several elements are brought together in a flow cytometer: a flow cell where hydrodynamic focussing occurs, a laser or lasers, lenses and optical filters, electronics including detectors. Sample preparation is as important to the success of flow cytometry as the instrument itself, and this may use multiple monoclonal antibodies conjugated to fluorescent dyes.

2.15.1 Flow cell

It is within the flow cell that the hydrodynamic focusing occurs, which takes particles from a random three-dimensional suspension singly to a specific point where they can be intersected by an illuminating beam (Ormerod 2000).

2.15.2 Laser

The laser produces a plane polarised intense narrow beam of light at specific selectable wavelengths. The Beckman Coulter Navios used in the current study has a blue and red laser and they emit light at wavelengths 488nm and 638nm respectively. The laser is directed at the flow cell through which the cells are passing singly.

2.15.3 Voltages and colour compensation

During protocol creation, voltages for each channel were set so that negative events were displayed in the first log decade of the flow cytometry plots. Colour compensation software was then used to colour compensate for all the fluorochromes to be used in all the protocols: Fluorescein isothiocyanate (FITC) FL1 channel, R Phycoerythrin (PE) FL2 channel, R Phycoerythrin-Cyanine 5.5 (PC5.5) FL4 channel, R Phycoerythrin-Cyanine 7 (PC7) FL5 channel, Allophycocyanin (APC) FL6 channel and Allophycocyanin-Alexa Fluor 750 (APC Alexa Fluor 750) FL8 channel. The FL3 channel was used for Trucount™ beads in all protocols. Fluorophore excitation and emission data are shown in table 2.1.

Table 2.1 Fluorophores, excitation and emission maxima

Fluorophore	Excitation maxima (nm)	Emission maxima (nm)
Fluorescein (FITC)	488	525
R-phycoerythrin (PE)	488	575
R Phycoerythrin-Cyanine 5.5 (PC5.5)	488	692
R Phycoerythrin-Cyanine 7 (PC7)	488	770
Allophycocyanin (APC)	633/638	660
Allophycocyanin-Alexa Fluor 750 (APC Alexa Fluor 750)	633/638	775

2.15.4 Fluorochrome selection

The Beckman Coulter Navios is an 8 colour flow cytometer so all protocols were limited to 8 colours. The blue laser provides for 5 channels (FL1 to FL5) and the red laser provides for 3 channels (FL6-FL8). The ranges for each channel are shown in table 2.2.

Table 2.2 Ranges for each FL channel in the Beckman Coulter Navios™

FL channel	Range (nm)
1	525 +/-40
2	575 +/-30
3	620 +/-30
4	695 +/-30
5	755LP
6	660 +/-20
7	725 +/-20
8	755 LP

LP = long pass filter.

FL channels 1, 2, 3, 4, 6 and 7 = band pass filters

The following criteria guided fluorochrome selection (a) Fluorescence profile (b) Relative brightness (c) Fluorescence overlap (d) Fluorochrome stability and (e) Reproducible conjugation to antibodies (Flores-Montero *et al.* 2019). The candidate fluorochromes suggested by this group for the Navios flow cytometer are shown in table 2.3. Fluorochromes used in the study based on these criteria were as advised by Beckman Coulter.

Table 2.3 Candidate fluorochromes for flow cytometry

Laser	Fluorochrome
Blue laser channel 1	FITC
Blue laser channel 2	PE
Blue laser channel 3	PE CF595
Blue laser channel 4	PerCP Cy5.5
Blue laser channel 5	PE Cy7 (PC7)
Red laser channel 1	APC
Red laser channel 2	APC Alexa Fluor 700
Red laser channel 3	APC Hilite 7 / Alexa Fluor 750

The greatest signal to noise ratio for 488 nm lasers is obtained from PE or the tandem conjugates of PE including PE-Cy5 and PE-Cy7 (also known as PC7). The greatest signal to noise ratio for 633 nm lasers is obtained from APC and its conjugates including APC Alexafluor 700 and APC-Hilite 7 (Hedley and Keeney 2013). Flores-Montero *et al.* (2019) state APC Alexa Fluor 750 may be used to replace APC-Hilite 7. The fluorochromes used in this study were as advised by Beckman Coulter and matched the recommendations by Flores-Montero *et al.* (2019), expect the study used PC5.5 (also known as PE-Cy5.5) a tandem conjugate of PE, which matched the recommendation by Hedley and Keeney (2013), instead of PerCP Cy5.5.

The pDC/myeloid dendritic cell 1 (mDC1) protocol used 7 out of 8 channels, the only unused channel being FL7. The blue laser fluorochromes were: BDCA-2/CD303-FITC, CD3/CD19/CD56-PE, Trucount™ beads, CD1c-PC5.5, CD11c-PC7 and the red laser fluorochromes were: HLA-DR-APC and CD14-APC-Alexa Fluor 750. FL7 is the 'middle' channel for the red laser and not using it helped to prevent crossover between red FL6 and red FL8.

The DC-10 protocol used 4 out of 8 channels, all were blue laser channels (FL2, FL3, FL4 and FL5): CD83-PE, Trucount™ beads, CD14-PC5.5 and CD11c-PC7.

The Tr1 protocol used 5 out of 8 channels. This was a mixture of blue and red laser channels: FL1, FL2, FL3, FL5 and FL6. The blue laser fluorochromes were: CD49b-FITC, Human LAG-3-PE, Trucount™ beads, CD45RA-PC7 and the red laser fluorochrome was CD4-APC. Having the gaps between the FL channels helped to prevent crossover.

Reagents and their suppliers used in flow cytometry are shown in table 2.4.

Table 2.4 Reagents used for flow cytometry

Conjugated monoclonal antibody	Manufacturer	Product code
CD3-PE	Beckman Coulter	A07747
CD19-PE	Beckman Coulter	A07769
CD56-PE	Beckman Coulter	A07788
HLA-DR-APC	Beckman Coulter	IM3636
CD14-APC-Alexa Fluor 750	Beckman Coulter	B92421
CD11c-PC7	Beckman Coulter	B96763
CD1c-PC5.5	Beckman Coulter	B46036
BDCA-2/CD303-FITC	BioLegend	354208
FcR Blocking Reagent	Miltenyi Biotec	130-059-901
Mouse IgG1-PC7 (isotype control)	Beckman Coulter	737662
Mouse IgG1-PE (isotype control)	Beckman Coulter	A07796
CD14-PC5.5	Beckman Coulter	A70204
CD83-PE	Beckman Coulter	IM2218U
Mouse IgG1-PE (isotype control)	R&D Systems	IC002P
Mouse IgG1-FITC (isotype control)	Beckman Coulter	A07795
CD4-APC	Beckman Coulter	IM2468
CD45RA-PC7	Beckman Coulter	B10821
CD49b-FITC	Beckman Coulter	IM1425
Human LAG-3-PE	R&D Systems	FAB23193P
CD45-FITC	Beckman Coulter	A07782
7-AAD	Beckman Coulter	A07704
CD34-PE	Beckman Coulter	A07776
Trucount™ Tubes	Becton Dickinson	340334
Lysing solution IOTest 3	Beckman Coulter	A07799
Phosphate buffered serology saline	Source Bioscience	No longer available

2.15.5 DNA-binding dyes and red cell lysis

DNA binding dyes can be used for analysing cell viability. DNA dyes, for example 7-Amino-Actinomycin D (7-AAD) or propidium iodide, are used on the basis that these dyes are impermeable to the plasma membrane, so can only enter cells with non-intact plasma membranes. Viable cells will exclude these dyes and will not stain giving a negative result, while dead cells will stain positively (Cossarizza *et al.* 2017). 7-AAD was used as a viability dye in this study.

Blood contains approximately 1000 times more erythrocytes than leukocytes. Commercially available lysis solutions were used to lyse the red blood cells to facilitate white cell analysis (Cossarizza *et al.* 2017).

2.15.6 Fc blocking/Isotype control reagent use

To prevent false positive staining of cells via Fc-receptor mediated antibody binding (Cossarizza *et al.* 2017) FcR blocking reagent (table 2.4) was used in the DC-10 and Tr1 protocols. This prevented false positive staining of cells via antibody binding to their Fc receptor (Gagliani *et al.* 2013 and Gregori *et al.* 2010 respectively).

Isotype controls can be used to define negative populations, especially when positive and negative populations are continuous and not readily identified. The isotype controls used are shown in table 2.4. Both the Tr1 and DC-10 protocols contained plots with continuous populations, so isotype controls were used, as they were used in the published papers from which the protocols were derived.

The pDC/mDC1 protocol obtained discrete positive and negative populations. When there are discrete populations, isotype controls are less important (Ormerod 2000), so they were not used in this protocol, as they were not included in the published paper the protocol was derived from (Autenrieth *et al.* 2015). The concentration of conjugates for the antibody / isotype control pairs used is shown in table 2.5. There is no significance to the difference in the concentrations of the paired antibodies used (Beckman Coulter – private correspondence).

Table 2.5. Concentration of conjugates for antibody / isotype control pairs.

Antibody	Concentration of Conjugate (µg/ml)
Mouse IgG1-PC7 (Used in DC-10 protocol)	50
CD11c-PC7 (used in DC-10 protocol)	20
Mouse IgG1-PE (Used in DC-10 protocol)	6.25
CD83-PE (Used in DC-10 protocol)	6.25
Mouse IgG1-PE (used in Tr1 protocol)	50
Human-LAG-3-PE (used in Tr1 protocol)	25
Mouse IgG1-FITC (used in Tr1 protocol)	50
CD49b-FITC (used in Tr1 protocol)	50

2.15.7 Single platform testing

Cell counts can be performed using single or dual-platform flow cytometric testing. Single-platform testing provides a direct absolute count without the need for a haematology analyser and is considered more reliable and reproducible than dual-platform testing (Noulsri *et al.* 2018). This is because in dual platform testing there are two sources of instrumentation variation: the haematology analyser as well as the flow cytometer.

Single platform testing is achieved by using a tube containing a lyophilized pellet with a known number of reference microbeads to which the blood sample is added. By comparing cellular events to bead events the absolute number of cells/µL is determined. An example of dual-platform testing would be obtaining a $CD4^+$ count by multiplying the lymphocyte count obtained from a haematology analyser by the percentage of $CD4^+$ T lymphocytes obtained by flow cytometry.

All protocols used in the study were single platform and used Trucount™ tubes (Becton Dickinson) to obtain a cell count/µl. The formula for calculating counts is as follows:

$$\frac{\text{Target events}}{\text{Bead events}} \times \text{Bead count} = \text{count}/\mu\text{l}. \qquad \text{Bead count} = \frac{\text{Number of beads/test}}{\text{Test volume}}$$

Each batch of Trucount™ tubes has a stated CAL (calculation) factor, which refers to the known number of fluorescent beads in the lyophilized pellet. Counts are calculated by obtaining the ratio of beads to target cells and multiplying this ratio by the number of beads in the tube (Nicholson *et al.* 1997).

Not all studies have shown that single platform testing reduces inter-laboratory variation. One study suggests the historical variation attributed to dual platform testing may have been a result of other

factors such as washing which was historically used in sample preparation (Hultin *et al.* 2010). Washing involves centrifugation which can cause cell loss. However, the authors did not recommend laboratories currently using single platform cell analysis to switch to dual platform testing.

Although Podgorny (*et al.* 2014) and Rajasekar (*et al.* 2010) published papers similar to this study, they obtained cell counts using a dual platform method. This study used a single platform method for cell enumeration as cell counts obtained using this method are considered more reliable and reproducible.

2.15.8 Gating strategies

The literature search identified papers that described different phenotypic definitions of pDC, DC-10 cells and Tr1 cells. The papers selected and their phenotypic definitions of the tolerogenic cells were as follows.

Plasmacytoid and mDC1 DCs (mDCs in human peripheral blood can be sub-grouped into mDC1 and mDC2) were identified following the protocol published by Autenrieth *et al.* (2015). This paper was selected because it was a recent paper published following an international workshop, this paper has been cited by over 10 papers (CrossRef, as of date 27/11/21). Plasmacytoid DCs were phenotypically defined as Lin^-, $HLA\text{-}DR^+$, $CD14^-$, $CD11c^{lo}$, $CD303^+$ and $CD1c^-$, while the mDC1 population could be identified by being $CD1c^+$ and $CD303^{lo}$, the latter dendritic cell type was not originally part of the study but was included. This protocol does not require an FcR blocking reagent or an isotype control. The pDC protocol created for this study can be seen in figure 2.1.

DC-10 cells were identified following the protocol published by Gregori *et al.* (2010). This paper was selected because it was a relatively recent paper from authors who had published many of the papers in the literature on DC-10 cells, this paper has been cited by over 360 papers (Web of Science, as of date 27/11/21). These cells were phenotypically defined as $CD14^+$, $CD83^+$ and $CD11c^+$. The DC-10 protocol created for this study can be seen in figures 2.2 and 2.3. This protocol used an FcR blocker and an isotype control.

Tr1 cells were identified following the protocol published by Gagliani *et al.* (2013). This paper was selected because it was a recent paper in *Nature Medicine*, and this paper has been cited by over 460 papers (CrossRef, as of date 27/11/21). These cells were phenotypically defined as $CD4^+$, $CD45RA^-$, $CD49b^+$ and $LAG\text{-}3^+$, which was a new and simpler phenotypic definition of these cells. The Tr1 protocol created for this study can be seen in figures 2.4 and 2.5. This protocol used an FcR blocker and an isotype control.

The study also performed analysis for $CD3^+$ and $CD4^+$ cells. There was a pre-existing $CD3^+$ protocol in use in the routine laboratory, and this was adapted to gain $CD3^+/CD4^+$ T helper cell counts. These cells were analysed as there are known associations with GvHD and their delayed recovery may have clinical consequences. The adapted protocol can be seen in figure 2.6.

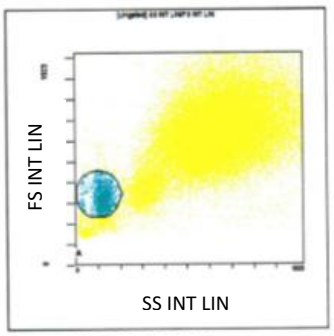
2.16 Colour compensation

Colour compensation was performed using the automated colour compensation module (Autosetup Scheduler) on the Beckman Coulter Navios™ flow cytometer (Navios™ Cytometer 1.3 software).

The colour compensation was performed using healthy donor peripheral blood using positive populations (CD3) for each individual fluorochrome. A panel was created for the automated colour compensation module, and as the samples were analysed, the software automatically calculated the compensation and built the compensation matrix. Figures 2.1 and 2.2 show the completed colour compensation module runs for FITC and PE respectively. The resulting voltage / gains / colour compensation settings created by the completed colour compensation module runs are shown below in table 2.6.

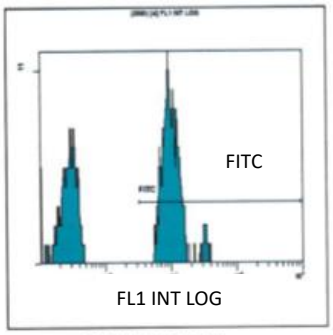
CD3 antibody was used as it gives clear positive and negative populations which aid colour compensation. The antigens used in a particular protocol could have been used, but if that antigen gave near 100% positive or negative populations only, that would not have aided colour compensation set up.

Figure 2.1 The completed colour compensation module run for FITC



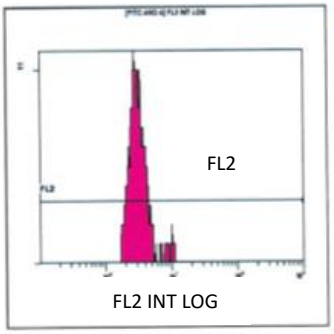
[Ungated] SS INT LIN:FS INT LIN

Region	Number	X-Mean
ALL	40640	604
A	1868	119



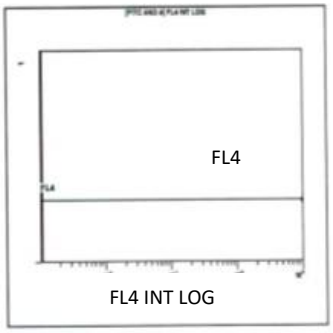
(2500) [A] FL1 INT LOG

Region	Number	X-Mean
ALL	1868	7.63
FITC	1089	12.9



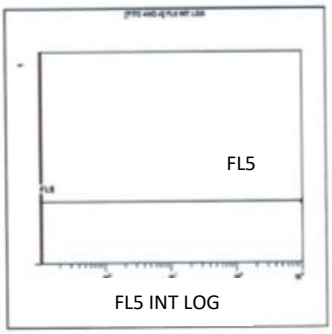
[FITC AND A] FL2 INT LOG

Region	Number	X-Mean
ALL	1089	3.95
FL2	1089	3.95



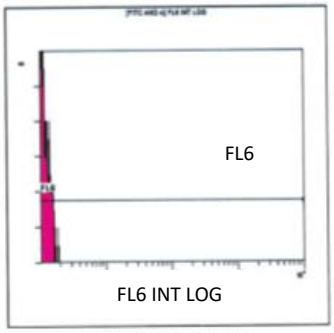
[FITC AND A] FL4 INT LOG

Region	Number	X-Mean
ALL	1089	0.103
FL4	1089	0.103



[FITC AND A] FL5 INT LOG

Region	Number	X-Mean
ALL	1089	0.102
FL5	1089	0.102



[FITC AND A] FL6 INT LOG

Region	Number	X-Mean
ALL	1089	0.111
FL6	1089	0.111

Figure 2.2 the completed colour compensation module run for PE

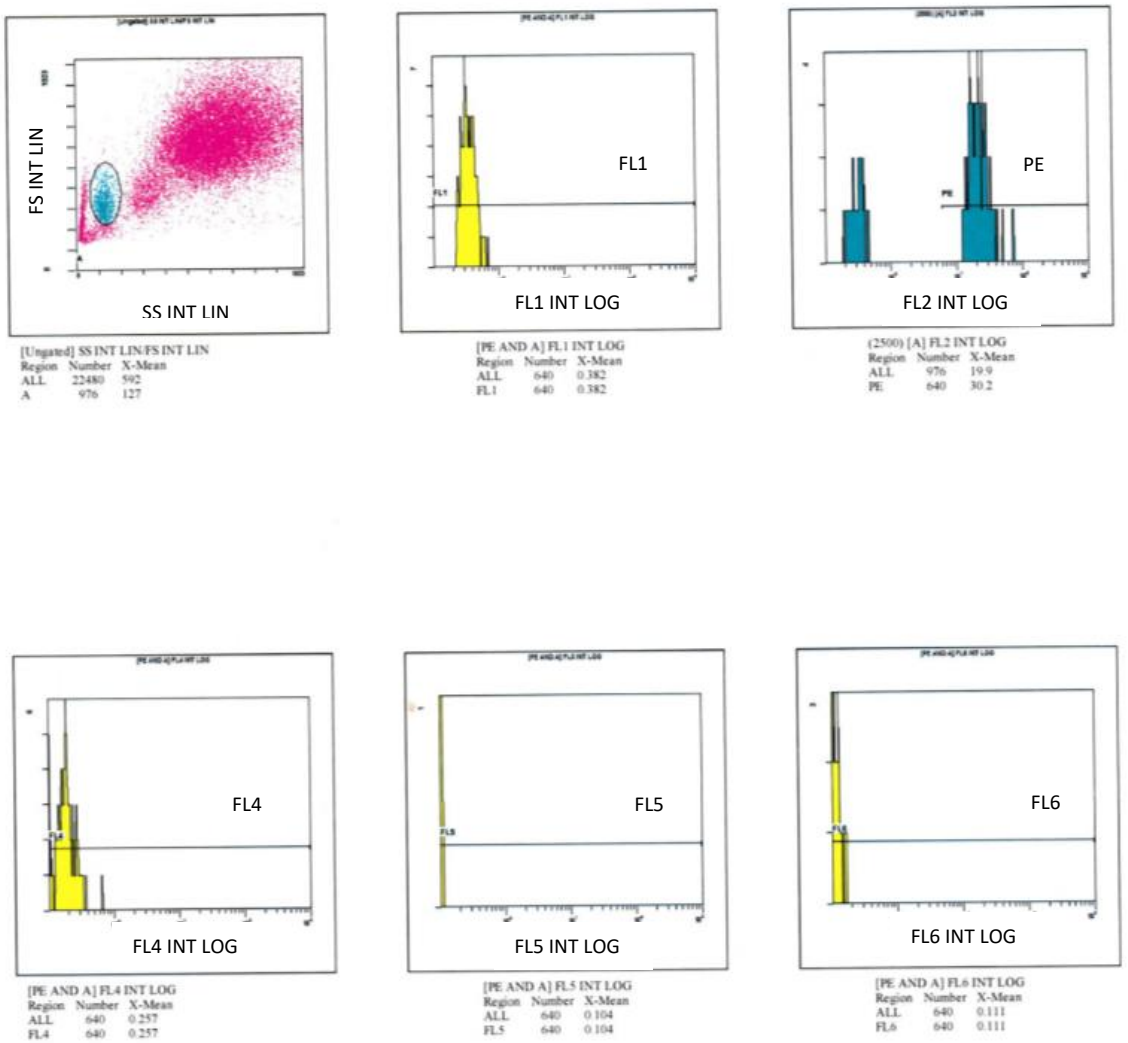


Table 2.6 Protocol voltages/gains and colour compensation.

	FS	SS	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8
Voltage	633	414	390	410	250	416	446	607	250	461
Gain	2.0	7.5	1	1	1	1	1	1	1	1
	FS	SS	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8
Disc.	100	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF
Col.comp.	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8		
FL1		0	0	0	0	0	0	0		
FL2	30		0	6.3	3.7	0	0	0		
FL3	0	0		0	0	0	0	0		
FL4	0	0.7	0		0	0	0	0		
FL5	0	0	0	36.3		0	0	0.4		
FL6	0.7	0.2	0	30.2	9.9		0	42.4		
FL7	0	0	0	0	0	0		0		
FL8	0	0	0	21.7	23.3	1.3	0			

FS = Forward scatter, SS = Side scatter, FL = fluorescence channel, Disc. = discriminator, Col.comp. = colour compensation

2.17 Staining peripheral blood stem cell collections and whole blood samples for tolerogenic cell and other cell counts

The flow cytometer used in the study was used in routine laboratory work and as such passed daily routine quality Flowcheck™ and Flowset™ checks (Beckman Coulter) prior to use.

G-CSF stimulated PBSC collections were collected from sibling and matched unrelated donors and transported to the SCI laboratory at NHSBT Leeds. Samples were taken from the collections in the SCI laboratory at NHSBT Leeds per routine procedure.

Patients' post-transplant peripheral blood samples were collected into EDTA blood tubes and transported from the SJUH, Leeds haematology laboratory to the SCI laboratory at NHSBT Leeds via the SJUH blood bank transport system.

Reagents and suppliers used in flow cytometric testing are listed above in table 2.4.

Samples from collections and from patients' post-transplant peripheral blood were analysed within 24 hours of being taken. If samples were stored overnight before analysis, they were stored at 4°C.

The patients' 6-month post-transplant follow up appointment was not fixed in time and could be at any point between 5 to 7 months, the samples obtained at this visit were treated as the 6-month samples.

The COVID-19 pandemic affected patient post-transplant sampling. As the pandemic developed patients were advised not to come to hospital if they could avoid doing so. All samples that were due from March 2020 onwards (the final patient's 6-month sample was expected May 2020) were lost to the study because of the pandemic.

Samples were tested before any clinical data had been returned to the SCI laboratory, and this helped prevent bias in the analysis.

2.18 General method for labelling of cells for flow cytometry analysis

Representative samples from PBSC collections or PB were analysed on a haematology analyser to obtain the WCC. Samples with a WCC > 10 x 10^6/ml were diluted in phosphate buffered serology saline (PBSS) to obtain a WCC of <10 x 10^6/ml.

All flow cytometry samples were set up following the manufacturer's instructions. Test samples were set up in duplicate, with an additional isotype control if applicable. 10µl of each of the test conjugated monoclonal antibodies, with isotype controls if applicable, were added to each Trucount™ tube. Each Trucount™ tube contained a lyophilized pellet with a known number of reference microbeads. 100µl patient/donor cell sample (diluted to a WCC <10 x 10^6/ml if applicable) was then added to the antibody cocktail. The tubes were mixed manually by flicking, and then incubated for 15 minutes at 22°C. After this time, 2ml of Beckman Coulter IOTest3 lysing solution (diluted 1:10 with distilled water) was added to each tube, mixed manually as before, and incubated for a further 15 minutes at 22°C. The samples were then analysed immediately on a Beckman Coulter Navios™ flow cytometer using Navios™ Cytometer 1.3 software. Gating of populations was performed manually. The stop conditions for the pDC/mDC1, DC-10 and Tr1 protocol were all 600 seconds or 2,500,000 events.

To obtain a desired CV of 10% in rare event analysis 100 target events should be obtained. The total number of events to be obtained is determined by the ratio of target events:total events. If the target event is 1 in 1000 (0.1%), then 100,000 total events should be obtained (Hedley and Keeney 2013).

The duplicate test samples were expected to give results within 10% of their mean, if the results did not meet this criteria the samples were retested.

2.18.1 Plasmacytoid Dendritic Cells/myeloid dendritic cell 1 cells

A seven-colour flow cytometry protocol as defined in Autenrieth *et al.* (2015) was selected. This group had published a protocol created in a recent international workshop. This protocol had the advantage of also enumerating mDC1 cells which could then be included in the downstream analysis. The antibodies used are shown in table 2.7. Autenrieth *et al.* (2015) used reagents from BioLegend. This study also used the BDCA-2 / CD303-FITC from BioLegend, while all other conjugated monoclonal antibodies were from Beckman Coulter. Fluorochromes selected were as recommended by Beckman Coulter.

Autenrieth *et al.* (2015) defined pDCs as Lin^{-}, $HLA\text{-}DR^{+}$, $CD14^{-}$, $CD11c^{lo}$, $CD303^{+}$, and $CD1c^{-}$. The mDC1 population was identified using the same markers but instead were $CD1c^{+}$ and $CD303^{lo}$. Autenrieth *et al.* (2015) do not state cell count ranges but Chevallier *et al.* (2013) reported that the median number of pDCs in PB was 7.5/µl (range 5 – 10/µl) and the median number of mDCs in PB was 10/µl (range 4 – 30.5/µl). The protocol created for this study gave a mean values of pDC 8.33/µl (range 6/µl – 12/µ) and mDC1 15.33/µl (range 14/µl – 17/µl), as shown in table 2.12.

Table 2.7 Antibody cocktail used for plasmacytoid and myeloid dendritic cell 1 analysis

Conjugated monoclonal antibody	Antigen cellular expression	Antigen function*
CD3-PE (Lineage marker)	T lymphocytes	Part of the complex that includes the TCR
CD19-PE (Lineage marker)	B lymphocytes	Involved in the regulation of B lymphocyte development
CD56-PE (Lineage marker)	Natural killer (NK) cells	Isoform of neural-CAM (N-CAM)
HLA-DR-APC	Antigen-presenting cells	MHC class II
CD14-APC-Alexa Fluor 750	Monocytes and macrophages	Receptor for the LPS and LBP complex
CD11c-PC7	Monocytes, macrophages and NK cells, weakly on dendritic cells	Associated with CD18 and both are associated with cytotoxic T cell killing
CD1c-PC5.5	Myeloid dendritic cells and a subpopulation of B lymphocytes	Involved in the antigen presentation of glycolipids
BDCA-2/CD303-FITC	Plasmacytoid dendritic cells	May act as a signalling receptor

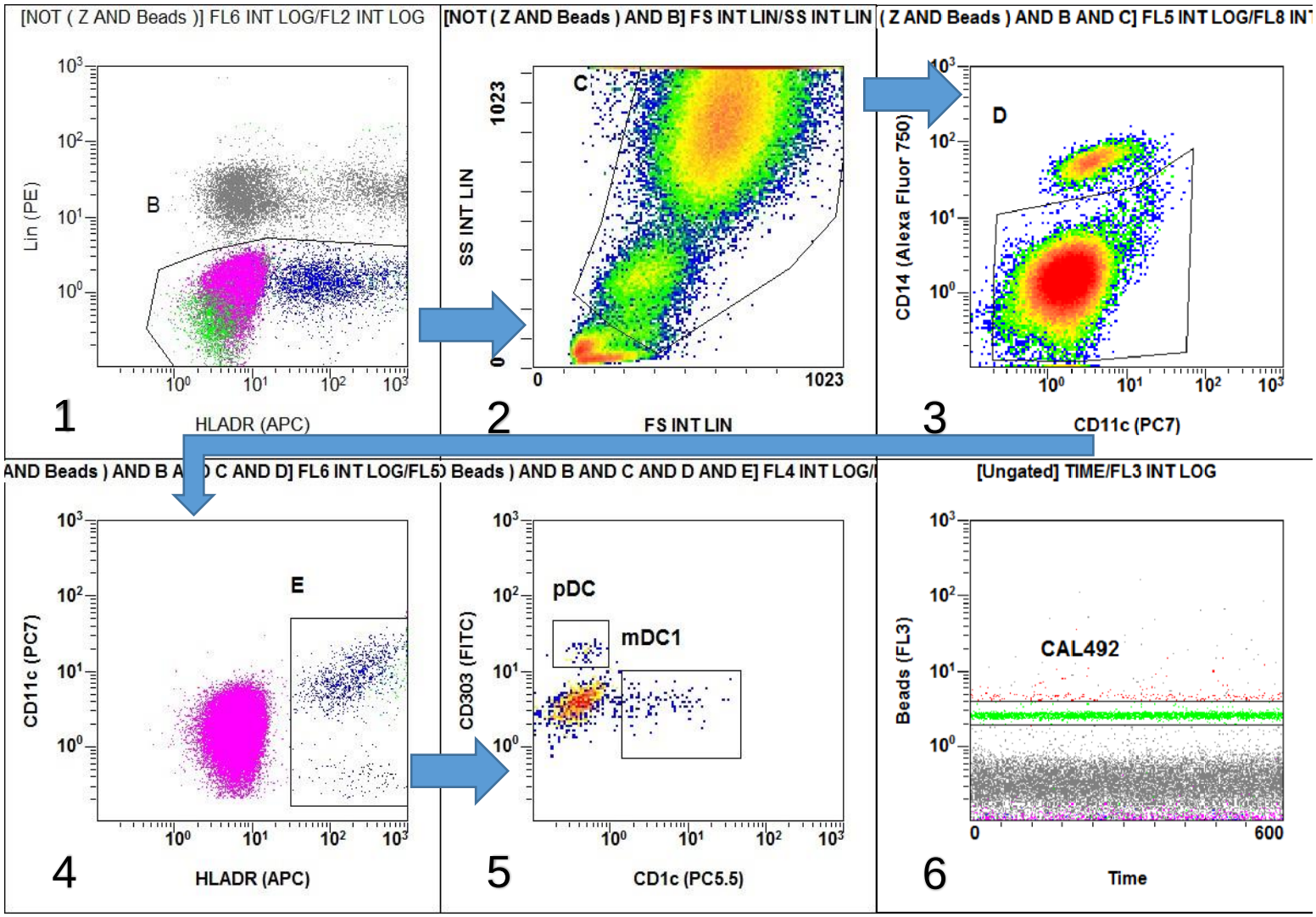
*https://www.mybeckman.uk/reagents/coulter-flow-cytometry

BDCA = Blood dendritic cell antigen

Sample preparation for flow cytometry was performed as described in 2.18.

Figure 2.3 illustrates the plots and gating strategy taken directly from the flow cytometry print out for identification of pDCs and mDC1 cells.

Figure 2.3 Plasmacytoid and myeloid dendritic cell 1 flow cytometry protocol



CAL = calculation factor

Plot (1) is HLA-DR vs Lineage markers (CD3, CD19 and CD56), events that are negative for lineage markers and positive for HLA-DR were gated into plot (2). Plot (2) is FS vs SS, all events except those with low FS/SS were gated into plot (3). Plot (3) is CD11c vs CD14, events that are negative for CD14 and positive for CD11c were gated into plot (4). Plot (4) is HLA-DR vs CD11c, events that were strongly positive for HLA-DR are gated into plot (5) which shows CD1c vs BDCA-2, $CD303^{+}CD1c^{-}$ are pDC and the $CD1c^{+}$ $CD303^{lo}$ population are mDC1. Plot (6) shows the Trucount™ beads. Gates positioned according to Autenrieth *et al.* (2015).

2.18.2 DC-10 dendritic Cells

A four-colour flow cytometry protocol as defined in Gregori *et al.* (2010) was selected. The authors of this paper had published many of the papers in the literature on DC-10 cells. While pDC have a low expression of CD11c (Autenrieth *et al.* 2015), DC-10 cells are CD11c positive. This protocol used an FcR blocker reagent and an isotype control and the antibodies used for DC-10 cells are shown in table 2.8. All conjugated monoclonal antibodies used for this protocol were purchased from Beckman Coulter. Gregori *et al.* (2010) did not state which manufacturers reagents they used, fluorochromes selected were as recommended by Beckman Coulter.

Gregori *et al.* (2010) reported that $CD14^+$, $CD83^+$ and $CD11c^+$ DC-10 cells are 0.3% (+/- 0.18%) of MNC in peripheral blood. The lymphocyte + monocyte count in peripheral blood has a range of 1.3 – 4.4 x 10^9/L (Punt *et al.* 2019) and 0.3% gives a DC-10 figure of 4 - 13/µl. This study obtained a mean of 7 DC-10 cells/µl (range 2/µl - 10/µl), as shown in table 2.12.

Table 2.8 Antibody cocktail used for DC-10 dendritic cell analysis

Conjugated monoclonal antibody	Antigen cellular expression	Antigen function*
Mouse IgG1-PC7 (isotype control)	N/A	N/A
Mouse IgG1-PE (isotype control)	N/A	N/A
CD14-PC5.5	Monocytes and macrophages	Receptor for the LPS and LBP complex
CD11c-PC7	Monocytes, macrophages and NK cells, DC-10 cells	Associated with CD18 and both are associated with cytotoxic T cell killing
CD83-PE	Dendritic lineage cells	Glycoprotein predominantly expressed by dendritic lineage cells

*https://www.mybeckman.uk/reagents/coulter-flow-cytometry.

N/A = not applicable

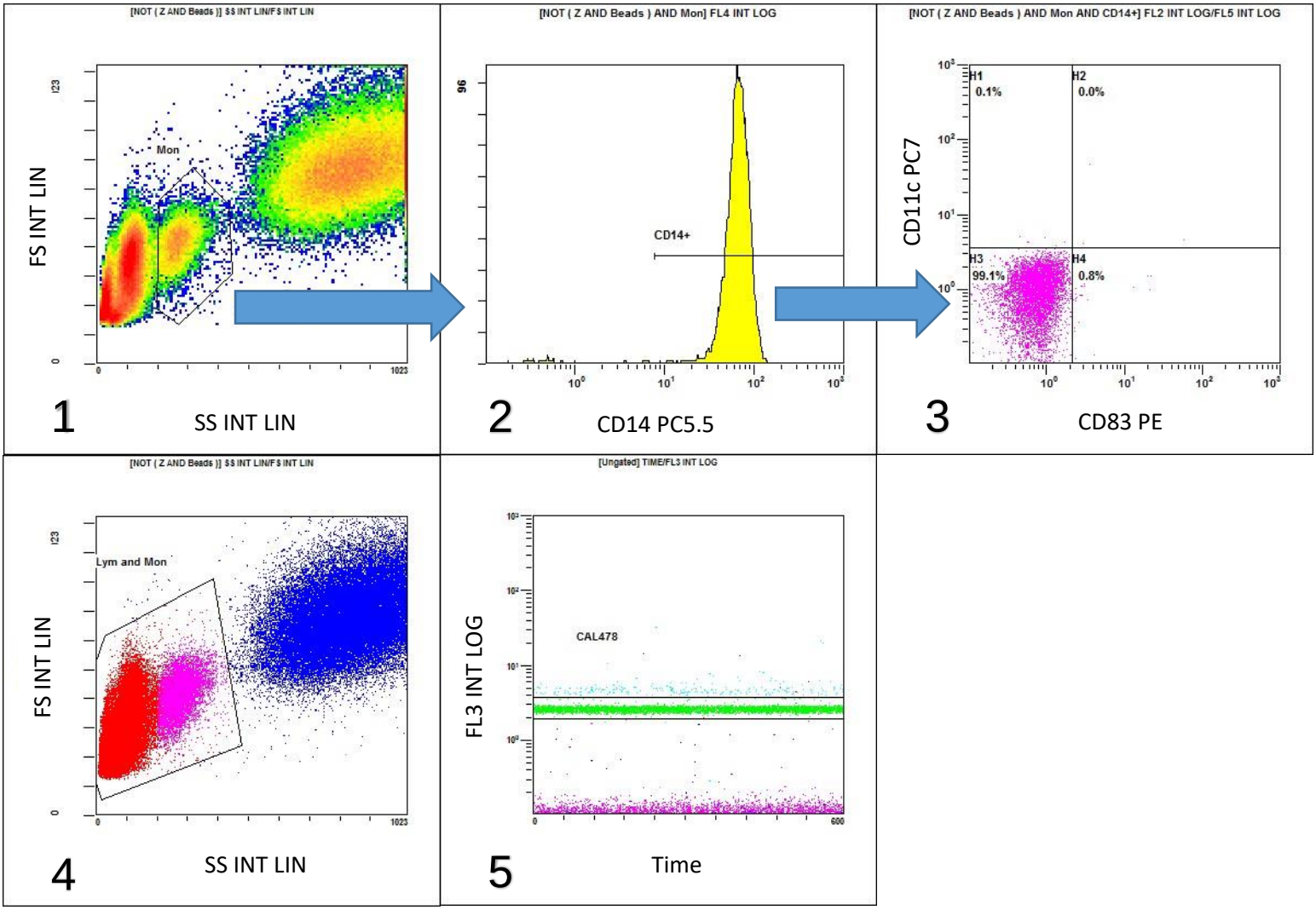
CD11c-PC7 and CD83-PE antibodies were IgG1 and IgG2 isotypes respectively and were manufactured by Beckman Coulter. Isotype controls from the same manufacturer were selected for

use. The Mouse IgG1-PC7 isotype control was the same isotype as the PC7 primary antibody. An IgG2 isotype control for the PE primary antibody was not available from the manufacturer, the manufacturer was asked for advice and product A07796 was recommended.

Sample preparation for flow cytometry was performed as described in 2.18. All three tubes also had 20µl FcR blocking reagent added. In addition to the duplicate test tubes, this protocol used an isotype control tube to define the negative population for CD83 and CD11c.

Figures 2.4 and 2.5 illustrate the plots and gating strategy taken directly from the flow cytometry print out.

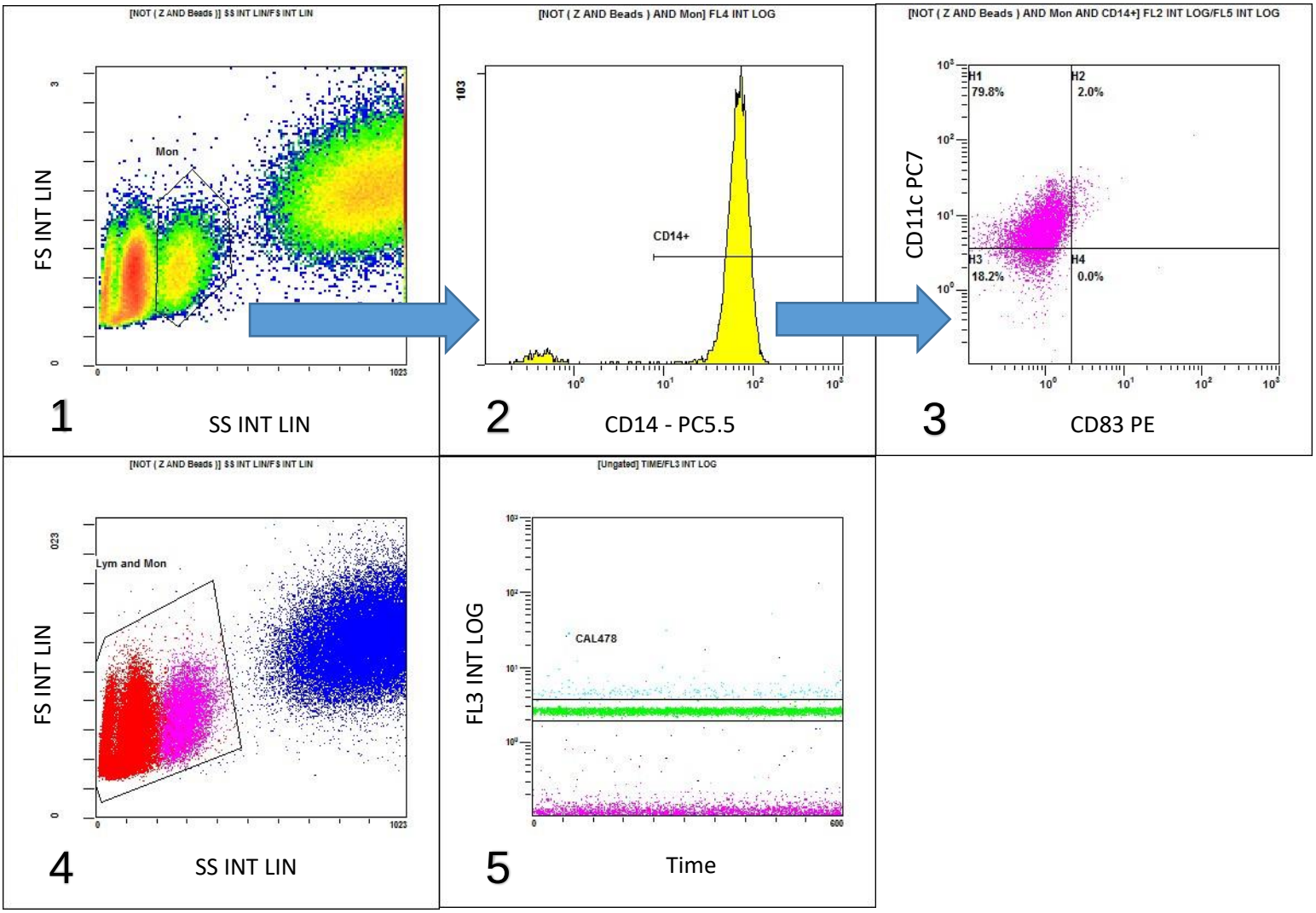
Figure 2.4 DC-10 cell (isotype control) flow cytometry protocol



CAL = calculation factor

Plot (1) is SS vs FS, monocytes were gated into plot (2). Plot (2) is CD14, CD14 positive are gated into plot (3). Plot (3) is CD83 vs CD11c, DC-10 cells were double positive. Plot (4) has the same axis as plot (1) but was not a density plot, and was interchangeable with plot (1) for gating into plot (2). Plot (5) shows the Trucount™ beads. Gates positioned according to Gregori *et al.* (2010)

Figure 2.5 DC-10 cell (test) flow cytometry protocol



2.18.3 Type 1 T regulatory cells

A five-colour flow cytometry protocol as defined in Gagliani *et al.* (2013) was selected as this paper had proposed a new and simpler phenotypic definition of these cells. This protocol used an FcR blocker reagent and an isotype control and the antibodies used for Tr1 cells are shown in table 2.9. Gagliani *et al.* (2013) used CD49b and CD45RA from BioLegend, CD4 from BD and LAG-3 from R&D Systems. The protocol in this study also used the LAG-3-PE and its isotype control from R&D Systems, all other conjugated monoclonal antibodies were from Beckman Coulter. Gagliani *et al.* (2013) do not state which fluorochromes they used, so fluorochromes selected in this study were as recommended by Beckman Coulter.

In the paper by Gagliani *et al.* (2013) the flow cytometry plots of a representative donor were provided and showed that 1.6% of $CD4^+CD45RA^-$ cells in peripheral blood were $CD49b^+LAG\text{-}3^+$ Tr1 cells. This study obtained a double positive percentage of this phenotype of 1.1%. Memory $CD4^+$ cells have a normal range of 250/µl – 810/µl (Stem Cell technologies, no date) and 1.6% of this gives a Tr1 cell number of 4/µl – 13/µl. This study obtained a mean of 7.67 Tr1/µl (range 3/µl – 12/µl), as shown in table 2.12.

Table 2.9 Antibody cocktail used for Type 1 regulatory T cells analysis

Conjugated monoclonal antibody	Antigen cellular expression	Antigen function*
Mouse IgG1-PE (isotype control)	N/A	N/A
Mouse IgG1-FITC (isotype control)	N/A	N/A
CD4-APC	T helper lymphocytes, low density on monocytes	Co-receptor on T_H for MHC class II
CD45RA-PC7	Naïve/resting peripheral $CD4^+$ T lymphocytes	Naïve/resting $CD4^+$ T lymphocytes
CD49b-FITC	Platelets and activated T lymphocytes	Forms the VLA-4 complex with CD29
Human LAG-3-PE	Activated $CD4^+$ and $CD8^+$ T cells, natural killer T cells, NK cells, plasmacytoid dendritic cells, and regulatory T cells	May be an inhibitory co-receptor

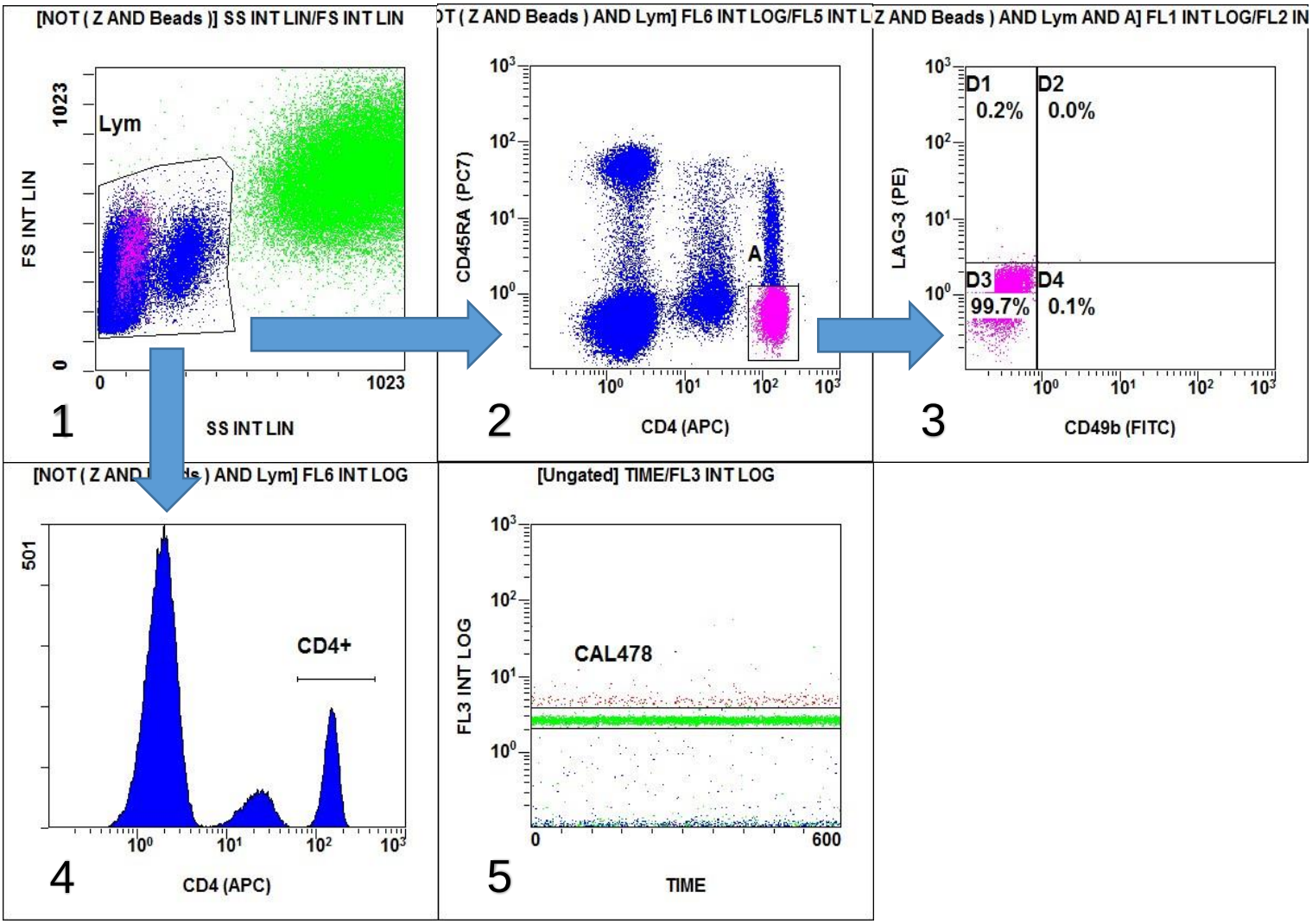
*https://www.mybeckman.uk/reagents/coulter-flow-cytometry. N/A = Not applicable.

Anti-hLAG-3-PE (R&D Systems) and anti-CD49b-FITC (Beckman Coulter) were both IgG1. Isotype controls from the same manufacturer were selected for use. Mouse IgG1-PE (R&D Systems) and MsIgG1-FITC (Beckman Coulter) were the same isotype as the primary antibodies.

Sample preparation for flow cytometry was performed as described in 2.18. All three tubes also had 20µl FcR blocking reagent added. In addition to the duplicate test tubes, this protocol used an isotype control tube to define the negative population for CD49b and LAG-3.

Figures 2.6 and 2.7 illustrate the plots and gating strategy taken directly from the flow cytometry print out for identification of Tr1 cells.

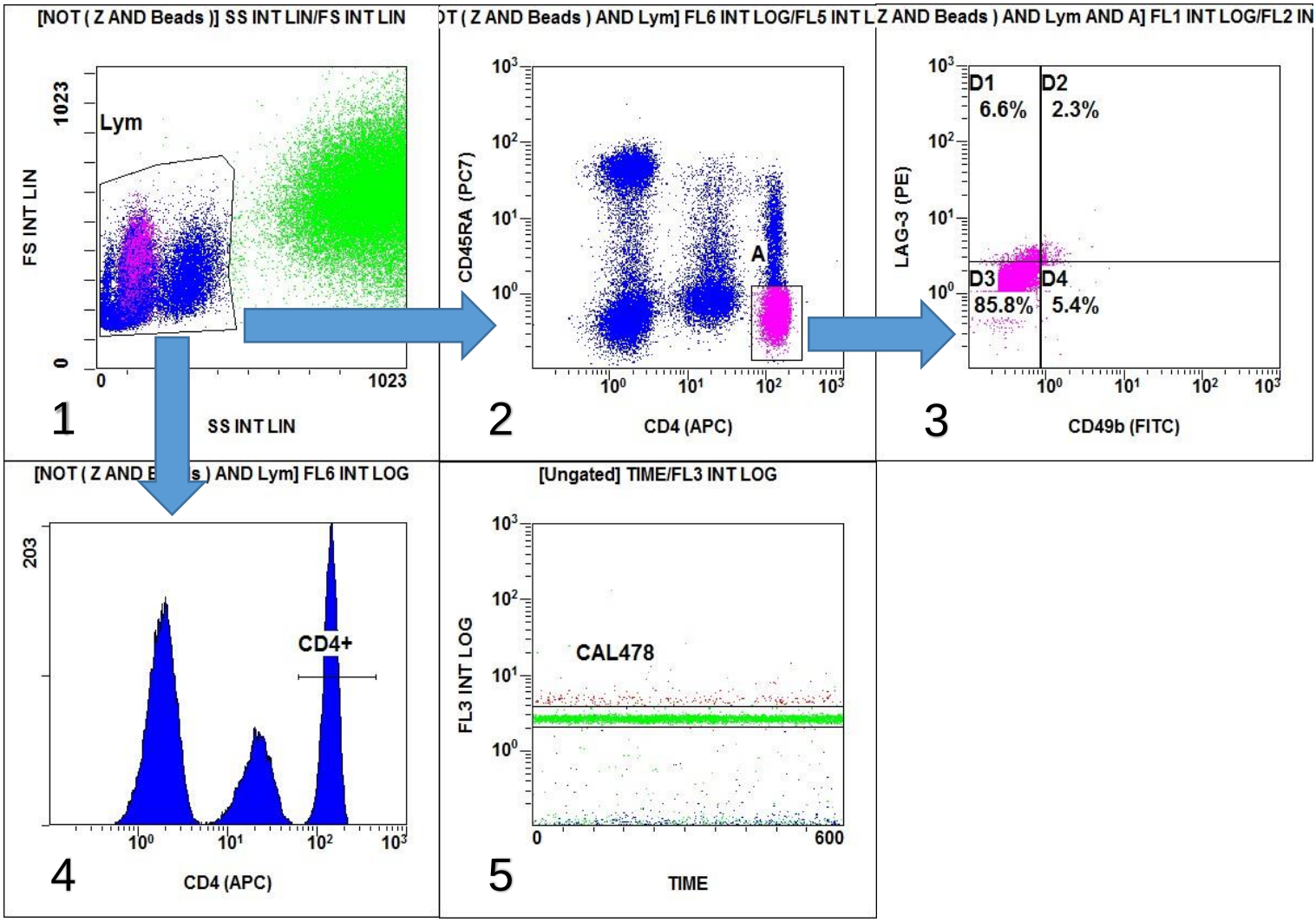
Figure 2.6 Type 1 regulatory T cell (isotype control) flow cytometry protocol



CAL = calculation factor

Plot (1) is SS vs FS, lymphocytes and monocytes were gated into plot (2). Plot (2) is CD4 vs CD45RA, events that were positive for CD4 and negative for CD45RA were gated into plot (3). Plot (3) is CD49b vs LAG-3, double positive events are Type 1 T regulatory cells. Plot (4) produces a $CD4^{+}$ count. Plot (5) shows the Trucount™ beads. Gates positioned according to Gagliani *et al.* (2013).

Figure 2.7 Type 1 regulatory T cell (test) flow cytometry protocol



2.18.4 $CD3^+/CD4^+$ T cells

A $CD3^+$ flow cytometry protocol already existed within the routine SCI laboratory and this was adjusted to a five colour protocol for $CD3^+CD4^+$ cell enumeration. The antibodies used for $CD3^+CD4^+$ cells are shown in table 2.10.

Table 2.10 Antibody cocktail used for $CD3^+/CD4^+$ T cell analysis

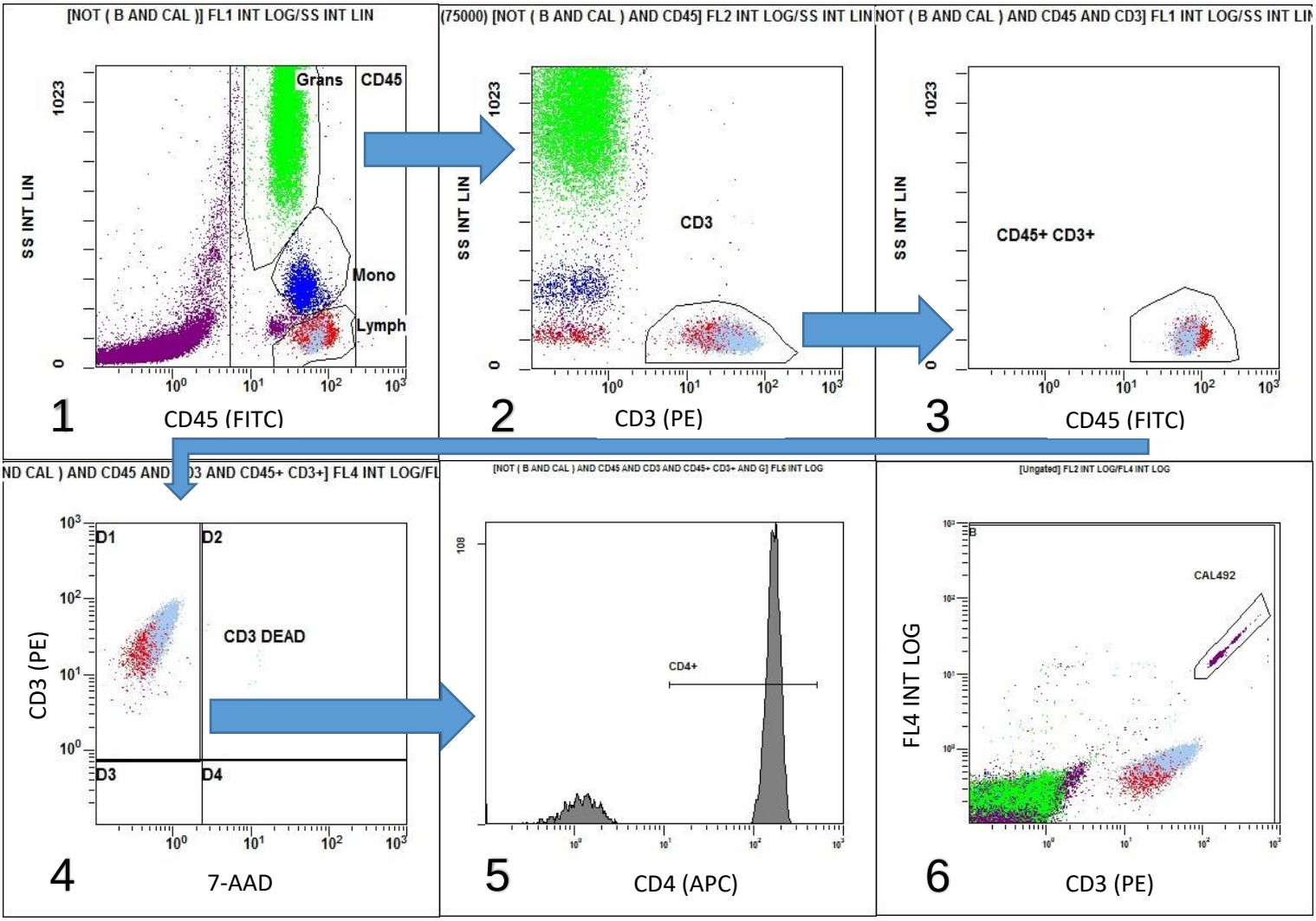
Conjugated monoclonal antibody	Antigen cellular expression	Antigen function*
CD45-FITC	Leukocytes	Leukocyte common antigen
CD3-PE	T lymphocytes	Part of the complex that includes the TCR
CD4-APC	T helper lymphocytes, low density on monocytes	Co-receptor on T_H for MHC class II
7-AAD	Intercalates between cytosine and guanine bases of DNA	DNA binding dye

*https://www.mybeckman.uk/reagents/coulter-flow-cytometry

Sample preparation for flow cytometry was performed as described in 2.18. This protocol also added 10µl 7-AAD viability dye to each tube.

Figure 2.8 illustrates the plots and gating strategy taken directly from the flow cytometry print out for identification of $CD3^+$ and $CD3^+/CD4^+$ cells.

Figure 2.8 $CD3^+/CD4^+$ T cell flow cytometry protocol



CAL = calculation factor

Plot (5) was added to a pre-existing $CD3^+$ protocol. Plot (1) is CD45 vs SS. CD45 positive events were gated into plot (2). Plot (2) is CD3 vs SS, CD3 positive events were gated into plot (3). Plot (3) is CD45 vs SS, positive events were gated into plot (4). Plot (4) is 7-AAD vs CD3, the negative events produce the $CD3^+$ count and are gated into plot (5). Plot (5) produces the $CD3^+CD4^+$ cell count.

2.18.5 CD34$^+$ Haematopoietic stem/progenitor cells

A four colour CD34 flow cytometry protocol already existed within the routine SCI laboratory and was used for this study. The antibodies used for CD34$^+$ count are shown in table 2.11.

Table 2.11 Antibody cocktail used for CD34$^+$ haematopoietic stem/progenitor cell analysis

Conjugated monoclonal antibody	Antigen cellular expression	Antigen function*
CD45-FTIC	Leukocytes	Leukocyte common antigen
CD34-PE	Haematopoietic progenitor cells of all lineages	HSC/HPC phosphoglycoprotein
7-AAD	Intercalates between cytosine and guanine bases of DNA	DNA binding dye

*https://www.mybeckman.uk/reagents/coulter-flow-cytometry

Sample preparation for flow cytometry was performed as described in 2.18. This protocol also added 10µl 7-AAD viability dye to each tube.

2.19 Testing the protocols with healthy donor peripheral blood samples

2.19.1 Protocol results with fresh healthy donor peripheral blood

Prior to starting the study with patient and donor samples, peripheral blood samples from three healthy donors were tested as internal procedure controls for the three tolerogenic cell phenotypes: pDCs, DC-10 cells and Tr1 cells and mDC1 cells. Three samples were selected so that an average could be obtained, while not delaying the start of the study by analysing many samples which would not have added value. The results are shown in table 2.12.

Table 2.12 Healthy donor peripheral blood tolerogenic cell counts.

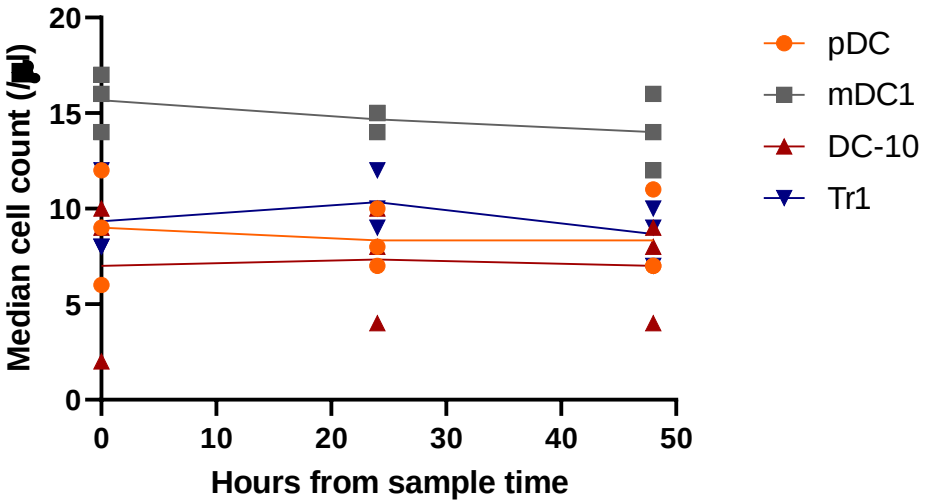
	Counts (/µl)			
Healthy donor	pDC	mDC1	DC-10	Tr1
1	6	17	9	12
2	12	14	10	8
3	7	15	2	3
Range	6-12	14-17	2-10	3-12
Test mean (n=3)	8.33	15.33	7.00	7.67
Test standard deviation (n=3)	3.21	1.53	4.36	4.51

The expected ranges of these cells in peripheral blood are: pDC 5-10/µl, mDC 4-30.5/µl, (Chevallier *et al.* 2013, DC-10 4-13/µl (Gregori *et al.* 2010) and Tr1 4-13/µl (Gagliani *et al.* 2013). The cell counts obtained using the created protocols were within the expected ranges for each cell type except pDC, one donor's pDC result being slightly higher than 10/µl. The protocols all obtained values that were within +/- 2 standard deviations of the mean, indicating that the created protocols were suitable for the study.

2.19.2 Impact of sample storage time on peripheral blood cell counts

Longevity storage studies were performed on three healthy donors' peripheral blood, with the same donor sample being tested on the date bled, and again after 24- and 48-hours following collection. Three samples were selected so that an average could be obtained, while not preventing the start of the study by analysing many samples, as well as the cost of more conjugated antibodies, which would not have added value. Samples were stored at 4^{o}C. This was performed to assess any effect of delay in sample analysis and the results are shown in figure 2.9. Friedman's test was performed for each tolerogenic cell type to test for any change in count over time. There was no statistically significant difference over time for any tolerogenic cell type: pDC $P = 0.889$, mDC1 $P = 0.389$, DC-10 $P > 0.999$ and Tr1 $P = 0.222$.

Figure 2.9 Median healthy donor peripheral blood tolerogenic cell counts over time from sample collection to analysis



Individual replicates with connected medians

The counts for the tolerogenic cells tested were consistent when the PB sample was stored for up to 48 hours at 4°C. Plasmacytoid DCs may have been reducing over time in storage, but as no samples analysed as part of the study were stored for greater than 24 hours the time in storage was not a factor in the cell number analysis.

2.20 Clinical and additional laboratory data collection (research nurse information)

Clinical data was obtained from several sources. Transplant timetables were supplied from the bone marrow transplant coordinator at SJUH, Leeds. The transplant timetables provided data on both donor and patient, risk factors, conditioning, and prophylaxis.

The PBSC collections were analysed for $CD34^+$ dose by the SCI laboratory at NHSBT Leeds as per routine procedure.

Neutrophil engraftment is defined as the first of 3 consecutive days where the neutrophil count is >0.5 x 10^9/L. Platelet engraftment is defined as the first of 3 consecutive days where the platelet count is >20 x10^9/L without transfusion. This was determined by the clinicians at SJUH, Leeds and data was shared with the SCI laboratory at NHSBT Leeds using NHSBT form 2G, as per routine procedure.

The post-transplant worksheet, provided by the PI (as shown in appendix XIV) was completed by a research nurse at SJUH, Leeds. This worksheet provided data on acute and/or chronic GvHD, disease

free survival time, progression free survival time, overall survival time, relapse dates and cause of death. These were determined by the clinicians at SJUH, Leeds.

2.21 Patient and donor recruitment

Twenty-four patients were recruited into the study for one year (November 2018 to November 2019). The 24 patients had the following donor sources: 8 German, 7 sibling, 5 Anthony Nolan, 2 USA, 1 French and 1 BBMR. At the 6-month time point only 4 donations from 15 transplants had been tested and only 5 eligible donors had been missed: 2 Anthony Nolan, 2 sibling and 1 BBMR. Recruitment did not match the estimates made before the start of the study, which was based on an analysis of transplants performed in the 3 years prior to the study.

The study was limited to one year due to the six months follow up period post transplantation to assess engraftment and to complete the analysis within the time frame of the two-year research phase of the DProf. The patient characteristics are summarised in table 2.13 and the patient's risk factors for GvHD are summarised in table 2.14. Diagnosis of GvHD, relapse, infection and regimen related toxicity were made by clinicians at SJUH, Leeds.

Five donors (2 from related donors and 3 from AN registry donors) were recruited into the study and their characteristics are summarised in table 2.15. Table 2.16 shows the average graft cell counts. Much fewer donors were recruited than anticipated and this was due to a large proportion of international donors being selected for transplant by the clinicians during the study period. The study's ethical approval excluded international donors as making research ethics applications to potentially many registries based abroad was not feasible in the time frame of this research.

Table 2.13 Characteristics of transplant recipients

Average age (years)		55 (range 22-74)
Gender M/F		14/10
Disease prior to transplant	AML	10
	MDS	6
	Hodgkin's lymphoma	3
	ALL	2
	Others	3
Disease status prior to transplant	CR/No evidence of disease	10
	Partial response	2
	Regenerating haematopoiesis	2
	MRD (persistent low-level disease)	3
	CMR (Deauville 1,2,3)	2
	Deauville (4)	1
	Hypoplastic marrow	1
	Ongoing disease	1
	Disease relapse	1
	MDS excess blasts	1

AML = Acute Myeloid Leukaemia, MDS = Myelodysplastic Syndrome, ALL = Acute Lymphoblastic Leukaemia, CR = complete remission, MRD = minimal residual disease and CMR = complete metabolic remission.

Table 2.14 Patients' risk factors for GvHD

		Number
Age (years)	< 40	2
	> 40	22
Related/unrelated donor		7/17
HLA match	12/12	9
	11/12	10
	10/12	4
	Haploidentical	1
Sex match	Matched	14
	Male into female	8
	Female into male	2
GvHD prophylaxis	Ciclosporin & Ursodeoxycholic acid	12
	Ciclosporin, Ursodeoxycholic acid & MMF	10
	Cyclophosphamide, Ursodeoxycholic acid, MMF & Tacrolimus	2
Conditioning	FluBuMethAlem	5
	FluCytAmsBuBuATG	2
	FluBuATG	7
	FluCycloAlemMethLDTBI	3
	FluCycloTBI	2
	FluMelAlem	3
	FluMel	1
	FluCycloAlem	1

MMF = Mycopenolate mofetil, Flu = Fludarabine, Bu = Busulfan, Meth =MethylPrednisolone, Alem = Alemtuzumab, Cyt = Cytarabine, Ams = Amsacrine, ATG = antithymocyte globulin, Cyclo = Cyclophosphamide, LDTBI = Low dose total body irradiation and Mel = Melphalan.

The recipient age threshold in transplantation is 40 (Dr J Clay, SJUH, Leeds – private correspondence). It was not feasible to perform analysis by age group as only two recipients in the study were under that age.

Table 2.15 Donor/donation characteristics

	Donors recruited into study	Donors ineligible for recruitment
Mean age (range)	29 (21-40)	36.7 (21-64)
Sex M/F	4/0	16/4
Average $CD34^+$ dose ($x10^6$/kg)	5.7 (range 3.6-9.6)	7.1 (range 2.9-10)
Related donor/MUD	1/3	6/14
Fresh/cryopreserved cells	2/2	16/4

MUD = Matched unrelated donor

Table 2.16 Mean graft cell counts

	$CD3^+$	pDC	mDC1	Tr1	DC-10
Mean dose ($x10^6$/kg) (range)	171.14 (104.6 – 236.1)	2.93 (1.2 – 4.7)	1.99 (1.1 – 2.7)	0.31 (0.12 – 0.8)	5.53 (2.8 – 8.7)
Standard deviation	52.8	1.21	0.58	0.24	2.25
Number	6	6	6	6	5

2.22 Analysis of the study results

Initially results were entered into Microsoft Excel 2016 and were transferred into GraphPad Prism 8. All data analyses and visuals shown in the results section were produced in GraphPad Prism 8.

Parametric tests assume that the data has a normal distribution, however it can be difficult to identify the shape of a distribution curve when n is less than 30 (Field and Hole 2006). The Kolmogorov-Smirnov test or quantile quantile (QQ) curves were used to test for normality. The Kolmogorov-Smirnov test used a significance level (alpha) of 0.05.

The independent t-test (parametric), Chi-square (non-parametric) and Mann-Whitney (non-parametric) tests were performed with a 95% confidence interval.

Groups were analysed using the independent t-test (unpaired two-tailed), Chi-square (two sided) and Mann-Whitney (two tailed) test. Results were considered significant if $P < 0.05$.

$CD34^+$ dose, neutrophil and platelet engraftment were tested for normality using QQ plots and were found to be normally distributed. Recipients were split into two groups depending upon if they received more or less than the average $CD34^+$ graft dose of 6.45 x 10^6/kg. The 'high' and 'low' dose groups had their engraftment times compared using the t-test.

Two groups were created according to GvHD status. These two groups were compared using Chi-square test against the (discrete) known risk factors for GvHD which were: related donor/unrelated donor, HLA mismatch, sex mismatch, conditioning with ATG vs alemtuzumab, and GvHD prophylaxis (tested as ciclosporin and ursodeoxycholic acid vs other). Chi-square tests were also performed on groups of patients, with complete remission at transplant patients vs other and relapse within 6 months of transplant patients vs other.

The tolerogenic cell counts versus GvHD status could be tested using a mixed ANOVA (parametric) or Mann-Whitney (non-parametric). If tolerogenic cell count(s) could be used as a biomarker for GvHD, evidence would come from the analysis of this data. The available resources unfortunately resulted in some sample time points being missed, and because a complete set of data was not available, it was not possible to perform a mixed ANOVA as its underlying assumptions were not met, and this is a limitation of the study. Because of this, the tolerogenic cell counts at each time point versus GvHD status were tested using the Mann-Whitney test which does not require a full set of data.

The study was a pilot study to collect the data which will enable the sample size (N) calculation to be performed to achieve a power of 0.8 for the principal research question. The study used the following website to calculate N - https://powerandsamplesize.com/Calculators/. The test selected compared two means, two sample, two sided equality. The 4-week tolerogenic cell count/µl mean and standard deviation was calculated for each group (GvHD and no GvHD), from which the pooled standard deviation was calculated and put into the online tool.

N was obtained for the 4-week time point, this was selected because it would give an early warning about patients potentially at risk of GvHD. At 4 weeks the sample size required to obtain a power of 0.8 with a significance criterion (α) of 0.05 was: pDC = 309, mDC1 = 101, DC-10 = 103 and Tr1 = 27. The sample size of 27 patients for Tr1 is a likely statistical quirk (Ellen Marshall, statistician, SHU, Sheffield – private correspondence) due to the very low Tr1 counts (only at the 8-week time point onwards were most patient's counts above <1cell/µl). However because Tr1 cells were slow to recover post allograft regardless of GvHD status, they would be a poor prognostic biomarker for GvHD and would therefore be excluded from any future study.

Advice on the appropriate statistical tests to perform was provided by Ellen Marshall and Dr Karen Kilner, Sheffield Hallam University.

Chapter 3 – Results

3.1 Transplant and post-transplant patient outcomes

The key research question addressed by this study was to determine whether there was an association between the numbers of tolerogenic cell types (pDC, DC-10 and Tr1) in PBSC collections and/or in recipients' PB during immune cell reconstitution following HSCT, and the development of GvHD. Reconstitution post HSCT was assessed at 2-, 4-, 8-weeks, 3- and 6-months by the enumeration of: WBC, $CD3^+$, $CD4^+$, pDC, mDC1, DC-10 and Tr1 cell counts, and neutrophil and platelet engraftment was also recorded. Any differences in the reconstitution of these cells in the recipient and transplant outcomes (e.g. GvHD) was analysed.

Twenty-four patients receiving HSCT were recruited into the study. At the final patient's 6-month follow up point; six patients had been diagnosed with aGvHD, one patient had been diagnosed with cGvHD, two patients had died, and one patient was in palliative care.

A complete set of five post-transplant samples was only obtained from 7 of the 24 patients. Details of obtained / missed samples at each sample time point are shown in table 3.1. Nine samples had only FBC analysis performed due to the available resources.

Table 3.1 Samples obtained/missed at each time point post HSCT

	Sample time point				
	2 weeks	4 weeks	8 weeks	3 months	6 months
Full analysis	17	19	19	19	11
FBC only	3	0	4	1	1
Missed	4	5	1	4	6
RIP/Palliative	0	0	0	0	3
Missed*	0	0	0	0	3

*Missed = due to available staffing and equipment resources

A summary of transplant details and post-transplant outcomes for patients in this study are shown below.

- The mean $CD34^+$ graft dose transplanted into recipients was 6.45 x 10^6/kg (range 2.9-10 x 10^6/kg, standard deviation = 2.05).
- All 24 patients had neutrophil engraftment within 28 days (no delayed engraftment in this patient cohort), with a mean engraftment time of 14.83 days (range 8 to 24 days, standard deviation = 3.09).

- 21/24 patients had platelet engraftment within 28 days, with a mean engraftment time of 17.36 days (range 9 to 44 days, standard deviation = 8.59). The three patients that did not have platelet engraftment within 28 days were patients 6, 14 and 19. Platelet counts for two patients never dropped below 20 (patients 4 and 8).
- Only 1/24 patients (patient 21) had an adverse reaction to the infusion which was recorded as blurred vision/seeing lights. This patient did not develop GvHD.
- All patients were alive at 100-days post-transplant.

At the end of the 6 months follow up period:

- Six out of the twelve patients who had 6-month samples analysed had WBC counts in the normal range (patients 5, 7, 9, 17, 20 and 22).
- 6/24 patients had been diagnosed with aGvHD (patients 1, 2, 3, 15, 17 and 20) and 1/24 patient had cGvHD (patient 19).
- 1/6 patients had steroid refractory aGvHD (patient 17)
- 6/24 patients had disease relapse (patients 12, 14, 15, 16, 19 and 23)
- Only patient 15 was diagnosed with GvHD and relapsed. Patient 15 was diagnosed with GvHD at 3 weeks and relapsed at 25 weeks.
- 22/24 patients were alive. One patient died of disease relapse (patient 14) and one patient died of GvHD and AML (patient 2). GvHD was therefore responsible for 1/24 deaths within the 6-month follow up period. One patient (patient 19 cGvHD) was in palliative care.

3.2 Pre transplant patient details by GvHD status

Pre transplant patient details were compared according to GvHD status, this is shown in table 3.2. There were 14 male and 10 female recipients. 18 patients received a 12/12 or 11/12 HLA matched donation. Excluding the single patient with cGvHD, the patients were of a similar age. Most transplants were sex matched or male into female, only two transplants were female into male. All patients received RIC. The most common diseases were AML and MDS. Ten patients were in complete remission/no evidence of disease status prior to transplant. The eight patients who had a level of disease present prior to transplant did not get diagnosed with GvHD within the 6-month follow up.

Table 3.2 Pre transplant patient details by GvHD status

		aGvHD group (n=6)	cGvHD group (n=1)	No GvHD group (n=17)
Mean recipient age (Standard deviation)		54.83 years (11.05)	65 years (N/A)	54.71 years (13.41)
Recipient gender	Male	4	1	9
	Female	2	0	8
HLA match	12	2	0	7
	11	3	1	6
	10	1	0	3
	Haploidentical	0	0	1
Sex match	Matched	4	1	8
	Male into female	1	0	7
	Female into male	1	0	1
GvHD prophylaxis	Ciclo & Urso	4	0	8
	Ciclo, Urso & MMF	2	1	7
	Cyclo, Urso, MMF and Tacrolimus	0	0	2
Conditioning (All RIC)	FluBuMethAlem	2	0	3
	FluCytAmsBuBuATG	1	0	1
	FluBuATG	1	1	5
	FluCycloAlemMeth LDTBI	1	0	2
	FluCycloTBI	0	0	2
	FluMelAlem	1	0	2
	FluMel	0	0	1
	FluCycloAlem	0	0	1
Disease prior to transplant	AML	2	1	5
	MDS	1	0	6
	Hodgkin's lymphoma	1	0	2
	ALL	1	0	1
	Others	1	0	3
Disease status prior to transplant	CR/No evidence of disease	3	0	7
	Partial response	1	0	1
	Disease present	1	1	8
	Others	1	0	1

Ciclo = Ciclosporin Urso = Ursodeoxycholic acid MMF = Mycopenolate mofetil, Flu = Fludarabine, Bu = Busulfan, Meth =MethylPrednisolone Alem = Alemtuzumab, Cyt = Cytarabine, Ams = Amsacrine, ATG = antithymocyte globulin, Cyclo = Cyclophosphamide, (LD)TBI = (Low dose) total body irradiation, Mel = Melphalan, AML = Acute Myeloid Leukaemia, MDS = Myelodysplastic Syndrome, ALL = Acute Lymphoblastic Leukaemia and CR = complete remission

3.3 Post transplant patient details by GvHD status

Post-transplant patient outcomes were compared according to GvHD status and is shown in table 3.3. The table shows that regardless of GvHD diagnosis, all patients received a similar $CD34^+$ graft dose. Excluding the single patient with cGvHD, the patients had similar neutrophil engraftment times, though the platelet engraftment was slightly later in the no GvHD group.

Table 3.3 Transplant and post-transplant factors

		aGvHD group (n=6)	cGvHD group (n=1)	No GvHD group (n=17)
Donor	Related	2	0	5
	Unrelated	4	1	12
Mean CD34 dose ($x10^6$/kg) (+/-Standard deviation)		6.76 (2.43)	6.8 (N/A)	6.35 (1.95)
Mean engraftment (Standard deviation)	Neutrophils	13.8 days (1.94)	24 days (N/A)	14.6 days (2.62)
	Platelets	14 (3.16)	33 (N/A)	17.7 (9.20)
Within 6 months of transplant	Relapsed	2	1	5
	Not relapsed	4	0	12

3.4 Cellular reconstitution post allogeneic haematopoietic transplantation

For the flow cytometry analysis only one post-transplant PB patient sample required dilution prior to analysis (patient 6 at 4-weeks, diluted 1 in 2), all other samples had a PB WBC count which allowed the sample to be tested neat. Reconstitution post HSCT was assessed at 2-, 4-, 8-weeks, 3- and 6-months. Individual colours assigned to each of the 24 patients included in the study are in the legend. Patients missed at the time points are as follows; Two weeks; 1, 13, 17 and 23. Four weeks: 4, 19, 20, 21 and 22. Eight weeks: 14. Three months: 4, 11, 14 and 17. Six months: 2, 3, 4, 6, 10, 12, 13, 14, 19, 21, 23 and 24. While the counts for WBCs and MNCs steadily rise following transplantation, the sub-groups of cells that constitute these groupings may present with a different pattern of reconstitution, thus these were investigated.

Figure 3.1 shows that the median WBC count reached the normal range at 3 months (GvHD group) and 6-months (no GvHD group) post allogeneic HSCT. The median MNC and neutrophil count did not achieve the normal range throughout the 6-month post HSCT follow up period. The median neutrophil count at 2-weeks was 0.88 x 10^9/L, which is above the threshold used to define engraftment (data not shown).

3.5 Tolerogenic cell count analysis and GvHD status

To answer the principal question of the study, which was whether there was an association between tolerogenic cell counts during immune reconstitution and development of GvHD, tolerogenic cell counts at the 5-sampling time points post-transplant were compared to GvHD status. Because of the missed samples due to limited resources within the laboratory, Mann-Whitney (non-parametric) analysis and not mixed ANOVA (which requires parametric data) was performed on the data. The mean age for patients diagnosed with aGvHD was 54.83, and the mean age of patients not diagnosed with aGvHD was 54.71.

Seven patients developed GvHD, six aGvHD (patients: 1, 2, 3, 15, 17 and 20) and one cGvHD (patient 19). Patients 2 and 14 were deceased before the 6-month follow up time point, patient 19 had been sent to palliative care before the 6-month sample point.

Scatter plots for tolerogenic cell counts for patients with and without GvHD at the five sampling time points are shown in figure 3.1 (WBC), figure 3.2 ($CD3^+$ T cells), figure 3.3 ($CD4^+$ cells), figure 3.4 (pDCs), figure 3.5 (mDC1s), figure 3.6 (DC-10 cells) and figure 3.7 (Tr1 cells). The scatter plots and discussion for each cell type are presented below.

3.5.1 White blood cell counts in patients split by GvHD diagnosis

Figure 3.1 shows the change in patients' PB WBC count post HSCT, over the 6-month study period. The normal WBC count range is 4.4 – 10.4 x 10^9/L (Chevallier *et al.* 2013), the highest WBC count obtained at any point in the 6-month follow up was 23.33 x 10^9/L (patient 6 at 4-weeks, no GvHD in the 6-month follow up period), the lowest was 0.01 x 10^9/L (patient 6 at 2-weeks). The median WBC counts achieved the normal range in the GvHD group at 3-months and in the no GvHD group at 6-months.

At 2 weeks post HSCT 16/20 of patients had a WBC count below the normal range. Patients 10, 12, and 21 (none of whom was diagnosed with GvHD in the 6 month follow up period) were in the normal range and patient 7 (no GvHD in the 6 month follow up period) had a count slightly above the normal range at 11.89 x 10^9/L. The no GvHD group has a slightly higher median WBC count than the no GvHD group (1.22 x 10^6/ml vs 0.86 x 10^6/ml respectively).

At 4 weeks post HSCT 14/19 patients had a WBC count below the normal range. Patients 16 and 24 (neither of whom was diagnosed with GvHD in the 6-month follow up period) were in the normal range, and patients 2, 6, 8 (all no GvHD in the 6-month follow up period) were above the normal range, patient 6 having a WBC of 23.33 x 10^9/L. Patients 7, 10 and 12 (all no GvHD in the 6-month follow up period) were now below the normal range. Samples for patients 20 and 21 were missed at

this time point. The GvHD group has a slightly higher median count than the no GvHD group (3.54 x 10^6/ml vs 3.21 x 10^6/ml respectively).

At 8 weeks post HSCT 16/23 patients had a WBC count below the normal range. Patients 2, 3, 4, 6, 8, 21 and 24 (only patient 3 was diagnosed with GvHD in the 6 month follow up period) were in the normal range, with patients 2, 6 and 8 being above the normal range at 4-weeks (of whom, only patient 2 was diagnosed with GvHD in the 6 month follow up period). Patient 16 (no GvHD in the 6 month follow up period) was now below the normal range. No patients had a WBC count above the normal range at the 8-week time point. The no GvHD group had the higher median count than the GvHD group (3.81 x 10^6/ml vs 3.03 x 10^6/ml respectively).

At 3 months post HSCT 9/20 patients had a WBC count in the normal range. Patients with a WBC count below the normal range were patients 6, 8, 10, 12, 13, 15, 16, 19, 20 and 23 (only patients 15 and 20 were diagnosed with GvHD in the 6 month follow up period). Patients 6 and 8 (no GvHD in the 6 month follow up period) had been in the normal range at 8-weeks. No patients had a WBC count above the normal range at this time point. The GvHD group had the higher median count than the no GvHD group (4.46 x 10^6/ml vs 4.25 x 10^6/ml respectively).

At 6 months post HSCT 6/12 patients had a WBC count in the normal range. Patients below the normal range were 1, 8, 11, 15, 16 and 18 (only patients 1 and 15 were diagnosed with GvHD in the 6 month follow up period). Patients 1 and 18 were in the normal range at 3-months. No patients had a WBC count above the normal range at this time point. At the 6 months the no GvHD group has a higher median WBC count than the GvHD group (4.77 x 10^6/ml vs 4.35 x10^6/ml).

Patients 1, 2, 3, 15, 17, 19 and 20 were diagnosed with GvHD. At 2 weeks patients 2, 3, 15, 19 and 20 were all below the normal range, though patient 20 only just below. Patients 1 and 17 were missed at this time point. At 4-weeks patients 1, 3, 15 and 17 were below the normal range, but now much closer to it, while patient 2 was above the normal range. Patients 19 and 20 were missed at this time point. At 8-weeks patients 2 and 3 are in the normal range, while patients 1, 15, 17, 19 and 20 are all below the normal range. At 3-months patients 1, 2 and 3 were in the normal range, while patients 15, 19 and 20 were below the normal range and patient 17 was missed at this time point. At 6-months patients 17 and 20 were in the normal range, patients 1 and 15 were below normal range, patient 3 was missed at this time point. Patient 2 was deceased and patient 19 was in palliative care at this time point.

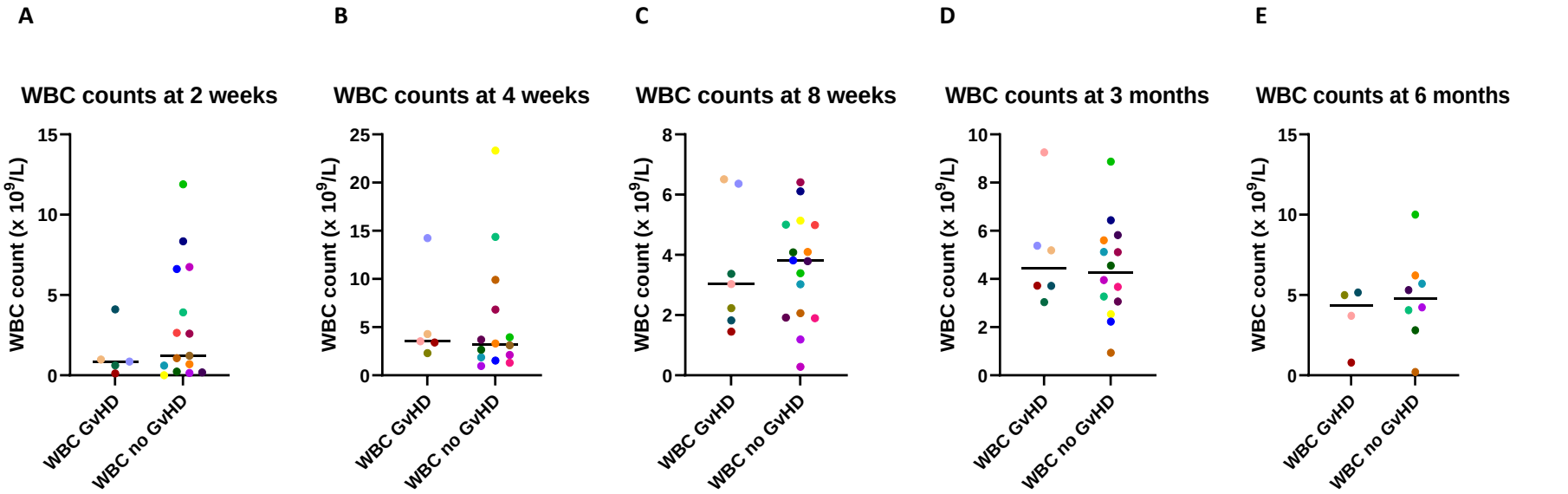
No GvHD group: For the WBC cell count at 2-weeks there appears to be two group of patients using a cut off value of 5 x 10^6/ml, with four patients with counts above that. This high group consists of

patient 7 (age 70), 10 (age 40), 12 (age 59) and 21 (age 60). At 4-weeks there also appears to be two groups of patients using a cut off of value 5 x 10^6/ml, with four patients with counts above that. This high group consists of patient 2 (age 48), 6 (age 49), 16 (age 58) and 24 (age 64). All the no GvHD 'high count' patients were alive at the 6-month follow up time point.

Patients 2 and 14 were deceased at the 6-month time point. Only 2- and 4-week samples were obtained from patient 14, there was no 6-month sample from patient 2. Patient 2 was not in the WBC normal range at 2 and 4-weeks but was at 8-weeks and 3-months. Patient 14 was below the normal range at 2 and 4-weeks and no more samples were received from this patient after that time point.

There were no statistically significant differences for peripheral blood WBC count in the GvHD group compared with the no GvHD group at any of the five sampling time points: 2-, 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.50, 0.50, 0.82, 0.60 and 0.46 respectively.

Figure 3.1 Timeline of peripheral blood WBC counts post HSCT in patients split by GvHD status



2-week GvHD median = 0.86 (n=5)	4-week GvHD median = 3.54 (n=5)	8-week GvHD median = 3.03 (n=7)	3-months GvHD median = 4.46 (n=6)	6-month GvHD median = 4.35 (n=4)
2-week no GvHD median = 1.22 (n=15)	4-week no GvHD median = 3.21 (n=14)	8-week no GvHD median = 3.81 (n=16)	3-months no GvHD median = 4.25 (n=14)	6-month no GvHD median = 4.77 (n=8)
Mann-Whitney *P* = 0.50	Mann-Whitney *P* = 0.50	Mann-Whitney *P* = 0.82	Mann-Whitney *P* = 0.60	Mann-Whitney *P* = 0.46

WBC normal range 4.4 – 10.4 x 10^9/L. (Chevallier *et al.* 2013). Each patient is represented by the following colours (GvHD patients in bold):

1 **2** **3** 4 5 6 7 8 9
10 11 12 13 14 **15** 16 **17** 18
19 **20** 21 22 23 24

3.5.2 $CD3^+$ T cell counts in patients split by GvHD diagnosis

Figure 3.2 shows the change in the patients' PB $CD3^+$ T cell count post HSCT, over the 6-month study period. The normal $CD3^+$ T cell count range is 540 – 1790/µl (Punt *et al.* 2019), the highest $CD3^+$ count obtained at any point in the 6-month follow up was 8381/µl (patient 7 at 6-months, no GvHD within the 6-month follow up time period), the lowest was <1/µl. (patient 11 and 12 at 2-weeks, and patient 11 at 4-weeks, neither patient was diagnosed with GvHD within the 6-month follow up time period). The only occasion the median $CD3^+$ T cell counts achieved the normal range (540-1790/µl, Punt *et al.* 2019) was at the 6-month follow up period for the GvHD group.

At 2 weeks post HSCT 16/16 patients had a $CD3^+$ T cell count below the normal range. The GvHD group and no GvHD groups have similar median $CD3^+$ cell counts (11/µl vs 18/µl respectively). This can be compared to the WBC count, which at 2-weeks post HSCT, 15/20 patients had a WBC count below the normal range.

At 4 weeks post HSCT 19/19 patients had a $CD3^+$ T cell count below the normal range. The no GvHD group has a higher median count than the GvHD group (44.5/µl vs 26/µl respectively). This can be compared to the WBC count, which at 4-weeks post HSCT 13/19 patients had a WBC count below the normal range.

At 8 weeks post HSCT 16/19 patients had $CD3^+$ T cell counts below the normal range, patients in the normal range were: 6, 7 and 18, none of these patients were diagnosed with GvHD in the 6-month follow up period. The no GvHD group maintains the higher median count than the GvHD group (133/µl vs 72/µl). This can be compared to the WBC count, which at 8-weeks post HSCT 16/23 patients had a WBC count below the normal range.

At 3 months post HSCT 13/19 patients have $CD3^+$ T cell counts below the normal range, patients in the normal range were: 2, 9, 18, 23 and 24, only patient 2 had GvHD in the 6 month follow up period. The no GvHD group maintains the higher median count than the GvHD group (345/µl vs 137/µl). Patient 7 exceeds the normal range with a count of 2515/µl (no GvHD in the 6 month follow up period). Patient 6's (no GvHD in the 6 month follow up period) $CD3^+$ T cell count had fallen below the normal range.

At 6 months post HSCT 6/11 patients have $CD3^+$ T cell counts below the normal range, patients in the normal range were: 1, 9, 18 and 20, only patient 1 had GvHD in the 6-month follow up period. At the 6 months the GvHD group has a higher median $CD3^+$ count than the no GvHD group (620/µl vs 189/µl). 620/µl is at the low end of the normal range (540-1790/µl) for $CD3^+$ counts. Patient 7 (no

GvHD in the 6 month follow up period) had a $CD3^+$ T cell count that exceeded the normal range with a count of 8381/µl.

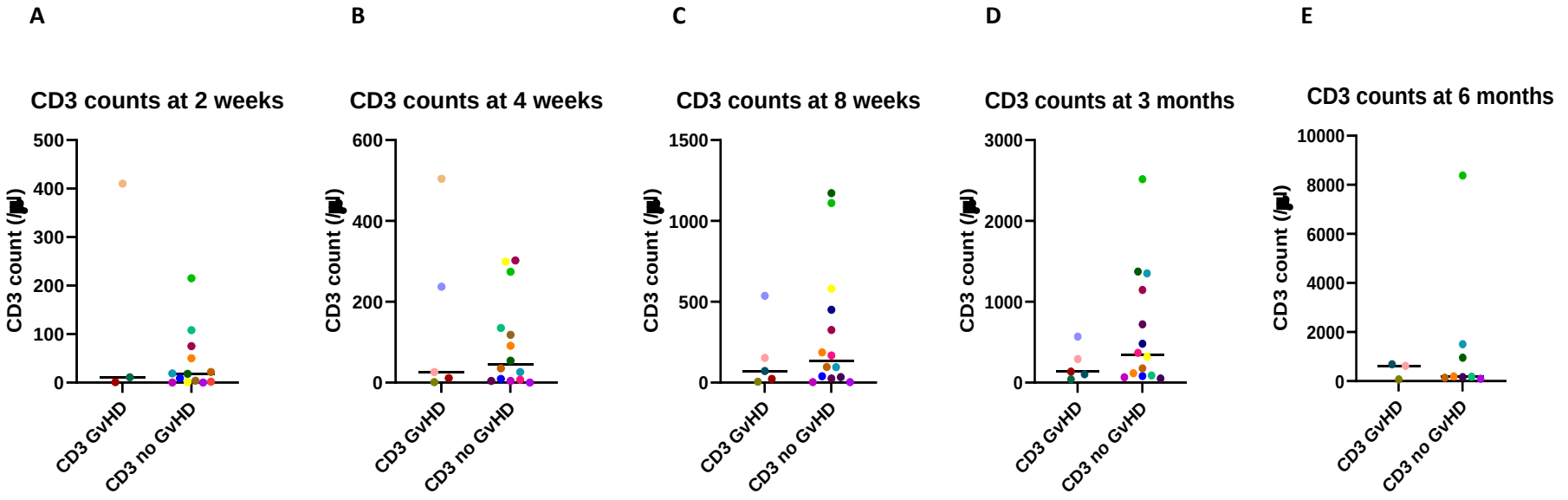
Patients 1, 2, 3, 15, 17, 19 and 20 were diagnosed with GvHD. At 2 weeks patients 3, 15 and 19 were below the normal range, while patients 1, 2, 17 and 20 were missed at this time point. At 4 weeks patients 1, 2, 3, 15 and 17 were all below the normal range, while patients 19 and 20 were missed at this time point. Patient 3 (age 53) had the highest $CD3^+$ cell counts at 2 and 4 weeks but was not sampled again during follow up. Patient 3 was alive at the 6-month follow up time point. At 8 weeks patient 2 was in normal range, patients 1, 15, 17 and 20 were below normal range and patients 3 and 19 were missed at this time point. At 3 months patient 2 was in the normal range, patients 1, 15, 19 and 20 were below the normal range, patients 3 and 17 were missed at this time point. Patient 2 (age 48) also a high $CD3^+$ cell count at 4-weeks and had the highest counts at 8-weeks and 3-months. At 6 months post HSCT, patients 1 and 20 had $CD3^+$ T cell counts in the normal range, patient 17 was below the normal range, patients 3 and 15 were missed at this time point. Patient 2 was deceased and patient 19 was in palliative care at this time point.

No GvHD group: For the $CD3^+$ cell count from 4-weeks onwards there appears to be two clusters of patients within the no GvHD group. However only patient 7 (age 70) is consistently present in the high $CD3^+$ T cell count group. Other patients in the high-count group are patient 6 (age 49) and patient 24 (age 64) at 4-weeks, and patient 18 (age 64) at 8-weeks. All the no GvHD 'high-count' patients were alive at the 6-month follow up time point.

Patients 2 and 14 were deceased at the 6-month post HSCT time point. Only the 2- and 4-week samples were obtained from patient 14, there was no 6-month sample from patient 2. Patient 2 is discussed above, patient 14 had $CD3^+$ T cell counts below the normal range at their available sample time points.

There was no statistically significant difference in the peripheral blood $CD3^+$ cell count between the GvHD and no GvHD groups at any of the five sampling time points: 2-, 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.83, 0.912, 0.488, 0.343 and 0.776 respectively.

Figure 3.2 Timeline of peripheral blood CD3⁺ T cell counts post HSCT in patients split by GvHD status



A	B	C	D	E
2-week GvHD median = 11.0 (n=3)	4-week GvHD median = 26.0 (n=5)	8-week GvHD median = 72.0 (n=5)	3-months GvHD median = 137.0 (n=5)	6-month GvHD median = 620.0 (n=3)
2-week no GvHD median = 18.0 (n=13)	4-week no GvHD median = 44.5 (n=14)	8-week no GvHD median = 133.0 (n=14)	3-months no GvHD median = 345.0 (n=14)	6-month no GvHD median = 189 (n=8)
Mann-Whitney *P* = 0.83	Mann-Whitney *P* = 0.912	Mann-Whitney *P* = 0.488	Mann-Whitney *P* = 0.343	Mann-Whitney *P* = 0.776

CD3⁺ T cell normal range 540-1790/µl (Punt *et al.* 2019). Each patient is represented by the following colours (GvHD patients in bold):

1 **2** **3** 4 5 6 7 8 9
10 11 12 13 14 **15** 16 **17** 18
19 **20** 21 22 23 24

3.5.3 $CD4^+$ cell counts in patients split by GvHD diagnosis

Figure 3.3 shows the change in patient's PB $CD4^+$ T cell count post HSCT, over the 6-month study period. The normal $CD4^+$ T cell count range is 300 – 1500/µl (Stem Cell Technologies, no date), the highest $CD4^+$ count obtained at any point in the 6-month follow up was 807/µl (patient 7 at 6-months, no GvHD diagnosis within the 6-month follow up period), the lowest was 1/µl (patient 12, 15 and 19 at 2 weeks, of these, only patient 15 was diagnosed with GvHD within the 6-month follow up period). At no time point did the median $CD4^+$ cell count for either the GvHD or no GvHD group reach the normal range of 300-1500/µl.

At 2 weeks post HSCT 17/17 patients had a $CD4^+$ T cell count below the normal range. The GvHD group median $CD4^+$ cell count was lower than the no GvHD group median count (3.5/µl vs 7/µl respectively). This can be compared to the $CD3^+$ T cell count post HSCT, where at 2 weeks post HSCT 16/16 patients had a $CD3^+$ T cell count below the normal range.

At 4 weeks post HSCT 19/19 patients had a $CD4^+$ T cell count below the normal range. The GvHD group median $CD4^+$ cell count was the same as the no GvHD group median count (24/µl). This can be compared to the $CD3^+$ T cell count post HSCT, where at 4 weeks post HSCT 19/19 patients had a $CD3^+$ T cell count below the normal range.

At 8 weeks post HSCT, 3/19 patients (patients 2, 21 and 24) had $CD4^+$ T cell counts in the normal range. Patient 2 had the highest cell count of 680/µl and was the only patient of these three to be diagnosed with GvHD within the 6-month follow up period. The other 16 patients had $CD4^+$ counts were below the normal range. The no GvHD group median $CD4^+$ cell count was 2.3 times higher than the GvHD group median count (88/µl vs 38/µl respectively). This can be compared to the $CD3^+$ T cell counts post HSCT, where at 8 weeks post HSCT 16/19 patients had $CD3^+$ T cell counts below the normal range.

At 3 months post HSCT, 8/19 patients (patients 1, 2, 7, 13, 18, 21, 23 and 24) had $CD4^+$ T cell counts in the normal range (only patients 1 and 2 developed GvHD within the 6-month follow up period). Patient 2's $CD4^+$ cell count had more than halved to 322/µl compared to the 8-week time point. All other patients were below the normal range. The GvHD group median $CD4^+$ cell count was 1.89 times higher than the no GvHD group (251/µl vs 133/µl respectively). This can be compared to the $CD3^+$ T cell counts post HSCT, where at 3 months post HSCT 13/19 patients have $CD3^+$ T cell counts below the normal range.

At 6-months post HSCT, 3/11 patients (patients 1, 7 and 18) had $CD4^+$ T cell counts in the normal range (only patient 1 developed GvHD within the 6-month follow up period). This compares to 4/11

patients that had a $CD3^+$ T cell count in the normal range (patients 1, 9, 18 and 20). The median $CD4^+$ cell count at 6-months is slightly higher than the $CD3^+$ T cell count, this is a mathematical quirk, at this time the mean $CD3^+$ T cell count (1181/µl) was higher than the mean $CD4^+$ cell count (287/µl). The GvHD group and no GvHD group median $CD4^+$ cell counts are similar (222/µl vs 205/µl respectively). This can be compared to the $CD3^+$ T cell count post HSCT, where at 6-months post HSCT 6/11 patients have $CD3^+$ T cell counts below the normal range.

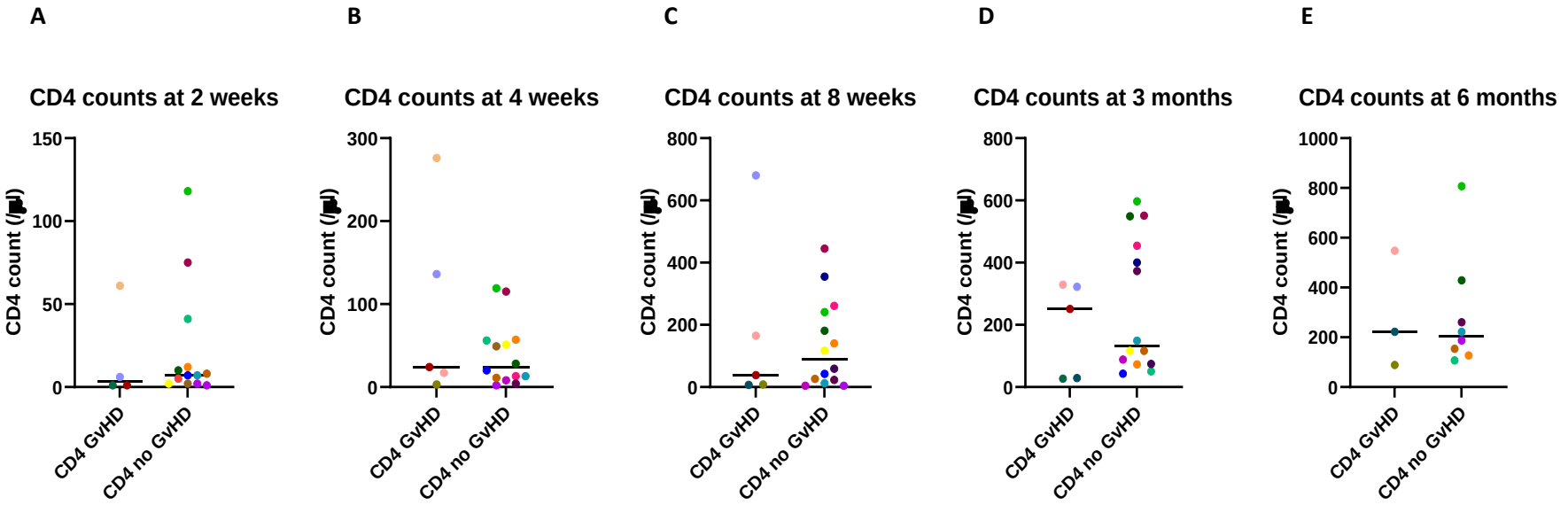
Patients 1, 2, 3, 15, 17 19 and 20 were diagnosed with GvHD. As expected, the $CD4^+$ T cell counts mostly mirrored the $CD3^+$ T cell counts. At 2 weeks patients 3, 15 and 19 were below the normal range, while patients 1, 2, 17 and 20 were missed at this time point. At 4 weeks patients 1, 2, 3, 15 and 17 were all below the normal range, while patients 19 and 20 were missed at this time point. Patient 3 (age 53) had the highest $CD4^+$ cell counts at 2- and 4-weeks but was not sampled again during follow up. At 8 weeks patient 2 $CD4^+$ T cell counts was in the normal range, whereas patients 1, 15, 17 and 20 had $CD4^+$ T cell counts below the normal range and patients 3 and 19 were missed at this time point. Patient 2 (age 48) had the highest $CD4^+$ cell counts at 8 weeks, was the other high count at 4-weeks, and the second highest count at 3 months. At 3 months patients 1 and 2 were in the normal range, patients 15, 19 and 20 were below the normal range, patients 3 and 17 were missed at this time point. At 6 months post HSCT, patient 1 had $CD4^+$ T cell counts in the normal range, patients 17 and 20 $CD4^+$ T cell counts were below the normal range, patients 3, and 15 were missed at this time point. Patient 2 was deceased and patient 19 was in palliative care at this time point. Patient 1 (age 73) had the highest $CD4^+$ cell count at 3- and 6-months.

No GvHD group: at 3- and 6-months there appears to be two clusters of patients within the $CD4^+$ cell counts for the no GvHD group. Patients 7 (age 70) and 18 (age 64) are present in both high-count groups, the other patients at 3 months being patient 13 (age 58), patient 21 (age 60), patient 23 (age 54) and patient 24 (age 64). All the no GvHD 'high-count' patients were alive at the 6-month follow up time point.

Patients 2 and 14 were deceased at the 6-month time point. Only 2- and 4-week samples were obtained from patient 14, there was no 6-month sample from patient 2. Patient 2 is discussed above, patient 14's $CD4^+$ T cell counts were below the normal range at the available time points.

There was no statistically significant difference in the peripheral blood $CD4^+$ cell count in the GvHD and no GvHD groups at any of the five sampling time points: 2-, 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.335, 0.547, 0.808, 0.333 and 0.964 respectively.

Figure 3.3 Timeline of peripheral blood CD4⁺ cell counts post HSCT in patients split by GvHD status



2-week GvHD median = 3.5 (n=4)	4-week GvHD median = 24.0 (n=5)	8-week GvHD median = 38.0 (n=5)	3-month GvHD median = 251.0 (n=5)	6-month GvHD median = 222.0 (n=3)
2-week no GvHD median = 7.0 (n=13)	4-week no GvHD median = 24.0 (n=14)	8-week no GvHD median = 88.0 (n=14)	3-month no GvHD median = 133.0 (n=14)	6-month no GvHD median = 205.0 (n=8)
Mann-Whitney *P* = 0.335	Mann-Whitney *P* = 0.547	Mann-Whitney *P* = 0.808	Mann-Whitney *P* = 0.333	Mann-Whitney *P* = 0.964

CD4⁺ cell normal range 300 – 1500 (Stem Cell Technologies, no date). Each patient is represented by the following colours (GvHD patients in bold):

1 **2** **3** 4 5 6 7 8 9
10 11 12 13 14 **15** 16 **17** 18
19 **20** 21 22 23 24

3.5.4 Plasmacytoid dendritic cell counts in patients split by GvHD diagnosis

Figure 3.4 shows the change in patient's PB pDC cell count post HSCT, over the 6 month study period. The normal pDC cell count range is 5-10/µl (Chevallier *et al.* 2013), the highest pDC count obtained at any point in the 6-month follow up period was 41/µl (patient 7 at 2-weeks, no GvHD diagnosis within the 6-month follow up period), the lowest was <1/µl (patients 6, 11 and 14 at 2-weeks, no GvHD diagnosis within the 6-month follow up time period). At 2 weeks the no GvHD group and at 4 weeks the GvHD group's median pDC count reaches the normal range of 5-10/µl, both groups at all other time points had pDC cell counts below the normal range.

At 2 weeks post HSCT, 9/17 of patients had a pDC count below the normal range. Six patients (4, 5, 8, 9, 18 and 19 – none of whom were diagnosed with GvHD within the 6-month follow up period) were within the normal range, and patients 7 and 10 (also not diagnosed with GvHD within the 6-month follow up period) exceeded the normal range with counts of 41/µl and 19/µl respectively. The no GvHD group had a pDC median count in the normal range while the GvHD group did not (7/µl vs 2/µl respectively).

At 4 weeks post HSCT, 10/19 of patients had a pDC count below the normal range. Patients 1, 3, 7, 8, 10, 11, 14 and 17 pDC counts were in the normal range (patients 1, 3 and 17 were diagnosed with GvHD within the 6-month follow up period, the other five patients were not). Patient 16's pDC count had exceeded the normal range (was not diagnosed with GvHD within the 6-month follow up period). The pDC count for patients 7 and 10 had dropped into the normal range. The GvHD group had a median pDC cell count in the normal range, while the no GvHD group did not (5/µl vs 3/µl respectively).

At 8 weeks post HSCT, 13/19 of patients had a pDC count below the normal range. Patients 10, 16, 17, 21 and 22 had a pDC cell count in the normal range (patient 17 was diagnosed with GvHD within the 6-month follow up period, the other four patients were not). Patient 1 had a pDC count that exceeded the normal range (was diagnosed with GvHD within the 6-month follow up period). While both medians had dropped, the GvHD group maintained the higher median pDC cell count compared to the no GvHD group (4/µl vs 2.5/µl respectively).

At 3 months post HSCT, 10/19 of patients had a pDC count below the normal range. Patients 10, 13, 16, 19, 20, 21 and 24 had pDC counts in the normal range (patient 20 was diagnosed with GvHD within the 6-month follow up period, the other six patients were not). Patients 7 and 9 had pDC cell counts that exceeded the normal range (neither patient was diagnosed with GvHD within the 6-month follow up period). Patient 1 pDC count was now below the normal range and was diagnosed

with GvHD within the 6-month follow up period. The GvHD and no GvHD group pDC cell count medians were the same (4/µl).

At 6 months post HSCT, 8/11 of patients had a pDC count below the normal range. Patients 9 and 22 pDC cell counts were in the normal range (neither patient was diagnosed with GvHD within the 6-month follow up period), while patient 7 had a pDC count above the normal range and was also not diagnosed with GvHD within the 6-month follow up period. At this time point the median pDC cell counts had dropped, with the no GvHD group having the higher median pDC cell counts (2.5/µl vs 1/µl respectively). The GvHD group median pDC cell count had dropped from week 4 onwards.

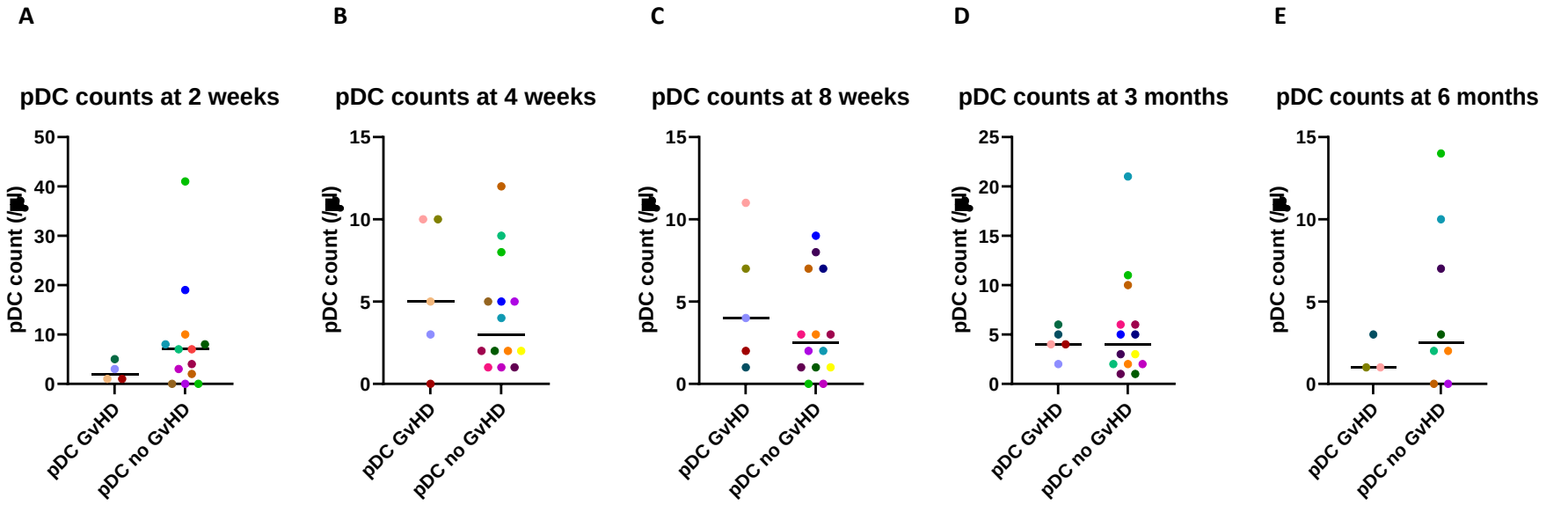
Patients 1, 2, 3, 15, 17, 19 and 20 were diagnosed with GvHD. At 2-weeks patients 2, 3 and 15 had pDC cell counts below the normal range, patient 19 was in the normal range, while patients 1, 17 and 20 were missed at this time point. At 4 weeks patients 1, 3 and 17 were in the normal range, patients 2 and 15 were below normal range and patients 19 and 20 were missed at this time point. At 8 weeks patient 17 was in the normal range, patients 2, 15 and 20 were below the normal range, patient 1 was slightly above the normal rage and patients 3 and 19 were missed at this time point. Patients 1 (age 73) and 17 (age 44) had the highest pDC count at both 4 and 8 weeks, both patients were alive at the 6 month follow up time point. At 3 months patients 1, 2 and 15 were below the normal range, patients 19 and 20 were in the normal range and patients 3 and 17 were missed at this time point. At 6 months, patients 1, 17 and 20 were below the normal range, patients 3 and 15 were missed, patient 2 was deceased and patient 19 was in palliative care at this time point.

No GvHD group: from 8 weeks onwards there appears to be two clusters of patients for the pDC cell counts within the no GvHD group. No individual patient is consistently present in the high-count group. Patients 7 (age 70), 9 (age 64), 16 (age 58) and 22 (age 34) are present at two of the three time points, the other patients being patient 10 (age 40) and 21 (age 60). All the no GvHD 'high-count' patients were alive at the 6-month follow up time point.

Patients 2 and 14 were deceased at the 6-month time point. Only 2- and 4-week samples were obtained from patient 14, there was no 6-month sample from patient 2. Patient 2 is discussed above. Patient 14 was below the normal range at 2 weeks but within the normal range at 4 weeks.

There was no statistically significant difference between the peripheral blood pDC cell count in the GvHD and no GvHD group at any of the five sampling time points: 2-, 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.303, 0.508, 0.409, 0.986 and 0.545 respectively.

Figure 3.4 Timeline of peripheral blood pDC counts post HSCT in patients split by GvHD status



A	B	C	D	E
2-week GvHD median = 2.0 (n=4)	4-week GvHD median = 5.0 (n=5)	8-week GvHD median = 4.0 (n=5)	3-month GvHD median = 4.0 (n=5)	6-month GvHD median = 1.0 (n=3)
2-week no GvHD median = 7.0 (n=13)	4-week no GvHD median = 3.0 (n=14)	8-week no GvHD median = 2.5 (n=14)	3-month no GvHD median = 4.0 (n=14)	6-month no GvHD median = 2.5 (n=8)
Mann-Whitney *P* = 0.303	Mann-Whitney *P* = 0.508	Mann-Whitney *P* = 0.409	Mann-Whitney *P* = 0.986	Mann-Whitney *P* = 0.545

Plasmacytoid DC normal range 5-10/µl (Chevallier *et al.*2013). Each patient is represented by the following colours (GvHD patients in bold):

1 **2** **3** 4 5 6 7 8 9
10 11 12 13 14 **15** 16 **17** 18
19 **20** 21 22 23 24

3.5.5 Myeloid dendritic cell 1 cell counts in patients split by GvHD diagnosis

Figure 3.5 shows the change in peripheral blood mDC1 cell count over the 6-month study period, post HSCT. The normal mDC1 cell count range is 4 – 30.5/µl (Chevallier *et al.* 2013), the highest mDC1 count obtained at any point in the 6 month follow up period was 42/µl (patient 8 at 4-weeks and patient 19 at 3-months, neither were diagnosed with GvHD within the 6 month follow up period), the lowest value was <1/µl (patient 6 at 2-weeks, and patient 15 at 4-weeks and patient 23 at 3-months, of these only patient 15 was diagnosed with GvHD within the 6 month follow up period). Both the GvHD and no GvHD groups had mDC1 median cell counts in the normal range at all time points.

At 2 weeks post HSCT, 12/17 of patients had an mDC1 count within the normal range. Patients 6, 11, 14 and 16 (all not diagnosed with GvHD within the 6-month follow up period) mDC1 counts were below the normal range and patient 10 mDC1 count exceeded the normal range with a count of 35/µl, and was also not diagnosed with GvHD within the 6 month follow up period. The no GvHD group had a higher median mDC1 cell count than the GvHD group (9/µl vs 6/µl respectively).

At 4 weeks post HSCT, 12/19 of patients had an mDC1 count with the normal range. Patients 1, 12, 13, 15, and 23 (patients 1 and 15 were diagnosed with GvHD within the 6 month follow up period, the other three patients were not) had mDC1 cell counts below the normal range and patients 6 and 8 had mDC1 cell counts that exceeded the normal range (neither patient was diagnosed with GvHD within the 6 month follow up period). Patients 10, 11, 14 and 16 mDC1 counts were within the normal range, and these patients were not diagnosed with GvHD within the 6-month follow up period. The median patient mDC1 cell counts were the same as at 2 weeks (9/µl and 6/µl).

At 8 weeks post HSCT, 11/19 of patients had an mDC1 count within the normal range. Patients 7, 9, 12, 13, 15, 18, 20 and 24 had mDC1 counts that were below the normal range (patients 15 and 20 were diagnosed with GvHD within the 6 month follow up period, the other patients were not). None of the patients sampled exceeded the mDC1 cell count normal range. This was the only time point where the GvHD group had a higher mDC1 median cell count than the no GvHD group (6/µl vs 4.5/µl respectively).

At 3 months post HSCT, 17/19 of patients had an mDC1 count within the normal range. Patient 23 was below the normal range and patient 19 exceeded the normal range, neither of whom were diagnosed with GvHD within the 6 month follow up period. At 3 months the no GvHD group had the higher median count than the GvHD group (9/µl vs 7.5/µl respectively).

At 6 months post HSCT, 9/11 of patients had an mDC1 count within the normal range. Patients 11 and 16 mDC1 cell counts were below the normal range (neither of whom were diagnosed with GvHD within the 6-month follow up period), none of the patients sampled exceeded the normal range at this time point. At 6 months the no GvHD group had the higher median count than the GvHD group (7/µl vs 4.5/µl respectively).

Patients 1, 2, 3, 15, 17, 19 and 20 were diagnosed with GvHD. At 2 weeks, patients 2, 3, 15 and 19 had an mDC1 cell count in the normal range, patients 1, 17 and 20 were missed at this time point. At 4 weeks patients 1 and 15 had an mDC1 cell count below the normal range, patients 2, 3 and 17 had an mDC1 cell count within the normal range and patients 19 and 20 were missed at this time point. Patients 1, 2 and 17 were in the normal range at 8 weeks, patients 15 and 20 were below the normal range and patients 3 and 19 were missed at this time point. Patients 1, 2, 15 and 20 were within the normal range, patient 19 was above the normal range and patients 3 and 17 were missed at this time point. Patients 1 (age 73) and 17 (age 44) had the two highest mDC1 counts at 8 weeks but were not the highest counts at 3 months, which were patients 15 (age 48) and 19 (age 65). All these patients were alive at the 6 month follow up point. At 6 months, patients 1, 17 and 20 had mDC1 cell counts in the normal range, patients 3 and 15 were missed, patient 2 was deceased and patient 19 was in palliative care at this time point.

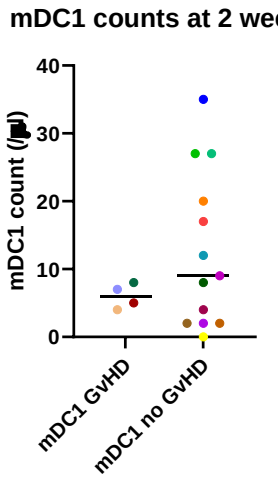
No GvHD group: at 8 weeks and 6 months there appears to be two clusters of patients for mDC1 cell counts within the no GvHD group. Only patient 22 (age 34) is present in both high cell count groups, the others being patients 6 (age 49), 10 (age 40), 16 (age 58) and 21 (age 60) at 8-weeks and patients 7 (age 70) and 9 (age 64) at 6-months. All the no GvHD 'high count' patients were alive at the 6-month follow up time point.

Patients 2 and 14 were deceased at the 6-month time point. Only 2 and 4 week samples were obtained from patient 14, there was no 6-month sample from patient 2. The mDC1 counts for patient 2 at all collected time points was within the normal range. At 2 and 4 weeks the mDC1 cell counts for patient 14 were below and then within the normal range respectively.

There was no statistically significant difference in the peripheral blood mDC1 cell count in patients with GvHD and no GvHD at any of the five sampling time points: 2-, 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.527, 0.277, 0.374, 0.987 and 0.444 respectively.

Figure 3.5 Timeline of peripheral blood mDC1 cell counts post HSCT in patients split by GvHD status

A

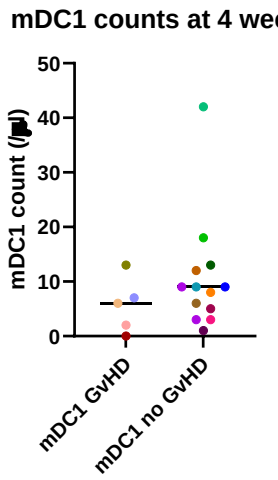


2-week GvHD median = 6.0 (n=4)

2-week no GvHD median = 9.0 (n=13)

Mann-Whitney *P* = 0.527

B

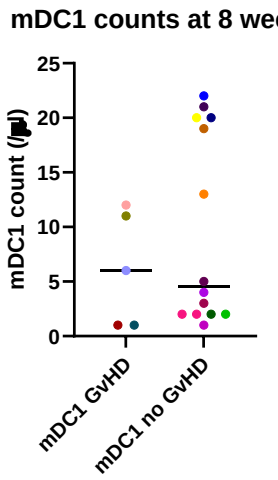


4-week GvHD median = 6.0 (n=5)

4-week no GvHD median = 9.0 (n=13)

Mann-Whitney *P* = 0.277

C

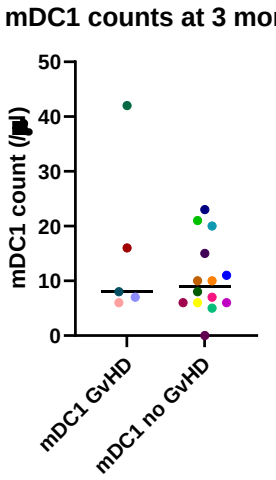


8-week GvHD median = 6.0 (n=5)

8-week no GvHD median = 4.5 (n=14)

Mann-Whitney *P* = 0.374

D

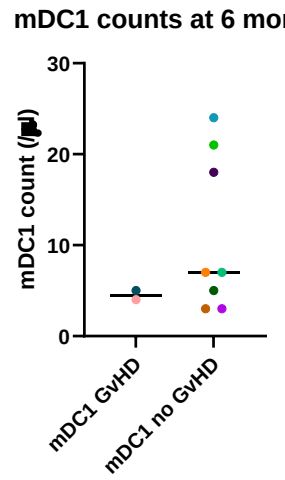


3-month GvHD median = 7.5 (n=6)

3-month no GvHD median = 9.0 (n=14)

Mann-Whitney *P* = 0.987

E



6-month GvHD median = 4.5 (n=2)

6-month no GvHD median = 7.0 (n=8)

Mann-Whitney *P* = 0.444

Myeloid DC1 normal range 4-30.5/µl (Chevallier *et al.*2013). Each patient is represented by the following colours (GvHD patients in bold):

1 **2** **3** 4 5 6 7 8 9

10 11 12 13 14 **15** 16 **17** 18

19 **20** 21 22 23 24

3.5.6 DC-10 dendritic cell counts in patients split by GvHD diagnosis

Figure 3.6 shows the change in patient's PB DC-10 cell count post HSCT, over the 6-month study period. The normal DC-10 cell count range is 4 – 13/µl (Gregori *et al.* 2010), the highest DC-10 count obtained at any point in the 6-month follow up was 207/µl (patient 2 at 3 months, diagnosed with GvHD and died of GvHD and AML within the 6-month follow up time period), the lowest was <1/µl. (patient 6 and 11 at 2 weeks, and patient 15 at 8 weeks, of these only patient 15 was diagnosed with GvHD within the 6-month follow up time period). Both the GvHD and no GvHD groups had mDC1 counts below the normal range at 2-weeks, at time points after that both groups were within the normal range, except for the GvHD group at 3 months which exceeded the normal range.

At 2 weeks post HSCT, 4/14 of patients had a DC-10 cell counts within the normal range. Patients 6, 11, 12, 14, 15, 16 and 18 had DC-10 cell counts below the normal range (patient 15 was diagnosed with GvHD within the 6-month follow up period, the other six patients were not). Patients 7, 10 and 24 had DC-10 cell counts that exceeded the normal range, none of whom were diagnosed with GvHD within the 6-month follow up period. The GvHD and no GvHD groups have similar DC-10 median counts (3/µl vs 3.5/µl respectively).

At 4 weeks post HSCT, 6/17 of patients had a DC-10 count in the normal range. Patients 11, 13, 15 are below the normal range (patient 15 was diagnosed with GvHD within the 6-month follow up period, the other two patients were not). Patients 3, 6, 7, 8, 10, 16, 18 and 24 had DC-10 cell counts that exceeded the normal range (patient 3 was diagnosed with GvHD within the 6-month follow up period, the other seven patients were not). The no GvHD groups have higher median DC-10 cell counts (13.5/µl vs 4/µl respectively).

At 8 weeks post HSCT, 7/19 of patients had a DC-10 cell count in the normal range. Patients 6, 7, 11, 12, 13, 15 and 17 had DC-10 cell counts below the normal range (patient 15 and 17 were diagnosed with GvHD within the 6-month follow up period, the other five patients were not). Patients 1, 2, 10, 21 and 23 had DC-10 cell counts that exceeded the normal range (patients 1 and 2 were diagnosed with GvHD within the 6-month follow up period, the other three patients were not). Patient 2 had a DC-10 cell count of 153/µl which was unusually higher than the normal range. Patients 6, 7 and 12 had DC-10 cell counts below the normal range, none of the patients was diagnosed with GvHD within the 6-month follow up period. The no GvHD groups maintain higher median DC-10 cell counts (7/µl vs 4/µl respectively).

At 3 months post HSCT, 11/19 of patients had DC-10 cell counts within the normal range. Patients 5 and 12 were below the DC-10 cell count normal range (neither patient was diagnosed with GvHD

within the 6-month follow up period). Patients 1, 2, 7, 8, 20 and 21 had DC-10 cell counts that exceeded the normal range (patients 1, 2 and 20 were diagnosed with GvHD within the 6-month follow up period, the other three patients were not). Patient 2's unusual DC-10 count had increased further to 207/µl. At 3 months the GvHD group median DC-10 cell count is higher than the no GvHD median DC-10 cell count (15/µl vs 7/µl respectively).

At 6 months post HSCT, 6/11 of patients had DC-10 cell counts within the normal range. Patients 1, 5, 11 and 16 had a DC-10 cell count below the normal range (patient 1, was diagnosed with GvHD within the 6-month follow up period, the other three patients were not). Patient 20 had a DC-10 cell count that exceeded the normal range and was diagnosed with GvHD within the 6 month follow up period. Both GvHD and no GvHD groups have similar DC-10 cell counts, (6/µl vs 5.5/µl respectively).

Patients 1, 2, 3, 15, 17, 19 and 20 were diagnosed with GvHD. At 2 weeks patient 15 had a DC-10 cell count below the normal range, patient 19 was within the normal range and the other patients were missed at this time point. At 4 weeks, patient 3 (age 53) had a DC-10 cell count above the normal range, patient 15 had a DC-10 cell count below the normal range and patient 17 had a DC-10 cell count within the normal range. The other patients' samples were missed at this time point. At 8-weeks, patients 1 and 2 had DC-10 cell counts above the normal range, patients 3 and 19 were missed and patients 15 and 17 had DC-10 cell counts below the normal range, while patient 20 had a DC-10 cell count within the normal range. At 3-months patients 1, 2 and 20 had DC-10 cell counts above the normal range and patients 15 and 19 had DC-10 cell counts within the normal range, samples from patients 3 and 17 were missed at this time point. Patient 2 (age 48) had an unusually high DC-10 count at 8 weeks and 3 months, which was very different to the slightly above/below normal range that many of the counts, not just DC-10 counts, were. Patient 2 was deceased by the 6-month follow up time point while patients 3 and 20 were not. At 6-months, patient 17 had a DC-10 cell count within the normal range, patient 20 had a DC-10 cell count above the normal range and patient 1 had a DC-10 count below the normal range. Patients 3 and 15 were missed at this time point. Patient 2 was deceased and patient 19 was in palliative care at this time point. The patient with the highest count at 6 months was patient 20 (age 63).

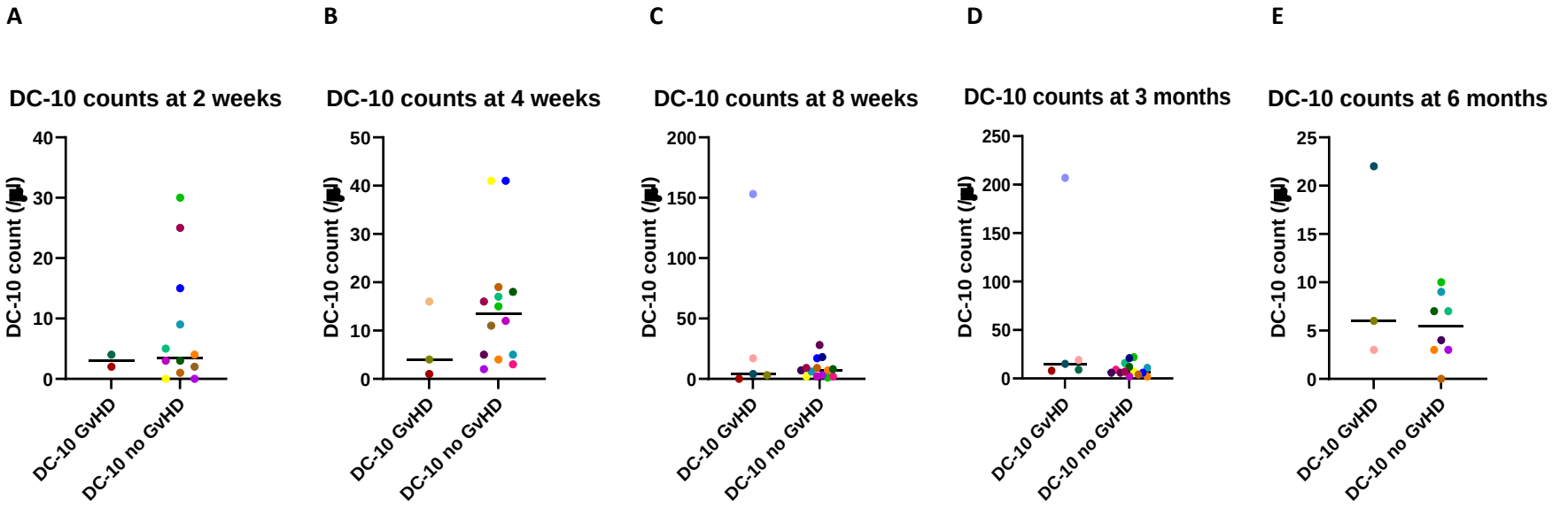
No GvHD group: at 4 weeks there appears to be two clusters of patients' DC-10 cell counts within the no GvHD group. Patients 6 (age 49) and 10 (age 40) are in a high cell count group, and both these patients were alive at the 6 month follow up point.

Patients 2 and 14 were deceased at the 6 month time point. Only 2 and 4 week samples were obtained from patient 14 and there was no 6-month sample from patient 2. Patient 2 had the

unusually high DC-10 count at 8-weeks and 3-months. Patient 14 had a DC-10 cell count below the normal range at 2-weeks and within normal range at 4-weeks.

There was no statistical significant difference for peripheral blood DC-10 cell count in patients with GvHD and no GvHD at any of the five sampling time points: 2-, 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.769, 0.234, >0.999, 0.09 and 0.733 respectively.

Figure 3.6 Timeline of peripheral blood DC-10 cell counts post HSCT in patients split by GvHD status



2-week GvHD median = 3.0 (n=2)	4-week GvHD median = 4.0 (n=3)	8-week GvHD median = 4.0 (n=5)	3-month GvHD median = 15.0 (n=5)	6-month GvHD median = 6.0 (n=3)
2-week no GvHD median = 3.5 (n=12)	4-week no GvHD median = 13.5 (n=14)	8-week no GvHD median = 7.0 (n=14)	3-month no GvHD median = 7.0 (n=14)	6-month no GvHD median = 5.5 (n=8)
Mann-Whitney *P* = 0.769	Mann-Whitney *P* = 0.234	Mann-Whitney *P* > 0.999	Mann-Whitney *P* = 0.090	Mann-Whitney *P* = 0.733

DC-10 normal range 4-13/µl (Gregori *et al.* 2010). Each patient is represented by the following colours (GvHD patients in bold):

1 **2** **3** 4 5 6 7 8 9
10 11 12 13 14 **15** 16 **17** 18
19 **20** 21 22 23 24

3.5.7 Type 1 T regulatory cell counts in patients split by GvHD diagnosis

Figure 3.7 shows the change in patient's PB Tr1 cell count post HSCT, over the 6 month study period. The normal Tr1 cell count range is 4-13/µl (Gagliani *et al.* 2013). The highest Tr1 count obtained at any point in the 6-month follow up was 10/µl (patient 2 at 8-weeks, diagnosed with GvHD and died of GvHD and AML within the 6 month follow up period), the lowest was <1/µl. (multiple patients at multiple time points). Both the GvHD and no GvHD groups had median Tr1 counts below the normal range at all time points. Due to the low counts obtained in both GvHD and no GvHD groups for Tr1 cells, the differences in median values discussed below need to be interpreted with caution.

At 2 weeks post HSCT 17/17 patients had a Tr1 cell count of 1/µl or lower, which is below the normal range. This mirrors the $CD4^+$ T cell count, which at 2 weeks post HSCT, 17/17 patients had a $CD4^+$ T cell count below the normal range.

At 4 weeks post HSCT 19/19 patients had a Tr1 cell count below the normal range. This mirrors the $CD4^+$ T cell count, which at 4-weeks post HSCT, 19/19 patients had a $CD4^+$ T cell count below the normal range. The no GvHD group and GvHD group both had median Tr1 cell counts of 0/µl.

At 8 weeks post HSCT, only 1/19 patients (patient 2, was diagnosed with GvHD within the 6-month follow up period) had a Tr1 cell count within the normal range. All the other patients analysed had Tr1 cell counts below the normal range. The no GvHD group and GvHD group had similar median Tr1 cell counts (1/µl vs 0.5/µl respectively).

At 3 months post HSCT, 19/19 patients had a Tr1 cell count below the normal range, (this includes patient 2) this can be compared with the $CD4^+$ cell counts where 8/19 patients were in the normal range. The median Tr1 cell count was slightly higher in the GvHD group than the no GvHD group (2/µ/ vs 1/µl respectively).

At 6 months post HSCT, 2/11 patients had a Tr1 cell count within the normal range, patients 1 and 7 (patient 1 was diagnosed with GvHD within the 6-month follow up period, patient 7 was not). The remaining analysed patient samples all had Tr1 cell counts below the normal range. The no GvHD group and GvHD group had similar median Tr1 cell counts (1.5/µl vs 1/µl respectively).

Patients 1, 2, 3, 15, 17, 19 and 20 were diagnosed with GvHD. At 2 and 4 weeks all these patients Tr1 cell counts were either below the normal range or missed. At 8 weeks patient 2 is in the normal range, patients 1, 15, 17 and 20 are below the normal range and patients 3 and 19 were missed. At 3 months patients 1, 2, 15, 19 and 20 were below the normal range, patients 3 and 17 were missed at this time point. Patient 2 (age 48) had the highest Tr1 cell count at 8-weeks and the joint highest at 4 weeks and 3 months. At 6 months, patient 1 was in the normal range, patients 17 and 20 were

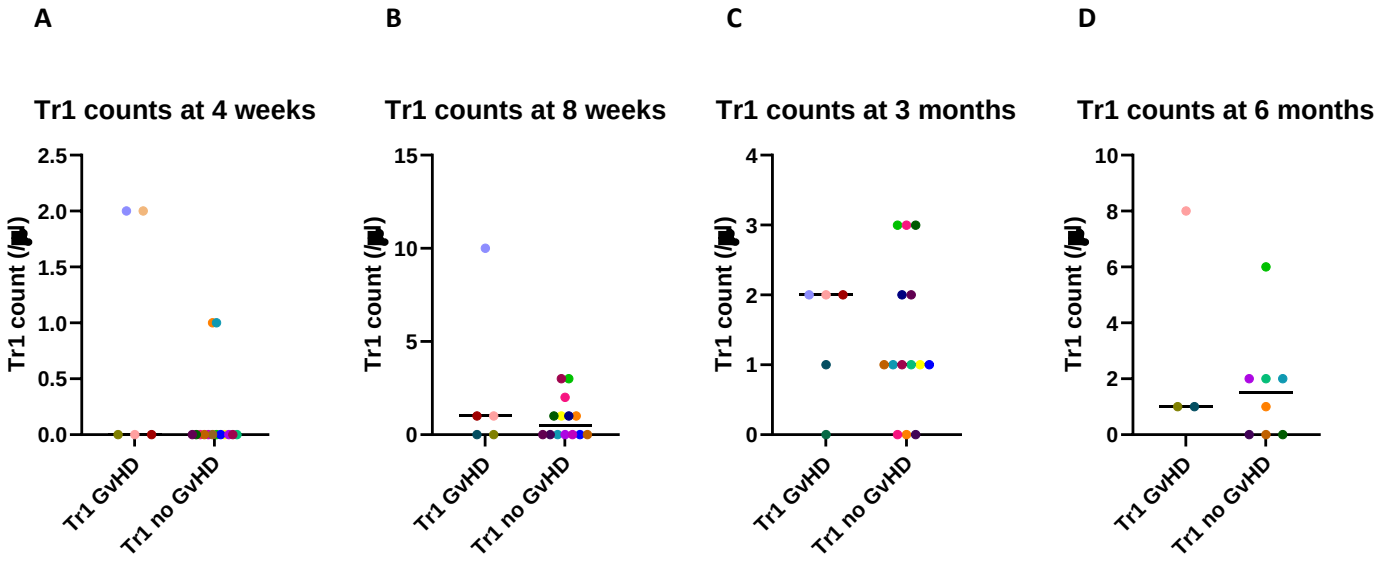
below the normal range, patients 3 and 15 were missed. Patient 2 was deceased and patient 19 was in palliative care at this time point. The highest Tr1 cell count at this time point was patient 1 (age 73).

No GvHD group: due to the low Tr1 counts, there does not appear to be different groups within this group.

Patients 2 and 14 were deceased at the 6-month time point. Only 2- and 4-week samples were obtained from patient 14, there was no 6-month sample from patient 2. Patient 14's 2- and 4-week Tr1 cell counts were below the normal range. Patient 2's Tr1 cell count was within the normal range at 8-weeks but was otherwise below the normal range.

Due to the slow recovery of Tr1 cells post HSCT there was no data for the 2 week samples. There was no statistically significant difference for peripheral blood Tr1 cell count in patients with GvHD and no GvHD at any of the four sampling time points: 4- and 8-weeks, 3- and 6-months with Mann-Whitney *P* values of 0.077, 0.712, 0.826 and 0.709 respectively.

Figure 3.7 Timeline of peripheral blood Tr1 cell counts post HSCT in patients split by GvHD status



4-week GvHD median = 0.0 (n=5)	8-week GvHD median = 1.0 (n=5)	3-month GvHD median = 2.0 (n=5)	6-month GvHD median = 1.0 (n=3)
4-week no GvHD median = 0.0 (n=14)	8-week no GvHD median = 0.5 (n=14)	3-month no GvHD median = 1.0 (n=14)	6-month no GvHD median = 1.5 (n=8)
Mann-Whitney *P* = 0.077	Mann-Whitney *P* = 0.712	Mann-Whitney *P* = 0.826	Mann-Whitney *P* = 0.709

Tr1 normal range 4 – 13/µl (Gagliani *et al.* 2013). Each patient is represented by the following colours (GvHD patients in bold).

1 **2** **3** 4 5 6 7 8 9
10 11 12 13 14 **15** 16 **17** 18
19 **20** 21 22 23 24

3.6 Summary of results for individual patient cell reconstitution following HSCT

None of the 24 patients undergoing HSCT consistently showed high cell counts in individual cell reconstitution as shown in figures 3.1 to 3.7, and patients who reached the normal range for one particular cell type at a specific time point did not always remain in the normal range after that time point.

Counts above and below the normal range were generally not dramatically above or below the normal range. Cell counts that were double or greater than the upper limit of the normal range were only achieved by six patients: Patient 2's DC-10 cells at the 8-week and 3-month time points, patient 6's WBC and DC-10 cell count at 4 weeks, patient 7's pDC count at 2 weeks and their $CD3^+$ count at 6 months, patient 9's pDC cell count at 3 months, patient 10's DC-10 cell count at 4 weeks and patient 23's DC-10 count at 8 weeks. Of these patients, only patient 2 was diagnosed with GvHD.

The highest $CD3^+$ T cell counts at 2 and 4 weeks were from patient 3 (though both were below the normal range, and no further samples were obtained from this patient), and this patient was diagnosed with GvHD at 20 weeks. Patient 18 had the highest $CD3^+$ T cell count at 8 weeks, and patient 7 had the highest $CD3^+$ cell count at 3 and 6 months, (and the second highest at 2, 4 and 8 weeks), neither patient 7 or 18 was diagnosed with GvHD in the 6-month follow up period.

The populations of the tolerogenic cells during recovery post HSCT were somewhat different to one another. The median counts (pre GvHD/no GvHD split) for Tr1 cells and pDCs were below the normal range at all time points, while the median mDC1 counts were in the normal range throughout. The median DC-10 cell count was below the normal range at 2 weeks but in the normal range thereafter.

In the GvHD and no GvHD group the pDC and DC-10 cells were the only two tolerogenic cell types that achieved counts higher than the normal range at the sampled time points.

In the no GvHD group the higher than normal range counts were obtained from: patient 7 pDC counts at 2 weeks, 3 and 6 months, and DC-10 counts at 2 and 4 weeks and 3 months. Patient 10 pDC counts at 2 weeks and DC-10 counts at 2, 4 and 8 weeks. Patient 16 pDC and DC-10 counts at 4 weeks. Patient 9 pDC count at 3 months. Other patients with higher than normal range DC-10 counts were: patient 6, 18 and 24 at 4 weeks, patient 8 at 4 weeks and 3 months, patient 21 at 8 weeks and 3 months and patient 23 at 8 weeks.

In the GvHD group there was only a single occasion when the pDC count achieved a count above the normal range, patient 1 at 8 weeks. DC-10 cell counts were above the normal range for patients 1 and 2 at 8 weeks and 3 months, patient 3 at 4 weeks, and patient 20 at 3 and 6 months.

Patients 2 and 14 were deceased by the 6 month follow up point, patient 2 is discussed above. Samples were only obtained from patient 14 at 2 and 4 weeks, at 2 weeks patient 14 was below normal range for all tolerogenic cells, and at 4 weeks had counts within the normal range for pDC, mDC1 and DC-10 cells, and was below normal range for Tr1 cells.

3.7 Summary of Mann-Whitney analysis of GvHD vs non GvHD patients

It can be difficult to identify the shape of a distribution when n is less than 30 (Field and Hole 2006), so data that is normally distributed may not appear to be so when numbers are low. As there were only 24 patients included in the study, and because parametric tests assume the data has a normal distribution, it was decided to use non-parametric statistical tests in this study. Non-parametric tests would also address any outliers that may be present in the data because when n is small, data can be vulnerable to misinterpretation due to outliers.

$CD3^+$ T cell, pDC and DC-10 cell populations all had large differences in median cell counts between the GvHD and no GvHD groups at the 2- and 4-week sampling points, but these values were not statistically significant, which may be due to the small patient numbers per group and the large difference in numbers of patients between the two groups. The closest factor to reaching significance was the DC-10 cell count at 3 months ($P = 0.090$) and the Tr1 cell count at 4 weeks ($P = 0.077$). Because the obtained results were not statistically significant between the GvHD and no GvHD groups, the null hypothesis - there is no association between studied cell number(s) in the graft and/or during host immune reconstitution and GvHD - was not rejected.

There is not a clear low PB tolerogenic cell count in GvHD patients - high PB tolerogenic cell count in patients not diagnosed with GvHD, which the experimental hypothesis was based on. There was also overlap of PB tolerogenic cell counts between the GvHD and no GvHD patients, so these results did not meet one of Edelstein's (2010) biomarker criteria, of producing no overlap in results between diseased and healthy patients.

3.8 Time course of the individual acute GvHD patients' tolerogenic cell counts post HSCT

Six patients were diagnosed with aGvHD (patients 1, 2, 3, 15, 17 and 20). They were diagnosed at between 3 – 21 weeks post-transplant, with an average onset time of 110 days. Patients 1, 3, 15, 17 and 20 were all alive at the 6-month follow up time point, the cause of death for patient 2 was GvHD and AML. Each of these patient's tolerogenic counts are shown in figure 3.8 below.

Patient 1 was diagnosed with aGvHD at 21 weeks, which was between the 3- and 6-month sample time points. There were no 2-week sample results due to resource issues. Patient 1 achieved the normal range at the following time points: pDC (4 weeks), mDC1 (8 weeks), DC-10 cells (higher than

normal range at 8 weeks) and Tr1 cells (6 months, and was the highest Tr1 count at 6-months). Between the 3- and 6-month sample time points the pDC, mDC1 and DC-10 cell counts dropped, though pDC and mDC1 counts had been dropping since 8 weeks. The Tr1 cell count increased through all the sampling time points.

Patient 2 was diagnosed with aGvHD at 15 weeks, which was between the 3- and 6-month samples. Patient 2 achieved normal range at the following time points: pDC (none of the tested time points), mDC1 (2 weeks), DC-10 cells (unusually high DC-10 cell counts at 8 weeks and 3 months, and was deceased at 21 weeks, cause of death GvHD and AML) and Tr1 cells (8 weeks, but dropped thereafter). The samples that were obtained and analysed for this patient were all before GvHD diagnosis and death. Both pDC and mDC1 counts were steady throughout the sampling time points.

Patient 3 was diagnosed with aGvHD at 20 weeks, which was between the 3 and 6 month samples. Due to resource issues this patient was only tested at 2- and 4-weeks. Patient 3 achieved normal range at the following time points: pDC (4 weeks), mDC1 (2 weeks), DC-10 cells (higher than normal range at 4 weeks) and Tr1 cells (none of the tested time points). Patient 3 had the joint highest Tr1 count at 4 weeks. The samples that were obtained and analysed for this patient were all before the aGvHD diagnosis, the limited data available shows the cell counts steadily increasing.

Patient 15 was diagnosed with aGvHD at 3 weeks, this was between the 2 and 4 week samples. Patient 15 achieved the normal range at the following time points: pDC and Tr1 (none of the tested time points), mDC1 (2 weeks) and DC-10 cells (3 months). Of the six patients with aGvHD, patient 15 was unusual in that they had the earliest onset of aGvHD (the other five patients being diagnosed with aGvHD had disease onset between 3 and 6 months). This patients' tolerogenic cell counts drop between 2 and 4 weeks, except for Tr1 cells which remained at <1/µl. Between the 4- and 8-week time point, pDC, mDC1 and Tr1 counts increase while DC-10 cell counts decrease. Between 8-weeks and 3-months all cell counts increase. The effect of diagnosis and treatment on cell counts is most visible in patient 15 due to the proximity of the sampling time points around the time of diagnosis. No 6-month sample was analysed for this patient due to resources.

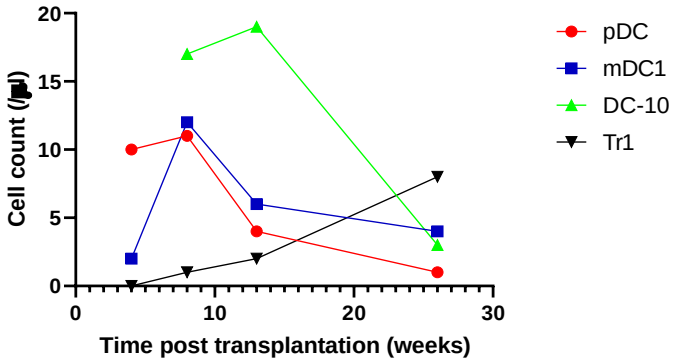
Patient 17 was diagnosed with aGvHD at 19 weeks, which was between the 3- and 6-month samples. The 2-week sample was missed due to available resources. Patient 17 achieved the normal range at the following time points: pDC and mDC1 (4 weeks), DC-10 cells (4 weeks) and Tr1 cells (none of the tested time points). This patients' pDC, mDC1 and DC-10 counts were dropping from 4 to 8 weeks. The 3-month sample was missed due to available resources, so there is therefore no data between 8 weeks and 6 months. At 6 months the pDC and mDC1 counts were lower than at 8 weeks, while the DC-10 and Tr1 counts were higher. Patient 17 was the only patient to have steroid refractory aGvHD.

Patient 20 was diagnosed with aGvHD at 16 weeks. There was no sample analysis at 2 and 4 weeks due to available resources. Patient 20 achieved the normal range at the following time points: pDC (3 months), mDC1 (3 months) and Tr1 (none of the tested time points) and DC-10 cells (8 weeks). Between the 3 and 6 month time point the pDC and mDC1 counts drop, while the DC-10 count dramatically increases and Tr1 counts slightly increase.

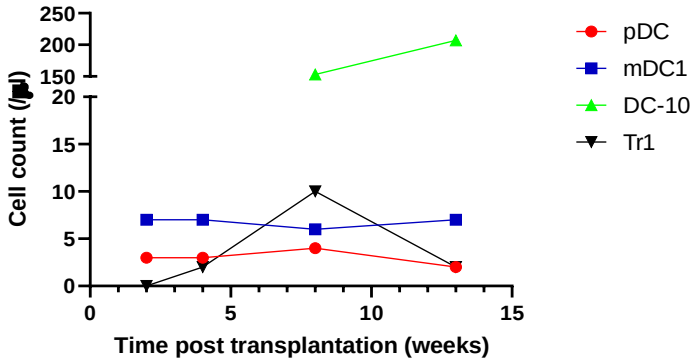
Figure 3.8 shows that there is little similarity in the timelines between aGvHD patients in their recovery of the tolerogenic cells post HSCT. All patients showed a steady increase in Tr1 cell counts over the time points except patient 2, whose count drops between 8 weeks and 3 months, this patient also had usual DC-10 counts at 8 weeks and 3 months. Patient 2 died of GvHD and AML at 21 weeks, all the other patients diagnosed with aGvHD were alive at the 6-month time point. Patient 15 has a dramatic drop in counts between 2 and 4 weeks and this likely due to the aGvHD diagnosis at 3 weeks and the immediate treatment, which was completed 7 weeks later. Patients 1 and 20 appear to have steadily decreasing pDC and mDC1 counts after 3 months, and this may have been the case for patient 17 but their 3-month sample was missed. Only 1 patient in the study died of GvHD, so it is not possible to know if the unusually high DC-10 count observed in patient 2 is of any significance, however, these unusual counts were obtained before the diagnosis of aGvHD.

Figure 3.8 Time course of reconstitution of tolerogenic cell counts in patients with aGvHD post HSCT

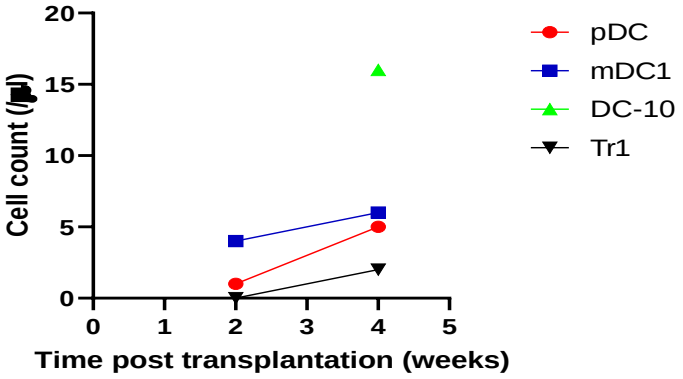
A. Patient 1 (diagnosed with aGvHD at 21 weeks)



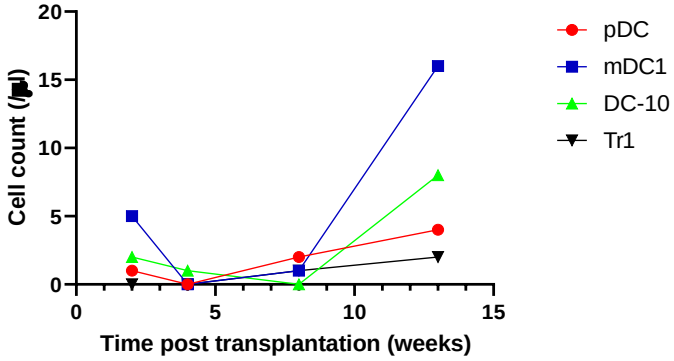
B. Patient 2 (diagnosed with aGvHD at 15 weeks)



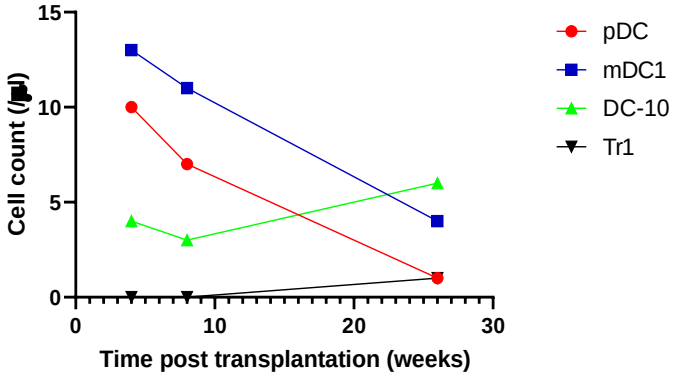
C. Patient 3 (diagnosed with aGvHD at 20 weeks)



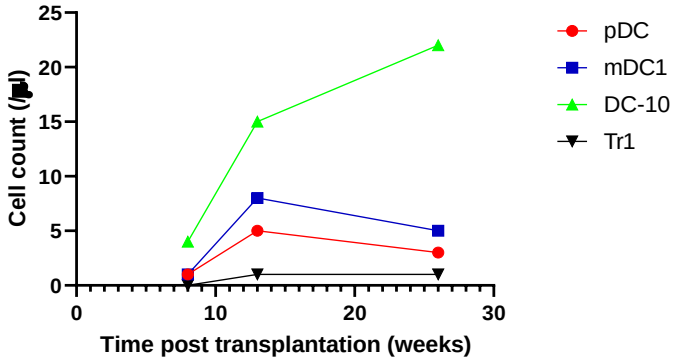
D. Patient 15 (diagnosed with aGvHD at 3 weeks)



E. Patient 17 (diagnosed with aGvHD at 19 weeks)



F. Patient 20 (diagnosed with aGvHD at 16 weeks)



Legend for figures 3.17 A to F

Plasmacytoid DC normal range 5-10/µl (Chevallier *et al.*2013).
Myeloid DC1 normal range 4-30.5/µl (Chevallier *et al.*2013).
DC-10 normal range 4 – 13/µl (Gregori *et al.* 2010).
Tr1 normal range 4 – 13/µl (Gagliani *et al.* 2013).

3.9 Chi-square analysis of GvHD and known risk factors

Chi-square analysis was performed on the presence or absence of GvHD and its known risk factors: MRD v matched unrelated donor (MUD), HLA matching, sex match and GvHD prophylaxis. Age (recipient under 40 vs over 40 years) was not statistically analysed as only two patients in this cohort were under 40 years of age. Complete remission (CR) vs no CR at time of transplant, relapse vs no relapse within the 6-month follow up period and conditioning with ATG vs conditioning with alemtuzumab were also tested for. This was performed by splitting the patients into (for example): HLA match GvHD, HLA match no GvHD, not HLA match GvHD and not HLA match no GvHD groups, etc. The results are shown in table 3.4 and indicate that there were no significant differences in these parameters between the GvHD group and the no GvHD groups. However, this may be due to the small total sample size (24) investigated in this study, and that only seven of these 24 patients developed GvHD. The 24 patients/donors were split for this analysis, which resulted in unequal splits and smaller numbers. It can be difficult to identify the shape of a distribution when n is less than 30 (Field and Hole 2006), so analysis of data when n < 30 is not ideal, but n in this study was limited due to the two-year time frame.

Table 3.4 Chi-square analysis for the known risk factors of GvHD in patients who developed GvHD and those that did not.

Parameter (all aGvHD [n=6] vs no GvHD [n=18])	Chi-square (2 sided)
Related donor (n=6) vs other (n=18)	*P* = 0.795
12/12 HLA match (n=9) vs other (n=15)	*P* = 0.404
Sex match (n=14) vs other (n=10)	*P* = 0.404
Ciclosporin and ursodeoxycholic acid (n=12) vs other (n=12)	*P* = 0.653
Complete remission at transplant (n=10) vs other (n=14)	*P* = 0.728
Relapsed within 6 months of transplant (n=8) vs other (n=16)	*P* = 0.525
Conditioning with ATG (n=9) vs conditioning with alemtuzumab (n=12)	*P >0.999*

ATG = anti-thymocyte globulin

Related and unrelated donors' comparison. The Chi-square result was not significant (*P* = 0.795) and therefore there was no association between using a related donor or not and GvHD. The two deaths within the 6-month follow up period were both in the unrelated donor group.

Human Leukocyte Antigen (HLA) matching comparison. The Chi-square result was not significant (*P* = 0.404) and therefore there was no association between being a 12/12 HLA match or not and GvHD. Both 12/12 match and the non 12/12 match groups contained one death at the 6-month follow up time point.

Sex matched and unmatched donors' comparison. The Chi-square result was not significant (*P* = 0.404) and therefore there was no association between receiving a sex matched donor or non sex matched donor with GvHD. Both the sex matched and the non sex matched groups contained one death at the 6-month follow up time point.

GvHD prophylaxis at time of transplant comparison. The Chi-square result was not significant (*P* = 0.653) and therefore there was no association between receiving ciclosporin and ursodeoxycholic acid prophylaxis or receiving the alternative prophylaxis and GvHD. The 2 patient deaths within the 6 months follow up were both in the alternative prophylaxis group.

Disease status at time of transplant comparison. The Chi-square result was not significant (*P* = 0.728) and therefore there was no association between whether the patient was in remission at the time of HSCT or not and GvHD. However, the two patient deaths within the 6-month follow up period were not in complete remission at transplant.

Relapsed patient comparison. The Chi-square result was not significant (*P* = 0.525) and therefore there was no association between relapsing within 6 months of transplant or not and GvHD. The 2 patient deaths within the 6-month follow up had relapsed in the 6-month follow up period.

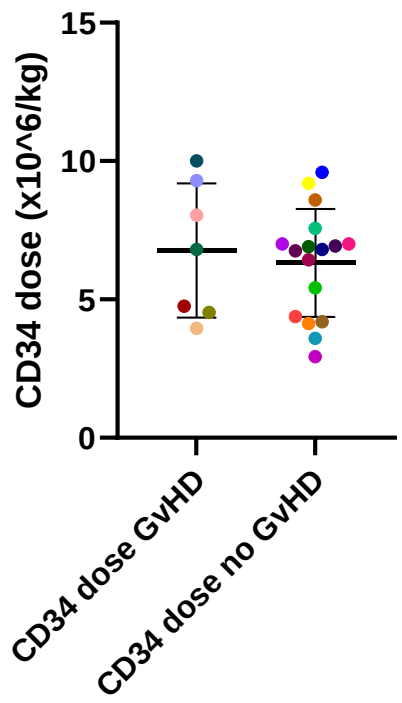
ATG versus alemtuzumab comparison. The Chi-square result was not significant (*P* > 0.999) and therefore there was no association between receiving conditioning that contained one of these drugs or the other and GvHD. No patient received both drugs in their conditioning and only 3 patients received conditioning that did not contain either of these drugs (patients 6, 21 and 24). The two patient deaths within the 6-month follow up period both received ATG within their conditioning.

The Chi-square analysis showed no association between any of the known risk factors of GvHD investigated here, and the occurrence of GvHD in the patients studied. These results may be due to the limited sample size of the study.

3.10 Transplanted $CD34^+$ graft dose and GvHD

The relationship between transplanted $CD34^+$ graft cell dose and GvHD was analysed for this transplanted patient cohort. It can be difficult to identify the shape of a distribution curve when n is less than 30 (Field and Hole 2006), and because of this Normal QQ plots were used. For all patients the $CD34^+$ graft dose was found to be normally distributed, Student's t-test could therefore be used to analyse this data. Figure 3.9 shows the mean and standard deviation for the two groups, and that there was no statistical significant difference (P = 0.68) between the $CD34^+$ graft dose in the GvHD and no GvHD groups.

Figure 3.9 Mean CD34$^+$ graft dose received by patients according to GvHD status



GvHD mean dose = 6.76 x 10^6/kg

No GvHD mean dose = 6.35 x 10^6/kg

Student's T test result *P* = 0.68

Error bars are the standard deviation.

Numbers of patients in GvHD group 6, no GvHD group 18.

Each patient is represented by the following colours (GvHD patients in bold).

1 **2** **3** 4 5 6 7 8 9

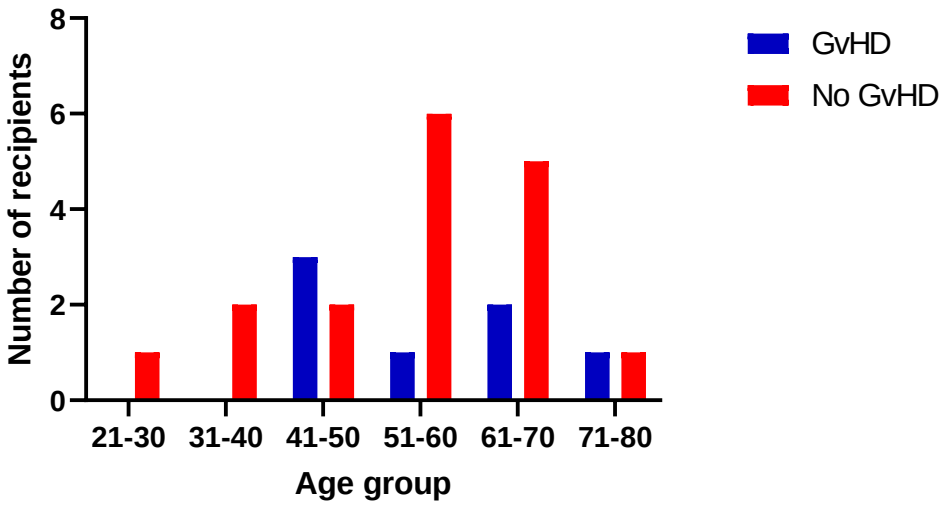
10 11 12 13 14 **15** 16 **17** 18

19 **20** 21 22 23 24

3.11 Recipient age and GvHD

The relationship between recipient age and GvHD diagnosis was compared by splitting recipients according to GvHD diagnosis and age group, and the results are shown in figure 3.10.

Figure 3.10 GvHD status by recipient age group



There was no GvHD diagnosis in the three recipients of 40 years of age and under. In this patient cohort the highest number of GvHD diagnosis (3) was in the 41-50 age group.

3.12 Transplanted $CD34^+$ graft dose and engraftment

The relationship between transplanted $CD34^+$ graft dose and engraftment was analysed for this transplanted patient cohort. Normal QQ plots were used and for all the patients $CD34^+$ graft dose, neutrophil and platelet engraftment were found to be normally distributed.

Two groups were created to allow statistical analysis to be performed to determine if receiving a high $CD34^+$ dose was advantageous. These groups were created by calculating the mean $CD34^+$ dose for the cohort (6.45 x 10^6/kg) and then placing recipients into one of two groups depending on whether they received a $CD34^+$ graft dose higher (the 'high' group) or lower (the 'low' group) than the mean graft dose. The characteristics of these groups are shown in table 3.5. This follows a similar approach performed by Mohty *et al.* (2005).

The two groups each had their $CD34^+$ graft dose, neutrophil and platelet engraftment tested using Normal QQ plots and they also were found to be normally distributed. Student's t-test could therefore be used to analyse this data.

Table 3.5 Patient and transplant characteristics of the 'high' and 'low' $CD34^+$ graft dose groups

Characteristic	Low Group (n=9)	High Group (n=15)
Patients (M/F)	6/3	8/7
Mean recipient age (range)	52 (22-70)	57 (34-73)
Sex matched/M→F/F→M	6/2/1	8/6/1
Related/unrelated donor/Haplo	2/7/0	4/10/1
Mean $CD34^+$ dose ($x10^6$/Kg) (range)	4.4 (2.93-6.43)	7.9 (6.75-10)
Mean days to neutrophil engraftment	14.60	14.93
Mean days to platelet engraftment	19.38	16.21
100-day survival status (alive/dead)	9/0	15/0

Haplo = Haploidentical transplant.

3.12.1 Student's t-test results for $CD34^+$ dose between 'high' and 'low' $CD34^+$ graft dose groups

The Student's t-test (equal variances assumed) was used to check whether there was a significant difference between the 'high' and 'low' $CD34^+$ graft dose groups. As expected, the two groups were significantly different with respect to $CD34^+$ graft dose, $P < 0.001$.

3.12.2 Student's t-test results for neutrophil and platelet engraftment between 'high' and 'low' $CD34^+$ graft dose groups

The Student's t-test was used to check whether there was a significant difference between the 'high' and 'low' $CD34^+$ graft dose groups for neutrophil and platelet engraftment time and the results are shown in table 3.6. There was no statistically significant difference for engraftment time between the 'high' and 'low' $CD34^+$ graft dose transplanted groups for either neutrophil engraftment (P = 0.7938) or platelet engraftment (P = 0.4197). Both 'high' and 'low' $CD34^+$ graft dose groups contained one death at the 6-month follow up time point.

Table 3.6 Student's t-test results (neutrophil and platelet engraftment between $CD34^+$ graft dose groups)

	t-test result (2-tailed)
Neutrophil engraftment ('high' v 'low')	*P* = 0.7938
Platelet engraftment ('high' v 'low')	*P* = 0.4197

Mean dose = 6.45 x 10^6/kg

Numbers of patients in 'low dose' group 9, 'high dose' group 15.

Chapter 4 – Discussion

4.1 Summary/overview

GvHD can cause significant morbidity and mortality in patients following HSCT. The study's hypothesis proposed that GvHD has an inverse relationship with the number of tolerogenic cells in mobilised PBSC grafts, and/or how quickly these cells emerge in the recipient's PB post allogeneic HSCT. If this was the case, low tolerogenic cell counts could potentially be used as a biomarker (medical signs that can be measured objectively, accurately, and reproducibly [Biomarkers Definitions Working Group 2001]), for GvHD, the count results potentially preceding any GvHD. This would be of use to clinicians treating patients undergoing allogeneic HSCT and may improve patient outcomes.

Immune cells circulate between the PB, lymphatic system, tissues and secondary lymphoid organs, and most white blood cells function in locations other than blood, blood is the transport system (Punt *et al.* 2019). As pDCs are present in the thymus and peripheral lymphoid tissues (as well as PB) they may be involved in tolerance to a greater extent than other DCs (Hadeiba *et al.* 2008). DC-10 cells are present in PB, and in the secondary lymphoid organs of healthy subjects, and accumulate in human decidua in the first trimester of pregnancy (Comi *et al.* 2018). T regs are predominant in the PB and in the lymph nodes and are believed to operate in two principal locations, secondary lymphoid tissues and peripheral tissues. (Shevyrev and Tereschchenko 2020). $CD4^+$ Tr1 cells are induced in the periphery (Gagliani *et al.* 2013). Therefore, these tolerogenic cells may only be present in the PB for a limited amount of time, during trafficking to tissue sites.

Obtaining samples from lymphoid tissues would require an invasive procedure, which is less suitable for a potential biomarker (Edelstein 2010) and would have been unlikely to have been approved by the ethical committee that reviewed the study and may have affected patient recruitment. Liver biopsies are rarely taken early after HSCT because thrombocytopenia increases the risks associated with biopsy procedures (Ferrara *et al.* 2009). Peripheral blood has ease of access and was unlikely to affect patient recruitment as these samples are part of routine care post HSCT.

Edelstein's (2010) biomarker requirements were matched in this study: he states that the sample should be non-invasive and from readily available sources e.g. blood or urine (this study used PB and mobilised PBSC). The sample should be easily measured, inexpensively and produce quick results, the method should have high sensitivity and specificity, allowing early detection (this study used flow cytometry). The biomarker should be biologically plausible, and this project enumerated known tolerogenic cells. The study aimed to discover if the remaining biomarker characteristics would be found i.e. that there would be no overlap in results between diseased patients and healthy controls

and potentially provide insight into the disease mechanism. The cells analysed in the study were pDC, mDC1, DC-10 cells and Tr1 cells.

This research study had two aims: to analyse the tolerogenic cell counts in the PBSC grafts and in the PB of patients post allogeneic HSCT. Restoration of normal immune homeostasis requires engraftment and expansion of donor T cells contained in the graft and the differentiation of immune cells from the donor haematopoietic progenitor cells (Lonial *et al.* 2013). PBSC grafts were analysed on receipt in the laboratory. The PB of HSCT patients is routinely sampled weekly post-transplant by LTHT. Because the first six months after allogeneic HSCT is the most sensitive time window for tolerance induction (Ukena *et al.* 2011) this period was targeted by the study. This study analysed recipient PB post HSCT at 2, 4, 8 weeks, 3 and 6 months. Sampling time points were selected at the early stages post-transplantation as any cell counts that were significant at the early time points could be useful potential biomarkers for GvHD risk, and could potentially be used to change clinical practice, in terms of early treatment intervention to prevent GvHD. The latter two sampling time points were selected around the definitions of acute and chronic GvHD. Acute GvHD typically occurs between the time of engraftment to 100 days (14.3 weeks) post-transplant and cGvHD typically occurs after 100 days.

Flow cytometry methodologies were initially established to identify and quantify the different tolerogenic cells, based on recent publications. The publications used for the study were: Autenrieth *et al.* (2015) – pDC and mDC1, Gregori *et al.* (2010) – DC-10 cells and Gagliani *et al.* (2013) – Tr1 cells. Published counts and percentages of these cells in PB were compared with the results obtained from the three protocols established at NHSBT Leeds as part of their validation.

Autenrieth *et al.* (2015) defined pDCs as Lin^-, $HLA\text{-}DR^+$, $CD14^-$, $CD11c^{lo}$, $CD303^+$, and $CD1c^-$. The mDC1 population was identified using the same markers but instead were $CD1c^+$ and $CD303^{lo}$. Autenrieth *et al.* (2015) do not state cell count ranges but Chevallier *et al.* (2013) reported that the median number of pDCs in PB was 7.5/µl (range 5 – 10/µl) and the median number of mDCs in PB was 10/µl (range 4 – 30.5/µl).

Gregori *et al.* (2010) reported that $CD14^+$, $CD83^+$ and $CD11c^+$ DC-10 cells are 0.3% (+/- 0.18%) of MNC in peripheral blood. The lymphocyte + monocyte count in peripheral blood has a range of 1.3 – 4.4 x 10^9/L (Punt *et al.* 2019) and 0.3% gives a DC-10 figure of 4 – 13/µl. This study obtained a mean of 7 DC-10 cells/µl, which matched the number reported by Gregori *et al.* (2010) which was indicative that the protocol was identifying the same population as previously reported.

In the paper by Gagliani *et al.* (2013) the flow cytometry plots of a representative donor were provided and showed that 1.6% of $CD4^+CD45RA^-$ cells in peripheral blood were $CD49b^+LAG\text{-}3^+$ Tr1 cells. This current study obtained a double positive percentage of this phenotype of 1.1%. Memory $CD4^+$ cells have a normal range of 250-810/µl (Stem Cell Technologies, no date) and 1.6% of this gives a Tr1 cell number of 4 – 13/µl. This study obtained a mean of 7.67 Tr1/µl, which provided validity of the protocol established here.

4.2 Donor and patient recruitment

The study recruited 24 patients who were undergoing allogeneic HSCT at LTHT between November 2018 and November 2019 and five donors. The number of transplants expected to take place during the 12 months of the patient recruitment in the study was calculated from data from the 3 years prior to study initiation, and it was estimated that 35 transplant recipients and 25 graft donors would be recruited into the study. The literature search found a paper by Rajasekar (*et al.* 2010) on allogeneic HSC graft analysis that recruited 69 patients in a 2-year timeframe, and this study as designed, would have contained a pro rata number. Ten fewer donor grafts were expected due to donor grafts coming from non-AN Trust, BBMR and DBC donors. It was initially hoped that since all international imports are organised by the AN Trust, that AN Trust research approval would enable all international donor grafts to be eligible for the study. However, the AN Trust made clear during the research application process that their approval would only cover AN Trust/BBMR/DBC donor grafts collected in the UK, and that if other registries were to be included separate research ethics applications would have to be made to each registry individually, and this was not feasible in the timeframe of this DProf study.

The recruitment phase of the study was limited to one year, due to the six months follow up period post-transplantation to enable completion of the study in the two-year research phase of the DProf. Several difficulties were encountered during the study, and this included the selection of non-sibling, non-AN Trust donors for the donor grafts by the clinicians. Patient recruitment during the summer months was limited due to study resources. Other patient recruitment issues included a patient lacking capacity and a second who it was not appropriate to approach due to their disease status. It was acknowledged before the start of the study that 35 transplant patients was a limited sample size and would not have the appropriate statistical power to provide evidence to either support or disprove the study hypothesis. This study was therefore a pilot study and would be used to inform a larger scale study in the future.

Twenty-four patients were recruited into the study. All five post-transplant time point samples were obtained from seven patients, while four post-transplant time point samples were obtained from 10

patients. This was due in part to resourcing issues, patients deciding to attend local clinics instead of LTHT, staff, equipment and the restrictions imposed at LTHT due to the COVID19 pandemic. Because of the pandemic, patients were advised not to come to hospital for routine follow up because of the risk of infection, this accounted for three 6-month samples. Because some sample time points were missed, and therefore a complete set of data was not available, it was not possible to perform a mixed ANOVA on the data obtained, ANOVA also requires parametric data. Therefore, the tolerogenic cell counts at each time point and GvHD status was tested using the Mann-Whitney test.

Only five donors (two from related donors, three from AN Trust registry donors [one donor gave two collections]) were recruited into the study and the graft arm of the study was abandoned due to the very low numbers of donor samples available. From the three-year analysis that preceded the study it was expected there would be many German donor grafts, but more German donor grafts than expected were collected for patients during the study. Future studies would want to approach the German registries to enable their donor grafts to be included.

4.3 GvHD, relapse and survival

Of the 24 patients recruited onto the study, six patients developed aGvHD (patients 1, 2, 3, 15, 17 and 20), and patient 19 was diagnosed with cGvHD, within the 6-month follow up period. Apperley *et al.* (2012) reported that moderate to severe acute GvHD occurs in approximately 40% of all recipients of allogeneic HSCT, and chronic GvHD occurs in 40%-70% of all recipients. The average time of onset of acute GvHD was 15.8 weeks or 110 days, which was just after the 3-month sample time point and between this study's penultimate and final post-transplant sample. This is slightly later than the traditional acute/chronic GvHD boundary of 100 days (Ghimire *et al.* 2017). Late acute GvHD is defined as persistent, recurrent, or new acute GvHD symptoms occurring after 100 days post-transplantation (Holten *et al.* 2016) and 4/6 patients with acute GvHD had late acute GvHD in the study. Late acute GvHD can arise with greater frequency after RIC (Ferrara *et al.* 2009) and all patients in this study received RIC protocols. Of the six patients with acute GvHD, only one was refractory to steroid treatment, patient 17. Pagliuca *et al.* (2020) reported findings that of patients with acute GvHD, 31% had steroid refractory acute GvHD.

Eight patients relapsed within the 6 month follow up period (patients 2, 12, 14, 15, 16, 18, 19 and 23). The GvHD group and relapse group were mutually exclusive except for patient 15 (diagnosed with GvHD at 3-weeks and relapsed at 25 weeks).

Two patients died within the 6 month follow up period, patient 2 (GvHD and AML) and patient 14 (relapse). Patient 19 had been sent to palliative care before the 6-month sample point.

4.4 Pre and post-transplant factors

Mean patient age was similar for patients diagnosed with GvHD and those that were not diagnosed with GvHD, 54.83 and 54.71 years respectively. Mean $CD34^+$ graft dose was similar between these two groups, 6.76 x 10^6/kg and 6.35 x 10^6/kg respectively. Mean neutrophil engraftment was also similar between the two groups, 15.3 days (GvHD) and 14.6 days (no GvHD) respectively, while the mean platelet engraftment was slightly quicker in the GvHD patients, 16.7 days versus 17.7 days.

4.5 Reconstitution post-transplantation

During conditioning patients' immune cell counts in PB drops close to zero, then post HSCT they recover, but different immune cells recover at different rates (Velardi *et al.* 2020). This study compared the patients' cell counts to the normal ranges. SJUH currently perform $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and NK cell analysis at 6-months post allogeneic HSC transplant, and they do not test for any T reg cells. The analysis performed in this study provides data on immune reconstitution before that 6-month time point and has new Tr1 data.

At LTHT patients are given G-CSF which stimulates the production of neutrophils, from day 14 until the PB neutrophil counts reach 1 x 10^9/L. Because neutrophils constitute most of the WBC population (50%-70% of WBCs in healthy individuals (Punt *et al*. 2019)), an increase in neutrophil numbers influences the overall WBC count.

The recovery of the pre-split median tolerogenic counts for each of the cell populations analysed showed similarities and differences. The median pDC and mDC1 counts mirrored each other through the sampling time points, but while the median mDC1 cells achieved the normal range throughout, the median pDCs were below the normal range throughout. The median DC-10 cell count was below the normal range at 2-weeks but was in the normal range thereafter. The median Tr1 cell count did not achieve the normal range at any point in the 6-month follow up period.

Patients were split into GvHD and no GvHD groups and their median counts were compared. Because the total number of patients in the study was 24, and because some time point samples were missed for some patients, once the patients were split into GvHD and no GvHD groups the number of patients within those groups was small. Sample size could have been increased by performing a multi-centre study in future studies.

The post-transplant patients' median PB WBC count increased dramatically between the 2- and 4-week time-point, and steadily increased thereafter, which was also true for neutrophil counts. The median WBC count achieved the normal range at the 6-month sample time point, but this did not necessarily reflect the pattern seen for all the WBC sub-types.

The post-transplant patients' median neutrophil count had achieved engraftment (defined as 0.5 x 10^9/L, Sohn *et al.* 2003) at 2 weeks (it is important for neutrophil counts to recover quickly as these cells phagocytose bacteria and secrete antimicrobial proteins providing protection from infections in patients (Punt *et al.* 2019)), however the median count did not achieve the normal range within the 6-month follow up period.

The median $CD3^+$ T cell and median $CD4^+$ T helper cell counts had not achieved the normal range at the 6-month time point. These findings match those reported by Velardi *et al.* (2020) who reported that innate cells such as neutrophils and monocytes recover relatively quickly, but T and B cells can take up to two years to recover.

The $CD3^+$ and $CD4^+$ cell counts in the GvHD and no GvHD groups had a similar pattern at the 2-, 4- and 8-week time points. But at 3 months the median $CD3^+$ cell count in the no GvHD group was higher than the GvHD group, while the median $CD4^+$ cell count in the GvHD group was higher than the no GvHD group. These time points were all pre this study's average onset of acute GvHD of 110 days. At the 6-month sample time point the GvHD group had a higher median $CD3^+$ cell count (which was the only sample time point to achieve the normal range), compared to the no GvHD group, while both groups had similar median $CD4^+$ cell counts.

At the 2-week time point the median pDC cell count in the no GvHD group was in the normal range, while the GvHD group median was below the normal range (7/µl vs 2/µl respectively), and the difference in counts between these groups was the largest of any of the following time points. At the subsequent time points the no GvHD group median pDC count was below the normal range. In the GvHD group the median pDC just achieved the normal range at 4 weeks but was below it at all other time points. The median pDC counts in both the GvHD and no GvHD groups dropped between the 3- and 6-month time points (average onset of acute aGvHD in this study was 110 days), and both were lower at 6 months than at 2 weeks.

Across the five-sampling time points the median mDC1 count in the GvHD group was consistent and at the bottom end of the normal range. The no GvHD group had slightly higher medians at all time points except for the 8-week sample, which had a median count lower than the GvHD group. The median counts for mDC1 cells dropped between the 3- and 6-month time points (average onset of acute GvHD of 110 days) in both the GvHD/no GvHD groups, and as with the pDC counts, both were lower at 6 months than they were at 2 weeks.

The median DC-10 cell counts in the GvHD and no GvHD groups were similar at 2 weeks and 6 months. The biggest difference in median cell counts was at 4 weeks where the no GvHD group

median was over three times higher than the GvHD group and exceeded the normal range. This gap closed at 8 weeks but the no GvHD group median was still nearly double the GvHD median. At the 3-month time point this had reversed and the GvHD group median DC-10 cell count was double the no GvHD group and exceeded the normal range. The median DC-10 cell count for the GvHD group fell back to normal range between the 3- and 6-month time points (average onset of acute GvHD of 110 days), while the no GvHD group median count only fell slightly.

The Tr1 counts were low and similar at 2 and 4 weeks in both the no GvHD and GvHD groups. At 8 weeks and 3 months the groups were opposite from the $CD3^+$ T cell count groups in that the GvHD group Tr1 median cell counts increased at a higher rate than the no GvHD group. Between 3 and 6 months (the average onset of acute GvHD of 110 days), the GvHD median Tr1 cell count dropped while the no GvHD median count increased.

Since the median T helper cell counts did not achieve the normal range in the 6-month follow up period it might be expected that type 1 regulatory T cells would also not achieve the normal range in the 6-months following transplantation (T reg cells are 5% of the total $CD4^+$ T cell population (Ukena *et al.* 2011)) and they did not.

Podgorny *et al.* (2014) analysed various cell subsets during immune reconstitution following allogeneic transplantation in 219 patients. They found that acute GvHD was preceded by high counts of $CD4^+$ T cells and $CD8^+$ T cells and were followed by low counts of myeloid cells and pDCs. These authors only display their data in scatter plots but the high count of $CD4^+$ T cells was a median of approximately 40 cells/µl at 28 days, which while this was higher than in the no-acute GvHD group it is still very low compared to the normal range of 300-1500 cells/µl. Low acute GvHD pDC counts reported by Podgorny *et al.* (2014) had a median of <0.5 cell/µl with the no-acute GvHD group having a median of approximately 2/µl at 56 days, which is very low compared to the normal range of 10-40/µl. It would be desirable to see the raw data in the paper so that readers could know how many flow cytometric events were present in a count that was <0.5 cell/µl.

Some of the Mann-Whitney scatter plots appeared to show two groups (high vs low count group) within either the GvHD group or the no GvHD group. Patient 7 was the most common to be found in a high-count group (no GvHD). Patient 7 did not have delayed engraftment and was alive at the 6-month follow up time point. The high-count groups within the GvHD and no GvHD group did not reflect a particular age range for the HSCT recipients, with a spread of ages found in each.

Mann-Whitney analysis of the tolerogenic cell counts between the GvHD and no GvHD groups found no statistically significant differences for any of the tolerogenic cell type counts at any of the five post-transplant time points on development of GvHD in recipients.

However, if a larger sample number had resulted in statistical significance being found for one or more of the tolerogenic cell types, at one or more time points, the data still would contain problems for these tolerogenic cells being used as biomarkers for GvHD. There was never a clear tolerogenic cell low count-GvHD, and high count-no GvHD pattern, which the experimental hypothesis was based on. The scatter plots show the tolerogenic cell counts in the GvHD and no GvHD groups overlap. One of Edelstein's (2010) proposed biomarker characteristics of 'no overlap in results between diseased patients and healthy controls' is not met. Without this separation of the cell counts for the GvHD and no GvHD groups, a cut off value for determining potential development of GvHD could not be determined, and without a cut off value the tolerogenic cell counts could not be used as a prognostic biomarker for GvHD.

Lugt *et al.* (2013) reported that ST2 (also known as the IL-33 receptor) was the best single biomarker of non-response to GvHD therapy and subsequent death. Patients with high ST2 concentrations in plasma at commencement of GvHD therapy responded poorly to treatment. Magenau *et al.* (2010) reported that Treg frequency (defined by the $CD4^{+}CD25^{hi}FoxP3^{+}$ phenotype) at GVHD onset had only modest diagnostic value with an AUC value of 0.69. Adom *et al.* (2020) reported that regulatory T cells as defined by the $CD4^{+}CD25^{hi}FoxP3^{+}$ phenotype can be a diagnostic and predictive biomarker of GvHD. Thus, PB tolerogenic cell counts have the potential to be used as biomarkers and has been investigated previously, although this work was published after the current research was initiated. However, no single biomarker has established itself in routine care, and biomarkers remain an active area of research (Magenau *et al.* 2016).

4.6 GvHD patients' tolerogenic cell counts

As the study was designed to analyse certain tolerogenic cell counts with the aim of acquiring evidence that one or more could have potential as a biomarker for GvHD, sampling time points were skewed to this goal. It was therefore not always possible to view what effect the diagnosis and subsequent treatment of GvHD had on these cell counts. Five out of six patients were diagnosed with late acute GvHD, between the 3- and 6-month sample, and these patients were not due their next sample until over a month post diagnosis. It is therefore difficult to determine what effect diagnosis and treatment had on their tolerogenic cell counts. If the purpose of the study had been to analyse the effect of diagnosis and subsequent treatment on tolerogenic cell counts it would have been designed differently, and the sampling time points would have been structured around GvHD

diagnosis and treatment. Samples could have been obtained on diagnosis but before treatment, during treatment and after treatment.

Only patient 15 (diagnosed with aGvHD at 3 weeks post HSCT) had sampling time points that were in proximity to the diagnosis and treatment of aGvHD. Their tolerogenic counts were at lower levels at 4 weeks compared to 2 weeks. The treatment for aGvHD was initiated immediately and had a 5 week duration, the patient was still having treatment at the 8-week sample time point where the counts were slightly higher than at 4 weeks. The counts then increased between 8 weeks and 3 months.

The six aGvHD patients showed little similarity in their recovery of the tolerogenic cells in PB post HSCT. The one patient who died of GvHD did have an unusually high DC-10 count prior to diagnosis of GvHD, but because this was only one patient it is not possible to know if this is of significance.

4.7 Known GvHD risk factors

Chi-squared analysis was performed on the presence or absence of GvHD in the patient cohort and its known risk factors. These were: related vs unrelated donor, HLA compatibility (analysed as 12/12 HLA match vs other), Sex matched vs mismatched transplants, GvHD prophylaxis at time of transplant (analysed as ciclosporin and ursodeoxycholic acid vs other), disease status at time of transplant (analysed as complete remission at transplant vs other) and relapsed within 6 months of transplant vs other (Apperley *et al.* 2012). Chi-squared analysis was also performed on conditioning with a cocktail containing ATG vs with a cocktail containing alemtuzumab. The analysis found no connection between those patients with a known risk factor vs those that did not and GvHD. This finding was probably due to the small number of patients recruited and a larger study in the future may find significance.

Only two HSCT recipients were under 40 years of age, and it was not possible to perform statistics on this low number. GvHD by age group was analysed and the highest number of GvHD diagnoses was found in the 41-50 (n=3) age group. There was no GvHD diagnosis in HSCT recipients in the 21-30 and 31-40 age groups.

4.8 Impact of $CD34^+$ graft cell dose on engraftment and GvHD

Because the patients could not be allocated into groups pre-transplant, they were allocated into groups post-transplant based on whether the patient received a higher or lower than mean $CD34^+$ cell graft dose of 6.45 x 10^6/kg. All patients had neutrophil engraftment within 28 days, while 21/24 patients had platelet engraftment within 28 days. The Student's t-test analysis performed in this study showed there was no statistically significant difference for engraftment time between the

'high' and 'low' $CD34^+$ dose groups. Rajasekar (*et al.* 2010) split according to their median $CD34^+$ cell graft dose of 10.3 x 10^6/kg and found none of the cellular subsets in the graft impacted neutrophil or platelet engraftment. Carvallo *et al.* (2004) analysed graft composition and reported a median $CD34^+$ graft dose of 7.6 x 10^6/kg, and found higher $CD34^+$ graft doses facilitated myeloid engraftment. Baron *et al.* (2005) reported a median allogeneic $CD34^+$ graft dose of 6.5 x 10^6/kg, and in a multivariate analysis that high levels of $CD34^+$ cells in the graft were associated with high levels of donor T cell chimerism.

This current study also split the HSCT transplant recipients into GvHD and no GvHD groups and analysed their $CD34^+$ graft dose using Student's t-test analysis. There was not a statistically significant difference between GvHD status and $CD34^+$ graft dose. Using multivariate analysis Baron *et al.* (2005) found no association between graft composition and GvHD, in agreement with the results reported here.

Rajasekar *et al.* (2010) analysed various cell types in grafts and in patient's PB following allogeneic transplantation. The authors found that patients that received a 'high' dose of pDCs within their grafts had significantly higher risk of relapse and lower overall survival and event free survival, and that this could possibly be used as a predictive tool or that graft manipulation could be performed to prevent GvHD. Because that arm of this study had to be abandoned, this study has no results to compare to the Rajasekar *et al.* (2010) paper using the Autenrieth (*et al.* 2015) pDC phenotypic definition.

4.9 Difficulties in making comparisons between studies from different laboratories

There are several difficulties in comparing studies of transplant patients from different laboratories. These include protocols using washed/unwashed cells, whole blood vs separated blood, single/dual platform cell enumeration and phenotypic definitions. Rajasekar *et al.* (2010) and Podgorny *et al.* (2014) washed cells and obtained cell counts using a dual platform method. A washing step will invariably mean loss of cells through all the washing stages which may even affect the different cell types differently, and single-platform testing provides a direct absolute count and is considered more reliable and reproducible than dual-platform testing (Noulsri *et al.* 2018). Chevallier *et al.* (2013) obtained a median number of pDCs in peripheral blood of 7.5 cells/µl, which was slightly below the mean obtained in this study of 8.33/µl, but unfortunately, they did not give details on their flow cytometry sample preparation.

The phenotypic differences between the DCs analysed in the study are mainly related to three markers. DC-10 DCs were $CD14^+$ while pDC and mDC1 were $CD14^-$ while pDC were $CD303^+CD1c^-$ and

mDC1 were $CD303^{-}CD1c^{+}$. Enumeration of these cell types in peripheral blood was performed by flow cytometry and the cell surface markers used by different laboratories to identify cell types can be different (Velardi *et al.* 2020). Different phenotypic definitions in published papers of pDC can be seen below in table 4.1, these papers were published before the Autenrieth (*et al.* 2015) paper. Similarities exist within the definitions; all have the Lin^{-} marker. All but the oldest paper have HLA-DR^{+} and $CD123^{+}$, the most recent two papers have the $CD11c^{-}$ marker.

Table 4.1 Different phenotypic definitions of pDC in the literature

Paper	pDC phenotype
Mohty *et al.* (2005)	Lin^{-}, $CD11c^{-}$ and $ILT3^{+}$
Rajasekar *et al.* (2010)	Lin^{-}, HLA-DR^{+} and $CD123^{+}$
Rogers *et al.* (2013)	Lin^{-}, $CD4^{+}$, HLA-DR^{+}, $CD11c^{-}$, $CD123^{+}$, $CD45RA^{+}$, $ILT7^{+}$, BDCA-2^{+}, $LAIR1^{+}$ and $CD2^{+}$
Podgorny *et al.* (2014)	Lin^{-}, HLA-DR^{+}, $CD11c^{-}$, $CD123^{+}$

4.10 Limitations of the study

This study was staffed by one part time researcher and was therefore limited by that. The total number of patients in the study was only 24, and because some time point samples were missed for some patients, once the patients were split into GvHD and no GvHD groups the number of patients within those groups was small. It would have been desirable to have data on at least 30 GvHD patients (Field and Hole 2006), but this was not possible due to available resources. Sample size could have been increased by performing a multi-centre study. Because some sample time points were missed, it was not possible to perform a mixed ANOVA on the data obtained (ANOVA also requires parametric data). Therefore, the tolerogenic cell counts at each time point versus GvHD status was assessed using the Mann-Whitney test.

Peripheral blood tolerogenic cell counts at other time points may have provided statistically significant data. Alternative sampling time points could have been weekly for the first five weeks post transplantation (which would have fitted into the hospital's routine sampling timeframe), or multiple sample time points within the first two weeks post transplantation (which would not have fitted into the hospital's routine sampling timeframe, so would have required samples specifically for this project, which may have affected its ethics application). It was decided that weekly samples for the first five weeks would not be selected due to the low WBC counts seen during this period. However, the tolerogenic cell counts may or may not be proportional to the low WBC counts seen

during this early post transplantation period. If tolerogenic cell production was high during the first few days post transplantation, this would have been missed.

Following production in the primary lymphoid organs the blood cells are released into the bloodstream. Immune cells circulate between the peripheral blood, lymphatic system, secondary lymphoid organs and tissues (Punt *et al.* 2019). The study did not analyse tolerogenic cell counts in the lymphatic system, secondary lymphoid organs and tissues, where the counts may have been higher, but obtaining samples from tissues would require an invasive procedure, which is less suitable for a potential biomarker. Invasive sampling of patients who are not currently showing signs of GvHD for a study may also not be clinically acceptable, but this could be initially investigated in animal models of HSCT.

The study's hypothesis proposed that GvHD has an inverse relationship with the number of tolerogenic cells in mobilised PBSC grafts and how quickly these cells emerge in the recipient's blood post allogeneic HSCT. However, PB is not a 'site of action' in GvHD, which in its acute setting is usually the skin, gut and liver. It is in these 'sites of action' and lymph nodes where the circulating tolerogenic cells may exert their influence. Therefore, the PB tolerogenic cell count was acting as a proxy for the tolerogenic cell count in the 'sites of action' and/or lymph nodes/spleen.

Any data on GvHD diagnosis or death that occurred after the patient's 6-month post HSCT time point was not included in the analysis. Therefore, patients who did not develop GvHD until after the 6-month post HSCT time point were categorised in the no GvHD group, which may only have been the case at that moment in time. Patient 19 had been sent to palliative care before the 6-month sample point and was in palliative care at the 6-month sample point, so was categorised as alive in the statistical analysis.

Protocols were created to count >100 target cell events, while this was possible on healthy volunteer donor PB, it was not always possible for post HSCT recipients. One hundred target events were not obtained for any cell at the 2-week post HSCT time point. The majority of HSCT recipients obtained one hundred target events for DC-10 cells and $CD3^+$ T cells at the 4-week time point, and mDC1 cells at the 8-week time point. One hundred target events were not obtained for pDC or Tr1 cells at any time point. One hundred target events were obtained for all cell types on all analysed PBSC collections. For those cell counts where 100 target events were not obtained, the CV is not 10% which is the target for rare event analysis. (Hedley and Keeney 2013).

In the results section cell counts are compared to the normal range. Counts above and below the normal range may not have been dramatically above or below the normal range. Cell counts that

were double or greater than the upper limit for the normal range were achieved by: Patient 2's DC-10 cells at 8 weeks and 3 months, patient 6's WBC and DC-10 cell count at 4 weeks, patient 7's pDC at 2 weeks and $CD3^+$ count at 6 months, patient 9's pDC cell count at 3 months, patients 10's DC-10 cell count at 4 weeks and patient 23's DC-10 cell count at 8 weeks. Of these patients only patient 2 was diagnosed with GvHD.

No chimerism analysis was performed on the analysed cells so it is not known if the enumerated pDCs, mDC1s, DC-10s and Tr1s were recipient or donor cells.

Finally, just because the cells are present in PB (or in the lymphatic system, secondary lymphoid organs or tissues) that does not mean they are contributing in a positive or negative direction to tolerogenicity and GvHD. This study could only have found an association with tolerogenic cell types and the development of GvHD, as it is difficult to resolve correlation and causation from a clinical study.

4.11 Further work and recommendations for change in practice in transplantation practice using tolerogenic cells and GvHD

The results showed that the Tr1 cells were the slowest of the cells analysed to recover post allogeneic transplantation, and because T cells recover at a slow pace (Velardi *et al.* 2020), and the median $CD4^+$ cell count never achieved the normal range during the 6-month follow up, this was probably to be expected. Since most patient's Tr1 cell count never reached the normal range of 4 – 13 cells/µl (only two patients had achieved this by the 6-month follow up time point), a longer follow up period might have shown when/if this would have been achieved. However, this would be of little use as a biomarker for GvHD which may already be present when they reach the normal range. Because of this, their use as a potential biomarker for patients at risk of GvHD is limited, so a future study should exclude Tr1 cells. Because of the unusually high DC-10 cell count prior to the diagnosis of GvHD in the one patient who died of GvHD, DC-10 cells should be included in a future study, to see if this observation is repeated. This result goes against the study hypothesis, which proposed that GvHD had an inverse relationship with the number of tolerogenic cells in the recipient's blood post allogeneic HSCT.

No chimerism analysis was performed on the analysed cells so it is not known if the enumerated pDCs, mDC1s, DC-10s and Tr1s are recipient or donor cells. It is also not known if/when a switch from recipient to donor tolerogenic cells occurs. If this was known, it may reveal factors at play regarding tolerogenic cells and GvHD. A future study would include chimerism analysis to determine if the tolerogenic cells are recipient or donor.

A future study would benefit from larger sample sizes for both donor collections and patients. To boost the number of participants a multi-centre approach to donor and patient recruitment would be required, and ideally aim for a three-figure number of patients and donors. This would require more resources than one part-time researcher. The logistical challenges of a multi-centre study would create would also have to be addressed. With more resources, individual international registries could be approached so that their donor grafts could be included in the study. Whether a large increase in patients would produce significant *P* values and enable Edelstein's (2010) proposed biomarker characteristics of 'no overlap in results between diseased patients and healthy controls' to emerge is questionable. Future studies could investigate other tolerogenic cell types including other subpopulations of T regs such as: NK T cells, γδ TCR T cells, $CD8^+CD28^-$ T cells, and $CD3^+CD4/8/56^-\alpha/\beta TCR^+$ T regs (McIver *et al.* 2008). A future study would also have a decision about DC-10 cells. A new phenotypic definition of DC-10 cells was published in 2020. Comi *et al.* (2020) propose $CD14^+CD16^+CD141^+CD163^+$ as surface markers for DC-10 cells (this study used $CD14^+$, $CD83^+$ and $CD11c^+$). The Comi group included researchers who published the phenotypic definition used in this study (this group also used the phenotypic definition of Tr1 cells as used in this study). A future study may move to this new phenotypic definition of DC-10 cells.

A future study could explore the analysis of suppressive cytokines for example IL-10 and TGF-β, and as T regs require exogenous IL-2 to function so this could also be analysed. Experimental GvHD has shown chemokines, for example: CCL2-CCL5, CXCL2, CXCL9, CXCL10, CXCL11, CCL17 and CCL27 enhance the movement of cellular effectors to target tissues, so analysis of these chemokines may be of use in assessing onset of GvHD (Ferrara *et al.* 2009). The expression of cytokine, chemokine, and chemokine receptors may change as GvHD develops, so the molecular interactions could change as weeks pass (Wysocki *et al.* 2005). However, cytokines and chemokines have a short half-life following release (Aziz *et al.* 2016), and their potential degradation when blood processing is delayed is a consideration for their use as biomarkers. A future study may require stability testing of the cytokines studied. These would be analysed using ELISA and cytokine levels in PB could be compared between GvHD and no GvHD groups using Student's t test or Mann-Whitney as appropriate. Since peripheral blood is not a 'site of action' in GvHD, which in its acute setting is usually the skin, gut and liver, cytokines and to a lesser extent chemokines, in PB would be a proxy just as cells are in PB. The future study may find that a biomarker for GvHD is a combination of cell, cytokine and chemokine results.

Future studies may obtain biopsies from affected tissue, draining lymph nodes and BM to analyse cell and cytokine levels in these locations. However, it is likely ethics committees would require

animal study results before they approved such a study in humans. Due to the thrombocytopenia that occurs early after HSCT, liver biopsies may not be possible (Ferrara *et al.* 2009).

To improve T cell engraftment Dvorak *et al.* (2008) administered allogeneic PBSC megadoses, which they defined as 20 x 10^6 $CD34^+$ cells/kg, to severe combined immune deficiency (SCID) patients. The authors found that of patients with T cell engraftment, the median time to a $CD4^+$ count of 200/µl (note the $CD4^+$ T_H cell count normal range 300-1500/µl) was 1.2 months. At the 4-week time point this study had a mean $CD4^+$ count of 53/µl, mean dose 6.45 x 10^6/kg. The time to a median $CD4^+$ count of 400/µl was 3.7 months. At the 3-month time point this study had a mean $CD4^+$ count of 242/µl. To obtain a large dose of 20 x 10^6/kg for every patient would be logistically difficult to achieve and expensive.

From a clinical perspective the slow recovery of Tr1 cells indicates that for these cells to have a functional impact in preventing GvHD during its early stages, then Tr1 cells would need to artificially be made to recover quickly, either by the administration of drugs or by *in vitro* expansion and transfusion. Several approaches have been attempted to aid recovery of T cells (Velardi *et al.* 2020), and these include administration of IL-7 (to stimulate development and expansion) and thymosin (to increase T cell numbers). The transfusion approach is being investigated by Mfarrej *et al.* (2017) who reported on the development of a Tr1 rich product which the group were preparing to use in the field of kidney transplantation. Future studies could try this approach in the field of HSCT.

Chandran *et al.* (2017) transfused kidney transplant recipients with *ex vivo* expanded autologous $CD4^+CD127^{lo}CD25^+$ T regs that were labelled with deuterium to enable tracking. They found the infused T regs peaked at 2-8% of circulating T regs in the first week. A month following transfusion deuterium signals remained detectable, but after 3 months the signal was near the detection limit of 0.2%. However, this study's findings of a peak in the first week can be compared to the study's sample time points of 2, 4, 8 weeks, 3 and 6 months. Thus, there is no 1-week sample time point for tolerogenic cell counts, so an early increase at this time point would be missed. However, the WBC count is very low one week post HSCT, and it is unlikely that the very low proportion of tolerogenic cells within the WBC population would be detected.

Potential future work could be designed to provide evidence that the tolerogenic cells were actively involved in reducing GvHD. This could involve the use of animal models of GvHD with suicide genes inserted into tolerogenic cells so that the researcher can control their activity and lifespan.

4.12 Summary

SJUH currently perform $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and NK cell analysis at 6-months post allogeneic HSC transplant, and they do not test for any T reg cells. The analysis performed in this study provides data on immune reconstitution before that 6-month time point and has produced new Tr1 data.

Following splitting of the HSCT recipients into GvHD and no GvHD groups, Mann-Whitney analysis of the tolerogenic cell count data did not reveal any significant differences between the groups. The split also showed there was an overlap in tolerogenic cell counts between GvHD and no GvHD patients, so the data did not match Edelstein's (2010) biomarker characteristic of 'no overlap in results between diseased patients and healthy controls'.

Two patients who died within the 6-month follow up period. At transplant one patient had residual disease (cause of death GvHD and AML) and the other patient had evidence of relapse (cause of death AML). The one patient who died of GvHD had an unusually high DC-10 cell count prior to the diagnosis of GvHD, this was opposite to the experimental hypothesis of an inverse relationship between tolerogenic cell counts and GvHD. It is not possible to conclude anything from one patient, but DC-10 cells would be included in a future study, to see if this observation is reproduced.

A biomarker that matches Edelstein's (2010) biomarker characteristics for GvHD is still a desirable goal. It would enable clinicians to make treatment decisions at early time points, which ideally would be before the onset of GvHD. Identification of biomarkers for GVHD with diagnostic (and possibly prognostic) significance might even make treatment of GVHD pre-emptive rather than prophylactic (Ferrara *et al.* 2009). This could lead to reduced GvHD and improved patient outcomes following allogeneic HSCT.

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Appendix I Sheffield Hallam University study protocol

SHU Professional Doctorate

Protocol.

	Content
1	**Project details** Investigator details: [redacted] Tel:[redacted] [redacted] **Project title** The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation **NHSBT Reference number** Msc-17-03 **Protocol version number and date** 1. 01/08/17 **Leeds Teaching Hospital Trust (LTHT) programme affiliation** Yorkshire Blood and Marrow transplant programme (YBMTP).
2	**Research question** The first objective is to analyse for associations between three tolerogenic cell types present in mobilised peripheral blood stem cell collections, and in recipient peripheral blood post allogeneic haematopoietic transplant with graft versus host disease (GvHD). A secondary objective is to analyse for associations between these three tolerogenic cell types and other transplant outcomes, including engraftment.
3	**Is the research original or is it intended to fulfil taught course requirements?** This is a student project for a Professional Doctorate. The research is original and it is hoped it will make a useful contribution to the understanding of GvHD. The research will contribute to a doctoral thesis.
4	**Background** Graft versus host disease (GvHD) is a complication of allogeneic haematopoietic transplantation which can result in patient morbidity and mortality. (Appelbaum 2004). GvHD results from a complex interaction between donor T cells and host tissues in an inflammatory milieu. (Appelbaum 2004). Moderate to severe acute GvHD occurs in approximately 40% of all recipients of allogeneic HSCT (Apperley *et al* 2012). Chronic GvHD occurs in 40% of HLA identical sibling unmanipulated HSC transplants, more than 50% of HLA- non-identical related HSC transplants and in 70% of matched unrelated HSC transplants. (Apperley *et al* 2012). A strategy of transplant tolerance has been pursued in order to minimise GvHD (Ezzelarab and Thomson 2011). Dendritic cells are professional antigen presenting cells that are capable of both activating immune responses and inducing tolerance (Volchenkov *et al* 2013). A tolerogenic cell is a cell that is capable of producing immunological tolerance. The limited immunostimulatory characteristics of plasmacytoid dendritic cells (pDC) that distinguish them from conventional DC (cDC) make pDC an attractive target for promoting tolerance in hematopoietic stem cell transplantation (Rogers *et al* 2013). Gregori *et al* (2010) identified and characterised a subset of dendritic cells which they termed DC-10. The authors found that DC-10 were present *in vivo,* and that DC-10 isolated from peripheral blood were potent inducers of antigen-specific IL-10 producing type 1 regulatory T (Tr1) cells. Gergori *et al* (2010) propose that DC-10 are a novel subset of tolerogenic DCs that have the function to induce Tr1 cells.

	Tr1 cells are induced in the periphery and have a pivotal role in maintaining tolerance (Gagliani *et al* 2013). Tr1 cells are distinguished from T helper type 1 (T_H1), T_H2 and T_H17 by their unique cytokine profile. Forkhead box P3 (FoxP3) is not a marker for Tr1 cells, its expression is low/transient after activation. The main mechanisms of Tr1 mediated suppression are the secretion of high levels of interleukin-10 (IL-10 is an immune-modulatory cytokine, which controls inflammation and inhibits immune responses [Amodio and Gregori 2012]), and the killing of antigen presenting cells (APCs) by granzyme B (Gagliani *et al* 2013). Plasmacytoid dendritic cells have been quantified in PBSC grafts (Rajasekar *et al* 2010) and in the peripheral blood during immune reconstitution Horvárth *et al* (2008). DC-10 that have been isolated from peripheral blood and are potent inducers of Tr1 cells (Gregori *et al* 2010). Tr1 cells have also been enumerated in peripheral blood (Gagliani *et al* 2013). Plasmacytoid DC, DC-10 and Tr1 have therefore all been linked to transplantation tolerance and are present in peripheral blood. These cells would therefore be expected to be present in mobilised peripheral blood stem cell (PBSC) grafts. There may be an association between the number and/or ratio of these tolerogenic cells an allogeneic recipient receives in their PBSC graft and the occurrence of GvHD and other transplant outcomes. There also may be an association between how quickly these tolerogenic cells emerge - and/or their quantity - during immune reconstitution and the occurrence of GvHD and other transplant outcomes. No studies have shown associations between Tr1 cells or DC-10 cells and transplant outcomes. Some studies have investigated associations between pDC and transplant outcomes, but pDCs have been included in this study because of the evidence mentioned above with respect to the role of pDC in transplant tolerance.
5	**Plan of investigation** 1. Methodology <u>Measurement of tolerogenic cell types in donor and patient samples</u> Adult patients undergoing allogeneic haematopoietic stem cell transplantation (ASCT) at St. James's University Hospital will be recruited into this study. This cohort will include patients who are undergoing ASCT for a variety of haematological malignancies. It is intended that most these patient's grafts will also be analysed. For the purposes of this study, donors can be split into three groups: 1. Sibling donors. 2. Anthony Nolan (AN), British Blood Marrow Registry (BBMR) and Delete Blood Cancer (DBC) donors. 3. Unrelated donors from other worldwide registries. This project hopes to use donors from groups 1 and 2. The reason for this is explained in Ethical Issues, below. While the target number of transplants for the study is 35, it is estimated that only 25 grafts will be analysed due to the exclusion of unrelated donors from the other worldwide registries. A sample from the graft(s) and samples from the patient post ASCT at 2, 4 and 8 weeks, 3 and 6 months will be taken as part of normal routine diagnostic testing. These samples are routinely tested for total nucleated cell (TNC) count, mononuclear cell (MNC) count, red blood cell (RBC) content and $CD34^+$ count. Following consent this project will also test the samples for: $CD3^+$ count, plasmacytoid dendritic cell (pDC) count, dendritic cell-10 (DC-10) count and type 1 regulatory (Tr1) cell count. Haematology clinicians at St. James's University Hospital will obtain consent from patients and related donors. An R&D application will be made to the Anthony Nolan charity to enable the use of their donations. <u>Creation of donor and patient information sheets and consent forms</u> Patient and related donor patient consent forms and information sheets have been created. Patients will be approached initially and if they consent their related donors will be approached for consent. An R&D application has been submitted to the AN for their unrelated donors. If the AN approve the application, AN, BBMR and DBC donors will be

eligible for use in the study. Unrelated donors from other registries will be excluded from the study.

Development of pre and post ASCT worksheets

There are known risk factors for acute/chronic GvHD, so this data will be incorporated into the analysis. These are: HLA compatibility, sex match/mismatch, alloimmunisation, stem cell source, age of recipient, conditioning regimen and any prevention of GvHD. This data is routinely collected and reported by St. James's University Hospital in each patient's Transplant Planner. The laboratory currently receives each patient's Transplant Planner as routine.

A post-transplant worksheet will be created for all transplants. These will be completed by the chief investigator and will collect data on all post-transplant key events: Neutrophil and platelet engraftment, acute GvHD (if applicable / Glucksberg classification), chimerism, disease free survival, relapse, progression free survival, chronic GvHD (if applicable / National Institute of Health grade) and overall survival. Cause of death (if applicable) will be collected. Because any treatment that may administered for GvHD may affect the tolerogenic cells being studied in the project, the post-transplant worksheet will collect data on what treatment has been given for GvHD and the date(s) of administration. Because of the number of drug options for GvHD treatment this will be recorded as first line, second line, etc therapy. This will enable cell counts before and after GvHD treatment to be compared.

Development of flow cytometry protocols for pDC, DC-10 and Tr1 cells

Samples from healthy volunteers and from donations whose donors have consented to research via the NHSBT 2B form will be anonymised and used for the creation and optimisation of flow cytometry protocols for the three tolerogenic cell types. These protocols will use sequential gating and beads to quantify the cells of interest. Protocols for CD34 and CD3 already exist.

2. Design: type of study and justification

The project will analyse consecutive adult allografts undertaken in a 1 year period at St. James's University Hospital (SJUH). An average of recent years suggests the study will be able to analyse 35 transplants in the 12 month period. A 6 month follow up means samples will be analysed over an 18 month period.

It is not possible to calculate the amount of patients required for the principle research question (plasmacytoid dendritic cells, DC-10 cells and type 1 regulatory T cell and acute graft versus host disease) at the time of writing due to a lack of data. The secondary research questions include engraftment. Using a clinically relevant difference of 1 day (power of 0.8 and a significance of 0.05) the study would require 710 patients for neutrophil engraftment and 7500 patients for platelet engraftment. These numbers are not possible for a part time 18 month study. This is therefore a pilot study. The study aims to recruit all adult allogeneic transplant recipients in a 1 year period at Leeds Teaching Hospital Trust. It is estimated that the majority of donors will be related donors or unrelated Anthony Nolan, British Bone Marrow Registry or Delete Blood Cancer donors. Unrelated donors from registries other than these will be excluded. It is therefore estimated that 35 transplant recipients and 25 donors will be recruited into the study.

The study is a pilot study which will collect the data which will enable N will be calculated for the principle research question.

3. Analysis including statistical methods, where appropriate

The independent variables for the study are the cell counts / doses in the graft and in recipient blood during immune reconstitution. The dependant variables in the study are GvHD, engraftment and other transplant outcomes. The diagnosis and grading of GvHD will be performed by the clinicians at SJUH.
Flow cytometry samples will be run in duplicate, with mean values used to calculate cell counts and doses.

Data will be tested using the Kolmogorov-Smirnov test to test if the data is normally distributed.

Groups will be created using the graft tolerogenic cell dose, tolerogenic immune reconstitution counts and GvHD diagnosis. Where the data is normally distributed they will be tested using the independent t-test or ANOVA. If data is not normally distributed the Mann-Whitney or Kruskal-Wallis test will be used. The Chi-square test will be used for categorical variables.

4. Outcome measures

The primary outcome measure will be to determine whether there are associations between pDC, DC-10 and/or Tr1 cell types present in mobilised peripheral blood stem cell collections, and/or in recipient peripheral blood post allogeneic haematopoietic transplant, with graft versus host disease (GvHD).

A secondary objective is to analyse for associations between pDC, DC-10 and/or Tr1 cells and other transplant outcomes, including engraftment.

5. Setting

Patients and related donors will be recruited by clinicians at the Leeds Teaching Hospital Trust. AN, BBMR and DBC donors will be recruited via the AN R&D application. Patients, donors and samples will be pseudonymised. Samples from grafts will be obtained at the laboratory and samples from recipients post ASCT will be obtained at SJUH and transferred to the laboratory. Samples will be analysed in the laboratory at NHSBT Leeds.

6. Participants

It is intended to analyse 35 consecutive ASCTs at SJUH in a 1 year period. Each transplant will produce samples for a 6 month period as part of the follow up. Due to the timescale of the project this is a pilot study. However, these participant numbers are comparable to previous studies in the literature.

Inclusion criteria

4. Adult ASCT patients undergoing transplantation at SJUH
5. Related donors
6. AN, BBMR and DBC donors

Exclusion criteria

4. Participants under the age of 18 years
5. Unrelated donors from non AN, BBMR and DBC registries

7. Recruitment

Pre study - Anonymised healthy volunteers and adults undergoing transplantation at LTHT.
Study - Pseudonymised adults undergoing ASCT at SJUH.

(i) Potential participants will be identified by the consultant haematologists at Leeds Teaching Hospital Trust.

(ii) Participants will be approached in person by the haematology clinicians during their routine consultations. Patient/donor information sheets will be provided. The clinicians will be able to answer any questions about the study while the patient/donor is considering the information. Potential participants will have at least 24 hours to decide if they wish to take part. Emphasis will be placed on the fact that participation is entirely voluntary and non-participation will have no bearing on their care or treatment.

(iii) Full voluntary consent will be obtained by the clinicians and the consent form will be completed.

8. Intervention. Flow chart indicating participant's involvement in the study:

Potential participants identified by SJUH consultants during ASCT work up

↓

Potential participants informed about the study given patient/donor information sheets and consent form

↓

Potential participants given at least 24 hours to consider the study

↓

Informed consent obtained by an SJUH clinician

Patients give a peripheral blood sample at the following routine post-transplant sample time points: 2, 4 and 8 weeks, 3 and 6 months. These samples will be analysed. After the 6 month post ASCT sample has been provided, the patient's involvement in the study ends.

Gantt chart:

Milestones:

- IRAS and all other approvals obtained
- Creation and optimisation of protocols for: pDC, DC-10 and Tr1 cells
- Patient samples analysis complete at 21 months
- Final proofing and editing of thesis at 23 months

Preliminary pDC, DC-10 and Tr1 analysis will be conducted in the first 2 months to create and optimise flow cytometry protocols.

6 **Project Management**

There will be regular contact between the chief investigator and the other key investigators/collaborators. The chief investigator will meet Prof N. Woodroofe and Dr Rebecca Leyland of Sheffield Hallam University (SHU) every 3 months for feedback and

	progress reports. The chief investigator will meet the clinical collaborators at Leeds Teaching Hospital Trust weekly for updates. SHU has an internal audit process for NHS IRAS projects. SHU is sponsor and will cover insurance and indemnity for the project.
7	**Expertise** Dr Maria Gilleece is the Consultant Haematologist and programme director of the Yorkshire Blood and Marrow Transplantation programme. Professor Nicola Woodroofe is the Head of Biomolecular Sciences Research Centre at SHU. Dr Rebecca Leyland is a lecturer in Bioscience and Chemistry at SHU specialising in immunology and immuno-oncology.
8	**Ethical issues** The risk to participants is not considered to be significantly greater than that normally associated with routine diagnostic procedures. There is no risk to the donor by sampling the collection(s), and the recipient peripheral blood samples will be taken at the same time as those taken for routine diagnostic purposes. The small risk of complications such as scratching, bruising and minor discomfort arising from taking blood are fully outlined in patient information sheets. Vulnerable adults and people under 18 years of age will be excluded from the study. Consent will be obtained via signed patient and related donor consent forms by clinicians at SJUH providing care for patients in the bone marrow transplant (BMT) unit. Consent for AN/BBMR and DBC donors will be obtained from the AN. Information will be provided orally and supported with patient/donor information sheets. The clinicians at SJUH will be able to answer any questions about the study. Donors from all other registries will be excluded from the study due to the logistical issues of applying for R&D consent from registries all over the world. All personal data concerning study participants will be kept strictly confidential, and medical records will only be accessed by the clinicians and the chief investigator. Personal data will be pseudonymised to allow for patient follow up. Electronic data will be stored on password protected computer database systems at NHSBT Leeds. Paper records will be kept behind locked doors within a controlled access building at NHSBT Leeds. Identifiable data will not be published. The study will sample over an 18 month period and the data it generates will be stored for up to 3 years from the first patient. Blood samples will be stored for 2 weeks and then disposed as per routine procedure.
9	**Service users** Dr Maria Gilleece and Prof Gordon Cook at SJUH have provided advice on the design of this project. Results will be verbally communicated to participants if requested and copies of any publications made available to them. Opportunities will be taken to present the results at scientific meetings such as the British Blood Transfusion Society annual general meeting.
10	**Dissemination** It is anticipated that results from this study will be reported in peer reviewed journals, in internal reports at LTHT, NHS Blood and Transplant (NHSBT) and at SHU, presented at relevant local and national conferences related to ASCT.
11	**Taking the work forward** If the study obtains positive findings, further research will be carried out if possible. Further funding would be sought to enable this.
12	**Intellectual property** No arrangements have been made as intellectual property issues do not arise with this project.
13	**Costing schedule** Year 1 consumables = £5,500 Year 2 consumables = £5,500

	Total costs for 24 months = £11,000
14	**Funding arrangements** An education support committee application within NHSBT was successful which resulted in NHSBT funding 75% course fees and 100% consumables expenses. Learning Beyond Registration have funded 100% course fees between Autumn 2013 – Summer 2017 (15 month suspension excepted).
15	**References** AMODIO G and GREGORI S. (2012) Human Tolerogenic DC-10: Perspectives for Clinical Applications. *Transplantation Research* **1** 1-10 APPELBAUM F.R, FORMAN S.J, NEGRIN R.S, BLUME K.G. (2004) Thomas' Hematopoietic Cell Transplantation *Wiley-Blackwell* Oxford APPERLEY Jane, CARRERAS Enric, GLUCKMAN Eliane, MASSZI Tamas (eds.) (2012) *The EBMT handbook* 6th ed. CHUGAI Sanofi-Aventis. Last accessed 24 May 2014 at: http://ebmtonline.forumservice.net/ EZZELARAB M and THOMSON AW. (2011) Tolerogenic Dendritic cells and their role in Transplantation. *Seminars in Immunology* **23** 252-263 GAGLIANI N, MAGNANI CF, HUBER S, GIANOLINI ME, PALA M, LICONA-LIMON P, GUO B, HERBERT DBR, BULFONE A, TRENTINI F, SERIO CD, BACCHETTA R, ANDREANI M, BROCKMANN L, GREGORI S, FLAVELL RA and RONCAROLO MG. (2013) Co-expression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nature Medicine* **19** 739-746 GREGORI S, TOMASONI D, PACCIANI V, SCIRPOLI M, BATTAGLIA M, MAGNANI C-F, HAUBEN E and RONCAROLO M-G. (2010) Differentiation of Type 1 T Regulatory Cells (Tr1) by Tolerogenic DC-10 requires the IL-10-dependant ILT4/HLA-G Pathway. *Blood* **116** 935-944 HOVÁRTH R, BUDINSKÝ V, KAYSEROVÁ J, KALINA T, FORMÁNKOVÁ R, STARÝ J, BARTŮŇKOVÁ J, SEDLÁČEK P and ŠPIŠEK R. (2008) Kinetics of Dendritic Cells Reconstitution and Co-stimulatory Molecules Expression after Myeloablative Allogeneic Haematopoetic Stem Cell Transplantation: Implications for the Development of Acute Graft versus Host Disease. *Clinical Immunology* **131** 60-69 RAJASEKAR R, LAKSHMI KM, GEORGE B, VISWABANDYA A, THIRUGNANAM R, ABRAHAM A, CHANDY M, SRIVASTAVA A and MATHEWS V. (2010) Dendritic Cell Count in the Graft Predicts Relapse in Patients with Hematologic Malignancies Undergoing an HLA-matched Related Allogeneic Peripheral Blood Transplant. *Biology of Blood and Marrow Transplantation* **16** 854-860 ROGERS NM, ISENBERG JS and THOMPSON AW. (2013) Plasmacytoid Dendritic cells: No longer an Enigma and Now Key to Transplant Tolerance? *American Journal of Transplantation* **13** 1125-1133 VOLCHENKOV R, KARLSEN M, JONSSON R and APPEL S (2013) Type 1 Regulatory T Cells and Regulatory B Cells Induced by Tolerogenic Dendritic Cells. *Scandinavian Journal of Immunology* **77** 246-254

16	**Abstract** Graft versus host disease (GvHD) is a complication of allogeneic haematopoietic transplantation, which can result in significant patient morbidity and mortality. Acute GvHD occurs in approximately 40% of all recipients of allogeneic haematopoietic transplantation, and can affect the skin, liver and gastrointestinal tract. Studies have suggested that plasmacytoid dendritic cells (pDC) can have a tolerogenic function in adaptive immunity; that dendritic cell-10 cells (DC-10) are potent inducers of antigen specific IL-10 producing type 1 (Tr1) T regulatory cells; and that Tr1 cells can have a pivotal role in maintaining tolerance. The study will measure the quantities of pDC, DC-10 and Tr1 in the grafts and in the transplant recipients at 2, 4, 8 weeks, 3 and 6 months post allograft. The study will analyse for associations between pDC, DC-10 and Tr1 cell numbers in the graft and/or in the recipient during immune reconstitution and GvHD, engraftment, disease-free survival, progression free survival, relapse and overall survival.
17	**Curriculum Vitae** [redacted] **Profile** I am a state registered Biomedical Scientist. I have experience in flow cytometry, clean room processing, cryostorage and product issue. I also have experience in organising laboratory workload, internal and external audit and collaborating with translational research partners. **Work History** <u>National Blood Service/NHS Blood and Transplant. 2006 – current. MLSO3 (Stem Cell and Immunotherapy Lab Manager)/BMS Advanced Specialist.</u> I have built good working relationships with colleagues and service users. I have organised the lab's workload during times that have occasionally been challenging. I have trained staff in new and updated procedures. I actively assisted in the introduction of an on call rota. I am responsible for the lab's invoicing, and I have made improvements in the system that was previously in place. For a 1 year period I had additional responsibilities as I covered a vacant lab director position. I have been involved in the introduction of LEAN methodology in the lab which has resulted in significant time and money savings. I have been actively involved in 5 HTA inspections and 3 JACIE inspections. I have presented the lab's work internally, locally and nationally. I was a member of the Leeds Blood Centre lunchtime lecture committee. I was a contributing member of the Leeds Blood Centre journal club. I have been a member of translational research teams. Our work has ranged from local phase 0/I studies to international phase III studies. Our collaborations with local groups have resulted in two publications. <u>National Blood Service 2002-2006. Stem Cell and Immunotherapy Lab. MLSO 2.</u> I transformed the lab's quality standards. I obtained flow cytometry testing skills such as $CD34^+$, $CD3^+$, $CD4^+$, $CD8^+$ and $CD133^+$ testing. I obtained processing skills such as bone marrow processing, $CD34^+$ selections, $CD8^+$ depletions, donor lymphocyte dosing and processing. <u>National Blood Service 1998-2002. Processing Lab. MLSO 2.</u> I obtained processing skills to produce a number of blood products including cryopreserved peripheral blood stem cells. I had a supervisory role and could be responsible for up to 12 people. In 1999 as a result of vCJD the lab underwent a major reconfiguration to enable 100% leucodepletion of blood products, and I was a member of the project team.

	Qualifications Sheffield Hallam University. 2012 – current. Doctorate in Professional Studies (Biomedical Science). Ongoing. University of Leeds. 1996-1997. Medical Mycology. MSc. Pass (68%) The University of Birmingham. 1993-1996. Biological Sciences (Microbiology). BSc. 2(ii) New College (Pontefract). 1991-1993. Biology, Chemistry, Mathematics. A Level. CCE. New College (Pontefract). 1991-1992. Biology, Chemistry, Mathematics. A/S Level. BDC. **Publications** CUTHBERT RJ, GIANNOUDIS PV, WANG XN, NICHOLSON L, **PAWSON D**, LUBENKO A, TAN HB, DICKINSON A, McGONAGLE D and JONES E (2015). Examining the Feasibility of Clinical Grade CD271^{+} Enrichment of Mesenchymal Stromal Cells for Bone Regeneration [online]. *PLoS ONE* **10** Article from PLoS ONE last accessed 07 May 2017 at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0117855 WEST E, MORGAN R, SCOTT K, MERRICK A, LUBENKO A, **PAWSON D**, SELBY P, HATFIELD P, PRESTWICH R, FRASER S, EVES D, ANTHONEY A, TWELVES C, BEIRNE D, PATEL P, O'DONNELL D, WATT S, WALLER M, DIETZ A, ROBINSON P and MELCHER A (2009). Clinical Grade OK432 Activated Dendritic Cells *In Vitro* Characterisation and Tracking During Intralymphatic Delivery. *Journal of Immunotherapy* 32 66-78 **Courses** Lean Enterprise Research Centre. 2013. Simpler Bronze Certification – LCS Fundamental Level 1c. Human Tissue Authority. 2009. E-learning package for Designated Individuals. Royal Microscopical Society. 2003. Flow Cytometry Course. NEBS Management. 2001. Introductory Certificate in Management. **State Registration number** [redacted] **Interests and hobbies** I am a keen amateur astronomer. I enjoy walking, restaurants and the theatre. I am interested in various sports, including cricket, golf and football. [redacted]
18	**Statistical analysis** The study is a pilot study. The obtained data will be analysed using SPSS. A *P*-value < 0.05 will be considered statistically significant. Graft dose ranges for each tolerogenic cell type (plasmacytoid dendritic cells [pDC], dendritic cell - 10 cells [DC-10] and type 1 regulatory T cells [Tr1 cells]) will be calculated. The graft dose range for each cell type will be split into groups, and these groups' outcomes will be compared. The recipients peripheral blood tolerogenic cell count range at the 5 post-transplant time points (2, 4, 8 weeks, 3 and 6 months) will be calculated and each will be split into groups and these groups' outcomes will be compared. Patients that are diagnosed with acute graft versus host disease (GvHD) will have their tolerogenic graft/peripheral blood cell counts compared to the counts in the patients who are not. The outcomes of interest are

	clinically significant / insignificant GvHD, engraftment, disease free survival, progression free survival, relapse and overall survival. The known risk factors for GvHD are: HLA compatibility, sex match/mismatch, alloimmunisation, stem cell source, age of recipient, conditioning regimen and any treatment for prevention of GvHD. This data will be collected and incorporated into the analysis. The Kolmogorov-Smirnov test will be used to determine if the engraftment data is normally distributed. This will determine whether the independent t-test/ANOVA (2/3+ groups) or the Mann-Whitney/Kruskal-Wallis (2/3+ groups) test is used to compare the groups. The Chi-square test will be used for categorical variables. The probability of developing acute GvHD will be calculated using the cumulative incidence. Probabilities of overall survival and progression free survival will be calculated using the Kaplan-Meier product limit estimates. The association of time to death for each of the tolerogenic cell types and other relevant variables will be calculated using Cox's regression model.

Appendix II Patient information sheet

NHS
Blood and Transplant

[redacted]

INFORMATION SHEET FOR PATIENTS

Study title: The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation.

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done, and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

This form should help you understand the possible risks and potential benefits of taking part, and seek your authorisation for the use, and disclosure, to researchers of your medical information, in connection with the study. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?

Patients undergoing allogeneic stem cell transplantation can suffer from a condition called graft versus host disease which is when donor cells attack host tissue(s). There is increasing evidence that a certain type of white blood cell - called dendritic cells - play an important role in the induction and maintenance of tolerance, preventing graft versus host disease. Another type of white blood cell - Type 1 T regulatory cells - have also been found to play an important role in promoting and maintaining tolerance between the donor cells and host. We will count the numbers of these tolerogenic cells in your blood following your transplant to discover if there is a link between the numbers, ratios and speed of production of these cells and any graft versus host disease that may develop. This research is being carried out to fulfil an educational qualification.

Why have I been asked to participate?

For the study we need patients who are undergoing allogeneic haematopoietic transplantation to volunteer. We can then examine the link between numbers of dendritic cells and type 1 T regulatory cells in the blood and graft versus host disease.

Do I have to take part in the study?

No, you are free to choose whether or not you wish to participate in the study. If you decide to take part, you will be given a consent form. You are free to withdraw from the study at any time without giving a reason. A decision not to take part, or to withdraw from the study, will not affect your medical care in any way.

What will be involved if I agree to take part in the study?

As part of your post allogeneic transplant care, peripheral blood samples will be taken at the following time points: 2, 4, 8 weeks, 3 and 6 months. This study will use these samples to determine the numbers of cells present. Since the study is using blood taken at routine post-transplant time points, it does not require extra blood to be taken or an increase in the number of times blood is taken. The blood samples will the sent to the laboratory at National Health Service Blood and Transplant in Leeds for analysis. You and your GP will not receive the results of any analysis, as this research will not provide any additional information relevant to your care.

Will my medical treatment be affected if I participate in the study?

No, your treatment is not altered by participation in the study.

Will there be any possible harmful effects from participating in the study?

We do not consider that there are any significant harmful effects likely to arise from volunteering to participate in the study, as the blood samples needed are taken as part of your routine post-transplant care. The physical risks of having blood taken may include brief discomfort, slight bruising, and very rarely nerve damage or infection where the needle was inserted. We use strict safety measures to prevent infection.

Are there any benefits to taking part in the study?

You will not receive any payment or direct benefits for participating in the study, but the results of this research may be useful to the medical and research communities striving to improve patient treatment and combat disease in allogeneic transplant patients.

What other information will be collected in the study?

We will collect some basic information from your medical notes including: the diagnosis of your condition, your age, whether you and your donor are a sex match/mismatch, tissue type match data, your conditioning regimen, your engraftment data and data on any graft versus host disease that may develop. We will also record information obtained during hospital follow up visits including, for example, your chimerism results.

Will the information obtained in the study be confidential?

All medical records and information recorded in this research study will be treated in the strictest confidence. Patients will not be named in any reports arising from the study.

What will happen to the results of the study?

It is hoped that the results from this research will be published in scientific journals. Individuals will not be able to be identified from the details in the published reports as the data will be anonymised. You will be able to get a copy of any published results if you so wish.

Will anyone else be told about my participation in the study?

No.

What if I change my mind about taking part?

You are free to withdraw from the study at any time without giving a reason for doing so. Your treatment will not be affected by any decision to withdraw, or not to take part.

What if I am harmed by participation in the study?

If you are harmed by your participation in the study, there are no special compensation arrangements, other than those which would apply if there was any negligence during standard medical care.

What if I have a complaint about the way the study was conducted?

If you have any cause to complain about your treatment whilst taking part in this research study, the normal complaints mechanisms available to anyone receiving care in the National Health Service apply, and you are not compromised in any way because you have taken part in a clinical research study.

Please feel free to speak with any member of staff regarding your concerns. Staff are happy to listen to all concerns and will respond to them quickly and efficiently. If you would prefer to speak with someone outside of the department you are concerned with you can contact the Patient Advice and Liaison Service (PALS) on[redacted]- available during normal working hours only. Outside of normal working hours PALS can be contacted on[redacted]- please leave a voicemail.

Who is funding the study?

This study is being funded by National Health Service Blood and Transplant.

Who can I contact for further information?

If you have any questions about this study, please contact[redacted]at National Health Service Blood and Transplant in Leeds on[redacted]

Appendix III Patient consent form

NHS
Blood and Transplant

[redacted]

Patient research consent form	
Title of project: The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation.	
The patient should complete the whole of this sheet himself/herself. If you agree with the sentence below, please initial the box:	Volunteer initials
I have read the patient information sheet?	
I have had an opportunity to ask questions and discuss this study?	
I have received satisfactory answers to all of my questions?	
I have received enough information about the study?	
I understand I am free to withdraw from the study: • At any time • Without having to give a reason for withdrawing • And without affecting my future medical care	
I agree that samples taken at the following time points post-transplant (2, 4, 8 weeks, 3 and 6 months) as part of my routine post-transplant care can be tested for the presence of tolerogenic cells.	
I understand that relevant sections of my medical notes and data collected during the study may be looked at by the chief investigator, where it is relevant to my taking part in this research. I give permission for this individual to have access to my records.	
I agree to take part in the study?	
Signed……Date…… Name (Block Capitals)……	

Name of physician taking consent……Date……

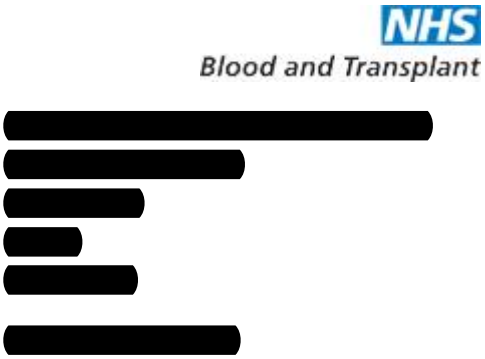
Name (Block Capitals)……

I confirm that I have received the above consent after I have explained the details of the study to the healthy donor as described in the healthy donor information leaflet. The healthy donor has confirmed that all explanations have been understood by him/her.

N.B. The donor must date his/her own signature.

One original signed/dated copy of the healthy donor information leaflet/consent form to be given to the donor and one original signed/dated copy to be retained in the investigator site file.

Appendix IV Donor information sheet



INFORMATION SHEET FOR PERIPHERAL BLOOD STEM CELL DONORS

Study title: The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation.

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done, and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

This form should help you understand the possible risks and potential benefits of taking part, and seek your authorisation for the use, and disclosure, to researchers of your medical information, in connection with the study. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?

Patients undergoing allogeneic transplantation can suffer from a condition known as graft versus host disease. There is increasing evidence that a certain type of white blood cell – called dendritic cells - play an important role in the induction and maintenance of tolerance, preventing graft versus host disease. Another type of white blood cell - type 1 T regulatory cells - have also been found to have an important role in promoting and maintaining tolerance. We will count the numbers of these tolerogenic cells in your peripheral blood stem cell donation(s) to discover if there is a link between the numbers and/or ratios of these cells and any graft versus host disease that may develop in the recipient following transplantation.

Why have I been asked to participate?

For the study we need donors of peripheral blood stem cells for transplantation to volunteer. To examine the link between dendritic cells and type 1 T regulatory cells and graft versus host disease we require donations that we can analyse before they are used for allogeneic transplantation.

Version 2

Version date 13/10/17 IRAS project ID 226012

Do I have to take part in the study?

No, you are free to choose whether or not you wish to participate in the study. If you decide to take part, you will be given a consent form. You are free to withdraw from the study at any time without giving a reason. A decision not to take part, or to withdraw from the study, will not affect your medical care in any way.

What will be involved if I agree to take part in the study?

A sample is routinely taken from peripheral blood stem cell donation(s) to count the number of stem cells present. This sample will also be used to also determine the number of specific white blood cells present in the donation(s). The study will use a sample of the donation that is taken anyway, so the study does not require any extra blood to be taken from the donation. The sample(s) will be tested at the Stem Cell and Immunotherapy laboratory at National Health Service Blood and Transplant in Leeds. You and your GP will not receive the results of any analysis, as this research will not provide any additional information relevant to your care.

Will my medical treatment be affected if I participate in the study?

No, your treatment is not altered by participation in the study.

Will there be any possible harmful effects from participating in the study?

We do not consider that there are any significant harmful effects likely to arise from volunteering to participate in the study, as the sample(s) needed are taken from your donation(s). The physical risks of donating peripheral blood stem cells will have been explained to you by a doctor, there are no additional risks to you by participating in the study.

Are there any benefits to taking part in the study?

You will not receive any payment or direct benefits for participating in the study, but the results of this research may be useful to the medical and research communities striving to improve patient treatment and combat disease in allogeneic transplant patients.

What other information will be collected in the study?

We will collect some basic information from your medical notes including: your age, whether you and the transplant patient are a sex match/mismatch and the tissue type match data.

Will the information obtained in the study be confidential?

All medical records and information recorded in this research study will be treated in the strictest confidence. Donors will not be named in any reports arising from the study.

What will happen to the results of the study?

It is hoped that the results from this research will be published in scientific journals. Individuals will not be able to be identified from the details in the published reports as the data will be anonymised. You will be able to get a copy of any published results if you so wish.

Will anyone else be told about my participation in the study?

No.

What if I change my mind about taking part?

You are free to withdraw from the study at any time without giving a reason for doing so. Your treatment will not be affected by any decision to withdraw, or not to take part.

What if I am harmed by participation in the study?

If you are harmed by your participation in the study, there are no special compensation arrangements, other than those which would apply if there was any negligence during standard medical care.

What if I have a complaint about the way the study was conducted?

If you have any cause to complain about your treatment whilst taking part in this research study, the normal complaints mechanisms available to anyone receiving care in the National Health Service apply, and you are not compromised in any way because you have taken part in a clinical research study.

Please feel free to speak with any member of staff regarding your concerns. Staff are happy to listen to all concerns and will respond to them quickly and efficiently. If you would prefer to speak with someone outside of the department you are concerned with you can contact the Patient Advice and Liaison Service (PALS) on [redacted] - available during normal working hours only. Outside of normal working hours PALS can be contacted on [redacted] - please leave a voicemail.

Who is funding the study?

This study is being funded by National Health Service Blood and Transplant.

Who can I contact for further information?

If you have any questions about this study, please contact [redacted] at National Health Service Blood and Transplant in Leeds on [redacted]

Appendix V Donor consent form

NHS
Blood and Transplant

[redacted]

Healthy donor research consent form	
Title of project: The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation.	
The donor should complete the whole of this sheet himself/herself. If you agree with the sentence below, please initial the box:	Volunteer initials
I have read the healthy volunteer information sheet?	
I have had an opportunity to ask questions and discuss this study?	
I have received satisfactory answers to all of my questions?	
I have received enough information about the study?	
I understand I am free to withdraw from the study: • At any time • Without having to give a reason for withdrawing • And without affecting my future medical care	
I agree that a sample from the peripheral blood stem cell harvest(s) I donate can be tested for the presence of dendritic cells and type 1 T regulatory cells	
I agree to take part in the study?	
Signed……Date…… Name (Block Capitals)……	

Name of physician taking consent……Date……

Name (Block Capitals)……

I confirm that I have received the above consent after I have explained the details of the study to the healthy donor as described in the healthy donor information leaflet. The healthy donor has confirmed that all explanations have been understood by him/her.

N.B. The donor must date his/her own signature.

One original signed/dated copy of the healthy donor information leaflet/consent form to be given to the donor and one original signed/dated copy to be retained in the investigator site file.

Appendix VI NHS Research Ethics Committee approval letter (Front page only)

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

Gwasanaeth Moeseg Ymchwil
Research Ethics Service

Ariennir gan Lywodraeth Cymru
Funded by Welsh Government

Wales REC 7
Building 1
Jobswell Road
St David's Park
SA31 3HB

Telephone : 01267 225045
E-mail : sue.byng@wales.nhs.uk
Website : www.hra.nhs.uk

Please note: This is an acknowledgement letter from the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

[redacted]

16 October 2017

Dear Mr Pawson

AMENDED

Study title:	**An investigation into tolerogenic cells and graft versus host disease in allogeneic haematopoietic stem cell transplantation.**
REC reference:	**17/WA/0288**
Protocol number:	**1**
IRAS project ID:	**226012**

Thank you for your email of 13 October 2017. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 13 September 2017

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Other [Email re minor changes]		13 October 2017
Participant consent form [Donor]	2	13 October 2017
Participant consent form	2	13 October 2017
Participant information sheet (PIS) [Donor]	2	13 October 2017
Participant information sheet (PIS)	3	13 October 2017

Approved documents

The final list of approved documentation for the study is therefore as follows:

Appendix VII HRA approval letter (Front page only)

NHS
Health Research Authority

[redacted]

Email: hra.approval@nhs.net

16 October 2017

[redacted]

Letter of HRA Approval

Study title:	**An investigation into tolerogenic cells and graft versus host disease in allogeneic haematopoietic stem cell transplantation.**
IRAS project ID:	**226012**
Protocol number:	**1**
REC reference:	**17/WA/0288**
Sponsor	**Sheffield Hallam University**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Appendix VIII NHSBT approval

From: R&D Office <research.office@nhsbt.nhs.uk>
Sent: 08 February 2021 09:39
To: [redacted] R&D Office <research.office@nhsbt.nhs.uk>
Subject: RE: NHSBT R&D Approval: Ref MSc-17-03

[redacted]

We usually issue an R&D approval letter now, so I thought I would find one of those when I was doing some digging, but I can't seem to find one so it must not have been needed back then. I can see in the way of approval from us that the Statement of Activities and Schedule of Events were signed on 22/09/2017 and the collaboration agreement that you've mentioned.

Best wishes,

Lucy

Lucy Roberts O-
Research Governance Manager
NHS Blood and Transplant
Mob 07471147961
Twitter @nhsbt_rd
Visit www.nhsbt.nhs.uk/research-and-development/

Appendix IX SHU ethics approval letter

Sheffield Hallam University

Registry Services
City Campus Howard Street
Sheffield S1 1WB

GT/RDC/DPS1 RESUB

Tel no: 0114 225 2045/ 4047/ 4053

E-mail: rdcadmin@shu.ac.uk

15 September 2017

[redacted]

Application for Approval of Research Project and Supervisory Team

The University Research Degrees Committee/ Chair of the RDC has noted receipt of the information requested, and I am pleased to inform you that it was satisfactory. Your application is now fully approved.

The next formal stage for you will be the approval of your Doctoral Project Report title and examining team. These details should be proposed on an RF3 by your Director of Studies, and submitted to rdcadmin@shu.ac.uk at least 4 months in advance of submission of your Doctoral Project Report. In your case we would expect to receive an RF3 by no later than 23 July 2020. Your registration details are also attached.

Please ensure that your final thesis conforms to the University's thesis layout and binding requirements. Details can be found on the Student Blackboard site.

If you have any queries, please contact Student Systems and Records (Research Degrees) based in Oneleven Building at City Campus, using the contact details above.

Yours sincerely

[redacted]

Secretary
University Research Degrees Committee

Director of Studies: Professor Margot Woodroofe

Enc

Appendix X LTHT approval

[redacted]

Subject: R&I No: HM18/107461 | Tolerogenic cells in allogeneic haematopoietic transplantation | LTHT Confirmation of Capacity and Capability

[redacted]

Re. Tolerogenic cells in allogeneic haematopoietic transplantation, R&I No: HM18/107461

This email confirms that the Leeds Teaching Hospitals NHS Trust has the capacity and capability to deliver the above research study, based upon Protocol version 1.0 (01/08/2017). You may now begin the study at this organisation.

Please find attached:

- signed agreement
- agreed statement of activities
- agreed schedule of events

It is the responsibility of the principal investigator to ensure that the study is conducted in accordance with the terms of the Health Research Authority approval and Leeds Teaching Hospitals NHS Trust policies and procedures including the requirements for research governance and clinical trials performance management. These are available at [redacted]

New requirement

Please note: If your study will involve the testing or use of an interventional procedure which is new to LTHT you must obtain the approval of the New Interventional Procedures Group (NIPG). Details and application form are available fr[redacted]ur study will involve an interventional procedure which is new to you as an individual (but not to LTHT) you must ensure you have agreement from your [redacted]

If you have any queries please do not hesitate to contac[redacted]

Appendix XI AN approval

From: [redacted]
Sent: [redacted]
Subject: RE: Research request APPROVED AN ID - RESDON062 Tolerogenic Cells and GvHD

[redacted]

I am pleased to let you know that your study (Anthony Nolan ID RESDON062) was approved at our Medical Affairs meeting today for Anthony Nolan (AN) donors. We will need to forward the study documents to BBMR and DKMSUK (DBC) letting them know we have approved for our donors and ask whether they have any objection to their donors being approached.
Donors will be consented at the time of their medical by their Consultant at the collection centre they will be donating at therefore we need to inform the CCs and gain their support in consenting the donor (we do not consent donors to studies here at AN).
WRT the donor information and consent forms, we will need to make some changes such as removing NHSBT letter heading, references to Leeds laboratory and contact details for further information. I will have these done by tomorrow and send to you for approval before forwarding to our CCs and Aligned Registry partners (BBMR and DKMSUK).
WRT alloimmunisation data; I raised this question during the meeting and was told that we do ask all donors if they have had any blood transfusions and pregnancy so we would have limited information.
Also David because we will be sharing donor confidential data with you we need to sign the attached confidentiality agreement.
Please let me know if you have any questions.

[redacted]

Appendix XII Material transfer agreement (Front page and signature pages only)

NHS
Blood and Transplant

COLLABORATON AGREEMENT

For an investigation into tolerogenic cells and graft versus host disease in allogeneic haematopoietic stem cell transplantation

AGREED by the Parties through their authorised signatories:

For: **NHS Blood and Transplant**

By: [redacted]

Full Name: [redacted]

Position: [redacted]

Date: [redacted]

For: **The Leeds Teaching Hospitals NHS Trust**

By: [redacted]

Full Name: [redacted]

Position: [redacted]

Date: [redacted]

For: **Sheffield Hallam University**

By: [redacted]

Full Name: [redacted]

Position: [redacted]

Date: [redacted]

For: **The Anthony Nolan Trust**

By: [redacted]

Full Name: [redacted]

Position: [redacted]

Date: [redacted]

Read and Acknowledged by Principal Investigator - I acknowledge receipt of a copy of this Agreement and confirm that I will abide by its terms insofar as those terms are applicable to me.

By: [redacted]

Full Name: [redacted]

Position: [redacted]

Date: [redacted]

Read and Acknowledged by Principal Investigator - I acknowledge receipt of a copy of this Agreement and confirm that I will abide by its terms insofar as those terms are applicable to me.

By: [redacted]

Full Name: [redacted]

Position: [redacted]

Date: [redacted]

Appendix XIII GDPR Transparency Statement

Privacy notice for research participants

On 25th May 2018 the General Data Protection Regulation (GDPR) replaced the Data Protection Act; and it governs the way that organisations use personal data. Personal data is information relating to an identifiable individual.

Transparency is a key element of the GDPR and this privacy notice is to inform you:

- What your rights are under GDPR
- How and why the study uses your personal data
- How to contact study personnel if you have questions or concerns about the use of your personal data

Your rights under Data Protection

The GDPR gives you the following rights:

The right to be informed
The right of access
The right to rectification
The right to erase
The right to restrict processing
The right to data portability
The right to object
Rights in relation to automated decision making and profiling.

Please note that many of these rights do not apply when the data is being used for research purposes, but we will always try to respond to concerns or queries that you may have.

Why is the study processing your personal data?

Data Protection laws allow researchers to use personal data for research with appropriate safeguards in place under the legal basis of research in the public interest.

The information sheets lists the basic information the study requires, and your consent for this can be recorded on the consent form.

Collecting and Using Personal Data

The study only collects information that is essential for its purpose. The research data is pseudonymised so that your privacy is protected.

Who do we share your data with?

Your pseudonymised data may be shared with research supervisors and/or research auditors. To communicate the research to the public and the academic community your fully anonymised data is likely to form part of a research publication or conference presentation or public talk.

Storage and Security

[redacted] takes a robust approach to protecting the information it holds with dedicated storage areas for research data with controlled access. Alongside these technical measures there are comprehensive and effective policies and processes in place to ensure that users and administrators of [redacted] information are aware of their obligations and responsibilities for the data they have access to.

Retention

Your information will not be kept for longer than is necessary and will be kept in a pseudonymised format. The length of time for which we keep your data will depend on a number of factors including the importance of the data, the funding requirements, the nature of the study, and the requirements of the publisher.

Contact information

You should contact the Data Protection Officer if:

- You have a query about how your data is used by NHSBT/the University
- You would like to report a data security breach (e.g. if you think your personal data has been lost or disclosed inappropriately).
- You would like to complain about how the NHSBT/the University has used your personal data [redacted]Postal address[redacted]

You should contact the Head of Research Ethics SHU [redacted]if:

- You have concerns with how the research was undertaken or how you were[redacted]

Further Information and Support

The Information Commissioner is the regulator for GDPR and you have the right to raise concerns with the Commissioner. The Information Commissioner's Office (ICO) has a website with information and guidance for members of the[redacted]

The Information Commissioner's Office operates a telephone helpline, live chat facility and email enquiry service. You can also report concerns online. For more information please see the Contact Us page of their[redacted]

Appendix XIV Post-transplant worksheet

NHS
Blood and Transplant

[redacted]

Post Transplant Worksheet

Title of project: The Role of Tolerogenic Cells in Allogeneic Haematopoietic Stem Cell Transplantation.	
Patient details SURNAME FORENAME DATE OF BIRTH NHS NUMBER GENDER	
ALLOGRAFT DATE	
Date and days to neutrophils (>0.5 x 10^9/L)	
Date and days to platelets (>20 x 10^9/L)	
Acute GvHD? (Date diagnosed? Site? Glucksberg classification? Treatment given? Start date of treatment?)	
Chimerism (2, 4, 8 weeks, 3 and 6 months?)	
Disease free survival (Date, if applicable)	
Relapse (Date, if applicable)	
Progression free survival (Date, if applicable)	
Chronic GvHD? (Date diagnosed? Site? National Institutes of Health consensus conference classification? Treatment given? Start date of treatment?)	
Overall survival (Date, if applicable)	

Cause of death (If applicable)	