Regulation of human graft versus host disease by innate lymphoid cells

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Abstract

Allogeneic haematopoietic stem cell transplant (HSCT) remains the only curative therapy for many malignant and non-malignant diseases. Its use, however, remains limited by the morbidity and mortality caused by graft versus host disease (GVHD). Treatment options, even where successful, often further immunosuppress the recipient and potentially reduce the effectiveness of the transplant.

IL-22, a member of the IL-10 family of cytokines, is an exciting potential therapy. Its receptor is found on the key target tissues of graft versus host disease, but not on leucocytes, thereby potentially separating GVHD from the graft versus tumour effect. The role of IL-22 in GVHD, however, remains controversial. Innate lymphoid cells (ILCs), found at many of the body's barrier surfaces, have been shown to be key producers of IL-22, but knowledge of their function in human GVHD is limited.

This project has further explored the role of IL-22 and ILCs in human stem cell transplantation. ILCs were depleted from the peripheral blood by transplant conditioning, and were predominantly of donor origin by Day 28. No difference was demonstrated in ILC recovery between patients who did and did not develop acute GVHD. No evidence was found of IL-22 induction by conditioning therapy, either full or reduced intensity, in the serum or skin, but serum IL-22 concentration was increased in GVHD. In addition, an IL-22 polymorphism study found a greater risk of death from GVHD where the donor had a 'high IL-22 producer' genotype. Finally rIL-22 was tested in a skin explant model of GVHD and supraphysiological concentrations of IL-22 reduced the GVHD Grade in 50% of experiments performed.

This project has further elucidated the role of ILCs and IL-22 in human GVHD and supports the potential for a therapeutic role for rIL-22 in this context.

Declaration

The material contained in this thesis is entirely the work of the author, except where otherwise stated, and has not previously been submitted for a degree to this or any other university.

Amy Publicover

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Dedication

‘What do you regard as the main principle – the, shall I say, the basic idea which you keep before you when you are exercising the practice of your profession?’

There was a pause while Andrew reflected desperately. At length, feeling he was spoiling all the good effect he had created, he blurted out:

‘I suppose – I suppose I keep telling myself never to take anything for granted.’

A.J. Cronin, The Citadel

This work is dedicated to Kim Orchard and Debbie Richardson, whose enthusiasm, support and encouragement inspired my love of transplantation.

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Glossary

ATG	Anti-thymocyte globulin
APC	Antigen presenting cell
BMT	Bone marrow transplant
CD	Cluster of differentiation
CILP	Common innate lymphoid cell precursor
CLP	Common lymphoid precursor
DC	Dendritic cell
DSS	Dextran sulphate sodium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FLAMSA	Fludarabine, amsacrine and cytarabine
GPI	Glycosylphosphatidylinositol
GVHD aGVHD cGVHD	Graft versus host disease (aGVHD, acute Graft versus host disease) Acute graft versus host disease Chronic graft versus host disease
GVL/GVT	Graft versus leukaemia/Graft versus tumour
HLA	Human leucocyte antigen
HSCT	Haematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
ID2	Inhibitor of DNA Binding 2
IFN	Interferon
IgG1	ImmunoglobulinG1
JAK	Janus kinase
IL-	Interleukin-
ILC	Innate lymphoid cells
IBD	Inflammatory bowel disease
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer
MLR	Mixed lymphocyte reaction
NFIL3	Nuclear factor IL-3 induced
NK cells	Natural killer cells
PBSC	Peripheral blood stem cells
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
RIC	Reduced intensity conditioning
RORγt	Retinoic acid-related orphan receptor γt
SCID	Severe combined immunodeficiency
SCT	Stem cell transplant
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TBI	Total body irradiation
Th	T helper
TNF α	Tumour necrosis factor alpha
t-SNE	t-distributed stochastic neighbour embedding
Tyk	Tyrosine kinase
UVB	Ultraviolet B

Chapter 1 Introduction

1.1 Stem cell transplantation

Stem cell transplantation, used primarily for the treatment of haematological disorders, can be performed using cells previously harvested from the patient (autologous) or an alternative donor, either related or unrelated (allogeneic). In the majority of clinical situations where autologous transplantation is performed, the aim is to allow the delivery of high doses of chemotherapy, from which the bone marrow might not be able recover, and then to 'rescue' the bone marrow by returning the previously harvested stem cells (Greb et al., 2008). The exception to this is where autologous transplant is used to treat autoimmune disease, which will be discussed further below.

In allogeneic stem cell transplant, in addition to allowing high dose chemotherapy to be given, the aim is to allow engraftment of a new, healthy immune system, which can be harnessed to fight the disease for which the transplant was performed (Kolb, 2008).

1.1.1 The transplant process

The patient undergoes conditioning with chemotherapy (in some cases with the addition of radiotherapy) with the aim of eradicating disease, followed by stem cell infusion. In allogeneic transplantation, the conditioning will include immunosuppressive agents to avoid rejection of the graft (Gyurkocza and Sandmaier, 2014), and may involve T cell depletion (discussed in 1.2.4). Originally allogeneic transplant conditioning was always performed with full intensity, or myeloablative conditioning. As the importance of the immune response was better understood, however, reduced intensity regimens were developed, that have made transplantation available to a much broader range of patients. Due to the high risk of infection during the transplant, patients often remain in isolation while awaiting engraftment.

1.1.2 Complications

The biggest risk common to allogeneic and autologous transplantation is infection. The type of infections most commonly seen shifts depending on the stage post-transplant (van Burik and Weisdorf, 1999, Leather and Wingard, 2001), with bacterial infections predominating in the early, neutropenic phase, but the risk of viral infections (both primary infection and reactivation) remaining for many months post allogeneic transplant, especially where there is on-going treatment with immunosuppressive therapy.

In allogeneic transplantation, graft versus host disease (GVHD), where the graft mounts a response to its new host, presents a specific challenge. GVHD forms the basis of this project, and is discussed in more detail in section 1.2.

1.1.3 Allogeneic stem cell transplantation

Allogeneic haematopoietic stem cell transplantation (HSCT), pioneered in the 1950s and 1960s, but entering more mainstream clinical practice in the 1970s, remains the only potentially curative therapy for many haematological malignancies and immunodeficiencies. The advent of new techniques for transplant conditioning and donor selection have increased the number of patients for whom allogeneic HSCT is a clinical option, with EBMT (European Society for Blood and Marrow Transplantation) reporting 15 717 allogeneic transplants registered in 2017 (EBMT Annual Report 2017).

1.1.4 Challenges in clinical application

The use of HSCT as a clinical option remains limited by the morbidity and mortality caused by GVHD (Wingard et al., 2011). A variety of alternatives for the prevention of GVHD, including T cell depletion and prophylactic immunosuppression are well established in clinical practice, but successful treatment of established GVHD remains limited, and a complete response to glucocorticoids is achieved in less than 50% of those requiring systemic treatment (Pidala and Anasetti, 2010). In addition, use of glucocorticoids post-transplant results in an increased risk of infection in an already vulnerable patient group (Thursky et al., 2004). An ability to reduce GVHD, while maintaining the graft versus leukaemia/graft versus tumour effect would potentially increase the clinical application of HSCT.

1.2 Graft versus host disease

Graft versus host disease remains a common and significant complication of HSCT. The reported incidence and severity of acute GVHD (aGVHD) vary widely, depending on a number of factors including conditioning intensity, use of T cell depletion, donor matching, stem cell source and prophylaxis, but has been reported to be as high as 80%. The prognosis is especially poor in steroid refractory GVHD (Martin et al., 1990, Weisdorf et al., 1990). The large number of agents trialled as second line therapy reflect the lack of a successful candidate (Pidala and Anasetti, 2010, Saliba et al., 2012). Originally termed ‘runt disease’, ‘wasting disease’ or ‘secondary disease’ (Billingham et al., 1960, van et al., 1959, Barnes et al., 1962) where ‘primary disease’ referred to the radiation-induced aplasia seen in

transplanted mice, it was Billingham who proposed a triad of requirements for the development of GVHD (Billingham, 1966).

- The graft must include cells that are immunologically competent
- There must be antigens present in the recipient that are not present in the donor
- The recipient must be unable to mount a response to reject the transplanted cells

1.2.1 Definitions, staging, grading and management

GVHD has classically been divided into acute (aGVHD) and chronic (cGVHD), where aGVHD occurred within the first 100 days after HSCT. Changes in methods of transplantation, however, have somewhat blurred this distinction, although the clinical findings themselves are often distinctive. For this reason, in 2005 the National Institutes of Health, proposed a new set of definitions (Filipovich et al., 2005):

Classical acute: Occurring within 100 days of HSCT or donor lymphocyte infusion (DLI), only features of acute GVHD
Persistent, recurrent or late-onset acute GVHD: Only features of acute GVHD, but occurring beyond 100 days of HSCT or DLI
Classic chronic: Features of chronic GVHD only
Overlap syndrome: Features of acute or chronic GVHD occurring together

Acute GVHD principally affects three target organs, the skin, liver and gastrointestinal tract. Each organ is staged separately, and the scores combined to give an overall grade. The definitions, using the modified Glucksberg criteria, are shown below (Glucksberg et al., 1974, Przepiorka et al., 1995):

Stage	Skin	Liver (Bilirubin)	GI (Diarrhoea)
1	<25% BSA	34-50umo/L	>500 mL/24hrs
2	25-50% BSA	51-102umol/L	>1000 mL/24hrs
3	Generalised erythroderma	103-255umol/L	>1500 mL/24hrs
4	With bullae formation and desquamation	>255umol/L	Severe abdo pain +/- ileus

Table 1.1 Staging of acute GVHD, according to the modified Glucksberg criteria

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

Table 1.2 Grading of acute GVHD, according to the modified Glucksberg criteria

The overall grade is important for research and transplant registry purposes to assess outcomes following HSCT, but is also used to guide treatment and prognosis in clinical practice. Grade I GVHD can usually be managed with optimisation of immunosuppressive agents and topical therapy, while Grade II or above will usually require systemic corticosteroids. Despite the advent of newer agents, corticosteroids remain first line therapy for GVHD. Complete response rates quoted differ from 20-70%, with worse responses seen in those with higher grades of GVHD. In patients who are steroid refractory, however, the outcome is very poor, with mortality rates of up to 80% (Pidala and Anasetti, 2010, Cesen Mazic et al., 2018, Luft et al., 2011).

Chronic GVHD can affect many organ systems, and the features are often similar to those seen in auto-immune conditions. The underlying pathophysiology is thought be distinct from that of aGVHD, the focus of this project. The manifestations of cGVHD, therefore, are not discussed in detail here.

1.2.2 Pathophysiology

Almost 50 years after Billingham, Ferrara proposed a three-phase model of GVHD development (Ferrara et al., 2009), illustrated in Figure 1.1. In the first phase, damage to the host tissues by transplant conditioning and the underlying disease causes activation of antigen presenting cells, resulting in the production of danger signals. In response to this, in phase two, donor T cells proliferate and differentiate. Phase three is then an effector phase, which results in damage of target tissues.

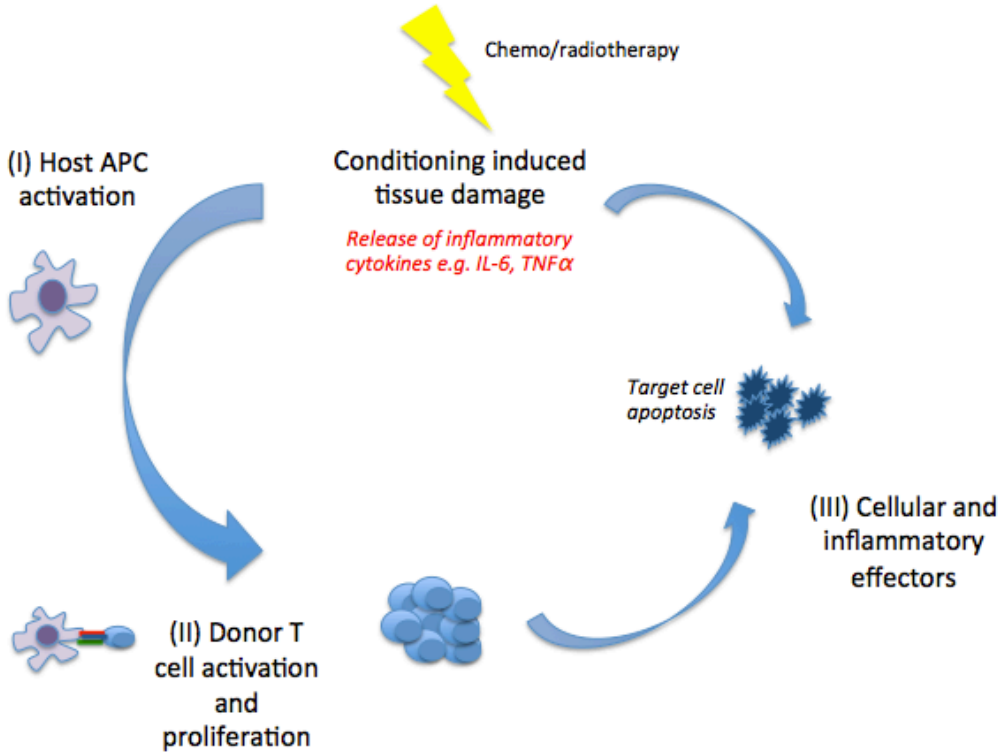

Figure 1.1 The three phase model of GVHD. Transplant conditioning results in tissue damage, with the release of pro-inflammatory cytokines. This leads to activation of host antigen presenting cells, which in turn activate donor T cells. These then produce further effectors, resulting in tissue damage. *Adapted from Ferrara et al., 2009.*

1.2.3 Transplant conditioning and graft versus host disease

Transplant conditioning refers to the chemotherapy, with or without radiotherapy given to the patient prior to stem cell infusion, necessary both to prevent rejection of the graft and, in some cases, for treatment of the underlying disease. Early transplant regimens were all myeloablative, and included total body irradiation (TBI) with high dose chemotherapy (Thomas, 2000). It is classically believed that conditioning results in tissue damage, with the release of inflammatory cytokines including TNFα, IL-1β and IL-6 (Hill et al., 1997, Henden and Hill, 2015). Ferrara's model, where the first phase is the result of conditioning-induced damage gives a rationale as to why full intensity conditioning has been observed to result in more severe GVHD than reduced intensity (Aoudjhane et al., 2005). The pathophysiology underlying aGVHD following reduced intensity conditioning (RIC) is less well understood than following myeloablative conditioning. It is believed, however, to be the result of a similar process, although the timing and key cytokines may be different (Mohty et al., 2005). It is worth noting that the presence of a cytokine storm as a result of conditioning has itself been challenged (Melenhorst et al., 2012).

1.2.4 T cell depletion

The importance of donor T cells in the pathogenesis of GVHD has led to the development of multiple methods, both physical and immunological, of T cell depletion. Although this has been shown to reduce the incidence of acute and chronic GVHD, this comes with an increased risk of relapse and graft rejection (Marmont et al., 1991, Maraninchi et al., 1987). Different mechanisms to overcome this through more selective depletion exist in clinical practice. These include the use of αβ T cell depletion and CD34+ selection (Saad and Lamb, 2017). For the samples analysed in this thesis, where the transplant was performed with T cell depletion, the patients have all received immunological *in vivo* T cell depletion with either Alemtuzumab (Campath-1H) or anti-thymocyte globulin (ATG). These agents are discussed in more detail below.

1.2.5 Alemtuzumab

Alemtuzumab, or Campath-1H is a recombinant, humanised immunoglobulin G1 (IgG1) monoclonal antibody, which targets human cluster of differentiation marker (CD) 52, resulting (*in vitro*) in complement and antibody-dependent cell mediated cytotoxicity (Hu et al., 2009). CD52, a glycosylphosphatidylinositol (GPI) anchored glycoprotein is expressed by most B and T lymphocytes (both healthy and malignant)(Ginaldi et al., 1998), with lower expression being seen in NK (natural killer) cells. Some expression has also been demonstrated on monocytes, basophils and dendritic cells, but, *in vitro* at least, T and B lymphocytes appear the most susceptible to treatment with Alemtuzumab (Rao et al., 2012). CD52 is also expressed in the male reproductive tract, including the epididymis and on the surface of mature spermatozoa (Hale et al., 1993). The function of CD52 remains unclear, but there is some evidence to suggest it acts as a co-stimulatory molecule for T cells (Watanabe et al., 2006), and plays a role in their migration (Masuyama et al., 1999).

The Campath antibodies (including CAMPATH-1M, CAMPATH-1G, and CAMPATH-1H) developed in Cambridge by Geoff Hale and Herman Waldmann (Riechmann et al., 1988, Bruggemann et al., 1987), showed initial clinical success in refractory non-Hodgkin lymphoma (Hale et al., 1988). Alemtuzumab is commonly used as part of the conditioning regimen for *in vivo* T cell depletion in allogeneic HSCT (in the United Kingdom). There is evidence for a reduction in both acute and chronic GVHD when Alemtuzumab is incorporated into the conditioning regimen (Kottaridis et al., 2000, Chakraverty et al., 2002). Not surprisingly, perhaps, this reduction in GVHD is accompanied by an increase in post-transplant infections (Chakrabarti et al., 2002a, Perez-Simon et al., 2002, Chakrabarti et al.,

2002b), slower immune reconstitution (Chakraverty et al., 2010, Morris et al., 2003) and an increase in mixed chimerism (Marsh et al., 2016).

1.2.6 Antithymocyte globulin

Antithymocyte globulin (ATG), purified immunoglobulin G is also commonly used for *in vivo* T cell depletion. The immunoglobulin is extracted from the sera of animals, usually rabbits or horses, which have been immunised using human thymocytes or the Jurkat cell line (similar to activated T cells). Unlike Alemtuzumab, ATG targets multiple antigens and various groups have investigated the likely functional targets (Mohty, 2007, Bonnefoy-Berard et al., 1991, Preville et al., 2001). The mechanism of action is believed to be through both the depletion of lymphocytes and interference with their functional surface molecules. The different products that are available are not identical, and findings from one should not be extrapolated to all.

1.2.7 T regulatory cells

While I have focused on the use of T cell depletion to reduce the incidence of GVHD, it is worth noting that several subsets of T cells exist. In addition to the activated effector cells discussed above, a subset of T cells, known as T regulatory cells, exist to control the immune response. Usually classified as CD4+CD25+Foxp3+, in 1995 it was shown in a murine model that these cells had the ability to transfer tolerance (Sakaguchi et al., 1995). The biology of T regulatory cells is beyond the scope of this thesis, but given their function, it is perhaps not surprising that trials of T regulatory cells for the prevention of GVHD have been performed, with some encouraging preliminary results (Di Ianni et al., 2011, Brunstein et al., 2011).

1.2.8 Graft versus tumour effect

One of the difficulties associated with the prophylaxis and treatment of GVHD is the potential loss of the associated graft versus tumour or graft versus leukaemia (GVT/GVL) effect. This effect, which is also reliant on donor T cells, is one of the key mechanisms by which allogeneic transplant exerts its anti-tumour effect, especially in the setting of reduced intensity conditioning. The increased risk of relapse seen with T cell depletion and the success of donor lymphocyte infusion, especially for conditions such as chronic myeloid leukaemia, have demonstrated the existence of GVL. Separation of GVL from GVHD, however, remains one of the major challenges in allogeneic SCT (Negrin, 2015, Soiffer et al., 2011, Bacigalupo et al., 1997).

1.3 Innate lymphoid cells

Innate lymphoid cells (ILCs) are a population of cells, with lymphoid morphology but without lineage specific markers or rearranged antigen-specific receptors. They exhibit many functional parallels with T helper (Th) cells in relation to cytokine production. This is discussed further below.

1.3.1 Innate versus adaptive immunity

The human immune system employs a number of different mechanisms to protect us against infectious agents. Immunity can be broadly divided into the innate and adaptive immune systems, where the innate immune system is not changed by having previously encountered the organism and does not rely on specific recognition of the pathogen. It therefore provides the initial response, while the adaptive immune system has the ability to 'remember' and so respond more rapidly to a repeat infection (Chaplin, 2006).

1.3.2 Innate immunity

The innate immune system includes physical barriers, such as epithelium, a mucous layer or acidic or bactericidal fluids (such as gastric acid or tears). It is also responsible for the production of cytokines and chemokines to orchestrate a further response. The innate immune system recognises danger signals (e.g. pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS)), through toll-like receptors (TLRs) (Beutler et al., 2003), rather than recognising the pathogen itself and responds through mechanisms including phagocytosis by neutrophils or macrophages. Another key component of innate immunity is complement, which has also been shown to interact with the adaptive immune system (Morgan et al., 2005). Phagocytosis is enhanced by coating of the pathogen with C3b (complement system), which increases the adherence of the pathogen to the phagocytes through their receptors. Activation of the complement system also results in development of the membrane attack complex, which inserts directly into the pathogen leading to cell lysis (Serna et al., 2016). A further cellular component of the innate immune system is the innate lymphoid cell family. The so-called 'helper ILCs' are discussed in more detail below. NK cells, acting through non-specific receptors, recognise and kill virally infected host cells, by secreting perforin and granzyme (Yokoyama, 2005).

1.3.3 Adaptive immunity

In contrast to the innate immune system, adaptive immunity is specific for the antigen, and develops a memory for pathogens previously encountered (Chaplin, 2006). The two major

subsets of cells are B (bone marrow derived) and T (thymus derived) lymphocytes. B cells each produce a unique antibody, which, when it binds its cognate antigen, results in differentiation to plasma cells, which continue producing the unique antibody, and memory B cells (Heesters et al., 2016). The antibody coats the pathogen, activating complement and enhancing phagocytosis. Both pre-existing antibodies and memory B cells, which are able to respond rapidly, form part of the immune response should the host be re-infected by the same pathogen (Kurosaki et al., 2015). An additional function of B cells is to present antigen to T cells through their MHC Class II.

T cells have unique receptors for antigen recognition (the T cell receptor, or TCR). T helper cells (CD4+) recognise antigen on the cell surface of professional antibody presenting cells, such as dendritic cells, in combination with MHC class II. Providing there is appropriate co-stimulation, this results in cytokine production by the T cell, that stimulates B cells to produce antibodies, in addition to other pro-inflammatory cytokines, thus further enhancing the immune response (Chaplin, 2006). Cytotoxic T cells (CD8+), in contrast recognise antigen in combination with MHC class I on infected cells. In both cases, this activation results in clonal proliferation and the production of effector T cells and memory T cells (Dimeloe et al., 2017).

1.3.4 Innate Lymphoid Cells

In the absence of specific antigen receptors, ILCs respond to stress signals and, through the production of cytokines, play a role in orchestrating the immune response. They have been shown to be important in early immune responses and in maintaining epithelial integrity (Spits et al., 2013, Hazenberg and Spits, 2014, McKenzie et al., 2014, Mjosberg and Spits, 2016). The earliest recognised subsets of ILCs were natural killer (NK) cells and lymphoid tissue inducer (LTi cells), first described in mice in 1975 (Kiessling et al., 1975) and 1997 (Mebius et al., 1997) respectively. More recently subsets that produce cytokines in a manner that mirrors the cytokine production profile of T helper subsets have been described. These are sometimes referred to as ‘helper’ ILCs Figure 1.2.

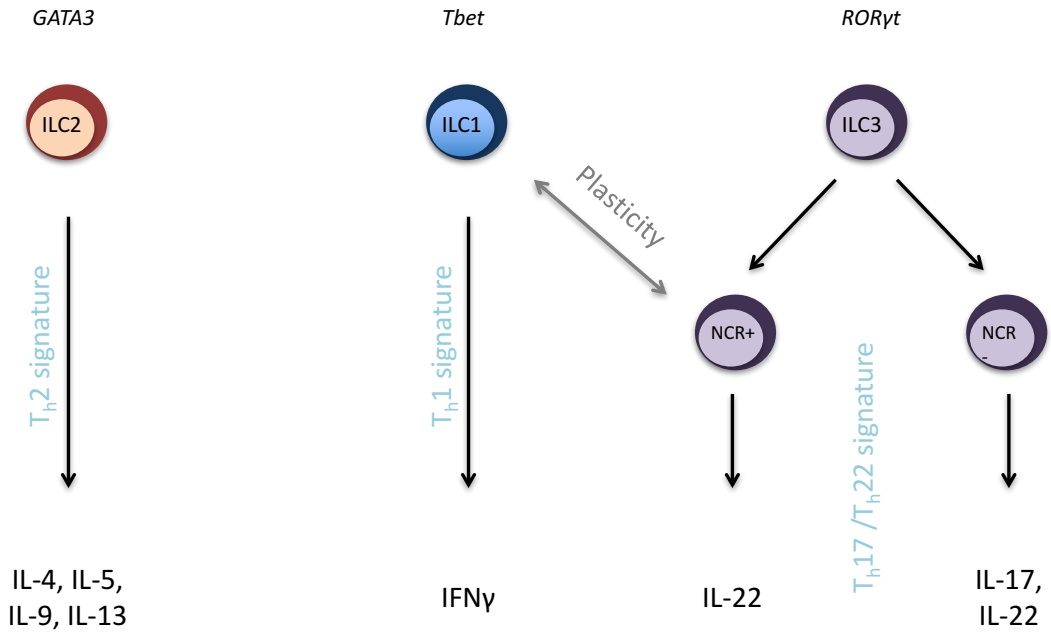

Figure 1.2 Key cytokine production by subsets of 'helper' ILCs. The major transcription factors required for the development of each subset is indicated above the cell type.

In healthy people, approximately 0.01%-0.1% of circulating lymphocytes are ILCs (CD127+) (Hazenberg and Spits, 2014). Tissue distribution is variable, but given the functions described above, it is not surprising that they are commonly found at the barrier surfaces. ILC2s predominate in the skin, where they account for approximately 3% of leucocytes, and are also the dominant ILC population in the lung (excluding NK cells) and the lamina propria of the large intestine. ILC3s predominate in the lamina propria of the small intestine (1-1.5% of leucocytes), but smaller populations are also found the large intestine, lung, liver and skin. ILC1s predominate in the intra epithelial compartment of the small and large (after NK cells) intestine, but small populations are widely distributed (Kim et al., 2016). It has been proposed that a further subset of ILCs, 'ILCregs', which produce IL-10, but do not express *Foxp3* are found in the intestine of both humans and mice (Wang et al., 2017). ILCregs have also been identified in the nasal and respiratory tract, where numbers are increased in chronic inflammation. In this study, up-regulation of retinoic acid resulted in conversion of ILC2s to ILCregs (Morita et al., 2019). It is believed these cells have a regulatory effect on the innate immune response.

1.3.5 ILC nomenclature

When initially described, ILC subsets were given multiple names by different groups. In an attempt to unify the definitions, a proposal for their nomenclature was made in 2013 (Spits et al., 2013). This defined ILC groups based on the pattern of cytokine production. The proposed nomenclature differentiates between 'Group X' and 'ILCXs'. For example Group 1 ILCs includes NK cells and ILC1s (all other cells that require Tbet and produce the Th1 cytokine IFNγ). ILC1s, unlike NK cells, have been shown not express perforin, granzyme B or IL-15R**α** (Bernink et al., 2013). Group 2 ILCs (which have been termed nuocytes, natural helper cells and innate helper 2 cells) require GATA binding protein 3 (GATA3) and produce Th2 cytokines, including IL-4, IL-5 and IL-13, when stimulated. It is suggested these cells are all termed ILC2s. Group 3 ILCs require retinoic acid-related orphan receptor γt (RORγt) for development and produce IL-17 and/or IL-22. The earliest cells in this class to be described were LTi cells, which are required for the formation of secondary lymphoid tissue in the embryo. The remaining RORγt dependent cells, ILC3s, can be divided into NCR+ ILC3s (expressing NKp44 in humans/NKp46 in mice) which produce IL-22, but not IL-17A and NCR-ILC3s, which produce IFNγ, IL-17A and IL-22.

1.3.6 Development

ILCs, like other lymphocytes derive from the common lymphoid precursor (CLP). Three developmental stages have been proposed using mouse models (Serafini et al., 2015). In stage one, the common ILC precursor (CILP) develops from the CLP. This process is dependent on IL-7, and the transcription factors ID2 (Inhibitor of DNA Binding 2), NFIL3 (nuclear factor IL-3 induced), and potentially GATA3. The three subsets of ILCs develop during stage two. Key transcription factors for each group have been discussed previously, with T-bet being the key transcription factor for ILC1s, GATA3 for ILC2s (although several other transcription factors including RORα have also been shown to be important) and RORγt for ILC3s. Stage three, maintenance of these groups, is thought likely to be the result of local regulation, with IL-7 again playing a key role. Whether ILCs develop in the bone marrow in adults, or peripherally remains unclear. Nor is it clear how numbers of ILCs increase in response to challenges – through recruitment and proliferation of mature cells, or through progenitors localising to the relevant site. It has, however, been proposed that final differentiation into ILC subsets may take place peripherally, dependent on cytokine signals produced by the local tissues (Lim and Di Santo, 2019). One example, in a mouse model, has demonstrated that increasing the availability of IL-7 improves survival and increases proliferation and generation of LTi cells (Schmutz et al., 2009).

1.3.7 Plasticity

There is evidence for plasticity between subtypes of ILCs. NCR+ ILC3s from foetal gut or tonsil, cultured with IL-2 and IL-12, will differentiate into (CD127+) ILC1s (Bernink et al., 2013). This may be (part of) the cause of the increased CD127+ ILC1s observed in the intestines of patients with a flare of Crohn's disease, which causes elevated levels of IL-12. CD127+ ILC1s will also differentiate towards ILC3s when cultured with IL-2, IL-1β and IL-23, and then produce IL-22 instead of IFN-γ, a process that is enhanced by retinoic acid. CD103+ ILC1s (low level CD127 expression) will not become ILC3s or produce IL-22. This plasticity may be induced by dendritic cells (DCs) *in vivo*. CD127+ ILC1s adopt an ILC3 phenotype when cultured with CD14- DCs, but maintain their original phenotype when cultured with CD14+ DCs. In contrast, NKp44+ ILC3s will differentiate into CD127+ ILC1s in the presence of CD14+ DCs, but maintain their original phenotype when cultured with CD14- DCs (Bernink et al., 2015). In addition, ILC2s in the lung have been shown to adopt and ILC1-like, IFNγ producing phenotype in the presence of IL-12 and IL-18. The relative reduction in frequency of ILC2s and increase in ILC1s has been shown to associate with disease severity in chronic obstructive airways disease (Silver et al., 2016, Bal et al., 2016). Plasticity has also been shown within ILC subsets. NCR- ILC3s isolated from human skin have been shown to differentiate to NCR+ ILC3s when cultured with IL-2, IL-23 and IL-1β (Teunissen et al., 2014).

This plasticity between subtypes poses both difficulties and opportunities for the use of ILCs as cellular therapy. Potential switches between subtype, with functional implications, must be well understood prior to their use as treatment.

1.3.8 ILCs in allogeneic stem cell transplant

In murine models of transplantation, it has been shown that ILCs in the lamina propria survive transplant conditioning, and that IL-22 production by recipient ILCs plays an important role in protecting the recipient from GVHD (Hanash et al., 2012). A human study, however, showed that circulating ILCs had achieved full donor chimerism in five of six patients by seven weeks post-transplant (Munneke et al., 2014). This paper demonstrates that NCR+ ILC3s, not normally found in the peripheral blood of healthy individuals are found following both induction chemotherapy and HSCT. Post induction chemotherapy but pre-HSCT, patients could be divided into two groups based on high or low expression of the activation marker CD69 on ILCs, with high CD69 expression associating with the development of less

severe mucositis and less acute GVHD, suggesting that initial protection from tissue damage might reduce the development of GVHD. At 12 weeks post allograft, patients who had not developed, and would not go on to develop acute GVHD had the highest number of circulating NCR+ ILC3s. It is proposed that recipient ILCs may give tissue protection during conditioning treatment and so reduce the risk of developing GVHD. Although IL-22 is not measured in this study, given the known function of NCR+ ILC3s, and what has previously been shown in murine models, this represents one potential mechanism.

1.3.9 Sensitivity to chemo/radiotherapy

That IL-22 producing ILCs were radio-resistant was initially inferred from their presence (although in reduced numbers) three days post BMT in a mouse model investigating the role of IL-22 in thymic regeneration (Dudakov et al., 2012), and Hanash and colleagues subsequently demonstrated ILCs of recipient origin in the lamina propria of mice post BMT (Hanash et al., 2012).

In patients with AML, numbers of circulating ILCs have been shown to fall following chemotherapy, with some recovery between cycles, although the authors note that this is incomplete in comparison to other innate cells, with differences between subsets, including an increase in NCR+ ILC3s. ILCs were 'decimated' by conditioning chemotherapy (Busulfan, Cyclophosphamide +/- ATG, Fludarabine, TBI +/- ATG or Fludarabine, Busulfan +/- ATG). This apparent greater resistance to radiotherapy than chemotherapy is important when considering the role of ILCs in murine models of BMT compared to human HSCT, as conditioning in mice is often performed with TBI alone, while this is not standard practice in humans.

1.3.10 Autoimmunity

Autoimmunity occurs when mechanisms for self-tolerance have failed. During T cell development, T cells that bind with too great an affinity to the MHC/self peptide complex should be negatively selected and undergo cell death. Why this process sometimes fails is poorly understood, but when these self-reactive T cells are not deleted, they may cause autoimmune diseases (Sakaguchi, 2000, Jessop et al., 2019).

Although GVHD results from allo-reactive, rather than auto-reactive T cells, the clinical manifestations are often similar. For this reason, many of the models discussed in this thesis, especially in relation to IL-22, draw on models from the field of autoimmunity.

Finally, transplantation (usually autologous) can itself be used in the treatment of severe autoimmune conditions, most commonly multiple sclerosis, systemic sclerosis, and Crohn's disease. The aim is to initially immunosuppress the patient to reduce inflammation, with the hope of then 'resetting' the immune system. Responses are variable, but can be of significant duration (Kapoor et al., 2007).

1.4 IL-22

IL-22, coded for in humans on chromosome 12 (12q15), is part of the IL-10 cytokine family. It has been shown to have both pro-inflammatory and regulatory effects, depending on the situation in which it is produced (Dudakov et al., 2015).

1.4.1 The IL-22/IL-22R system

IL-22 is produced by a number of leucocytes, including Th17 and Th22 cells, γδ T cells, NK cells mucosal-associated invariant T (MAIT) cells, and a subset of ILCs (Sabat et al., 2014, Lamarthee et al., 2016b, Toussirot et al., 2018). The IL-22 receptor is a heterodimer composed of the IL-10 receptor subunit IL-10R2 and the IL-22 specific IL-22R1 (Dudakov et al., 2015, Sabat et al., 2014). While IL-10R2 is relatively widely distributed, IL-22R1 is not expressed on leucocytes, but is constitutively expressed on non-haematopoietic cells of the skin (keratinocytes), liver and intestine, the key target organs of aGVHD, in addition to the pancreas, kidney and lung (Wolk et al., 2004, Boniface et al., 2005).

1.4.2 IL-22BP

In addition, and homologous to extracellular IL-22R1, there is a soluble receptor for IL-22, known as IL-22 binding protein (IL-22BP). IL-22BP has an inhibitory effect, as it prevents binding of IL-22 to the trans-membrane receptor. It is constitutively expressed in the lymph nodes and intestine, where DCs have been shown to be an important cellular source (Sabat et al., 2014, Lamarthee et al., 2016b, Wolk et al., 2007, Martin et al., 2014). In a dextran sulphate sodium (DSS) model of colitis, an inverse relationship between IL-22 and IL-22BP was shown, where at the time of peak disease, *Il22* expression had increased and *Il22bp* had fallen, with both subsequently returning towards baseline. This study also identified DCs as having high levels of IL-22BP expression (Huber et al., 2012). Interestingly, in a study of humans with inflammatory bowel disease, IL-22BP was found to be increased, thus reducing the protective function of IL-22. In addition, eosinophils were shown to be an important source of IL-22BP in this setting (Martin et al., 2016). This final study is of particular interest,

as the presence of eosinophilia is commonly seen in GVHD (Daneshpouy et al., 2002, Basara et al., 2002).

1.4.3 Downstream effects of IL-22 binding

Binding of IL-22 to the trans-membrane receptor activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), and so phosphorylates signal transducer and activator of transcription (STAT) 3 and to a lesser extent STAT1 and STAT5. A simplified representation of this axis is shown in Figure 1.3. The functions of IL-22 include the production of anti-microbial peptides, tissue repair and the induction of epithelial cells to increase the production of mucous associated molecules, thus increasing the mucous barrier and reducing gut translocation. In the skin, IL-22 also reduces the terminal differentiation of keratinocytes required to form the stratum corneum (Wolk et al., 2004, Lamarthee et al., 2016b, Sabat et al., 2014).

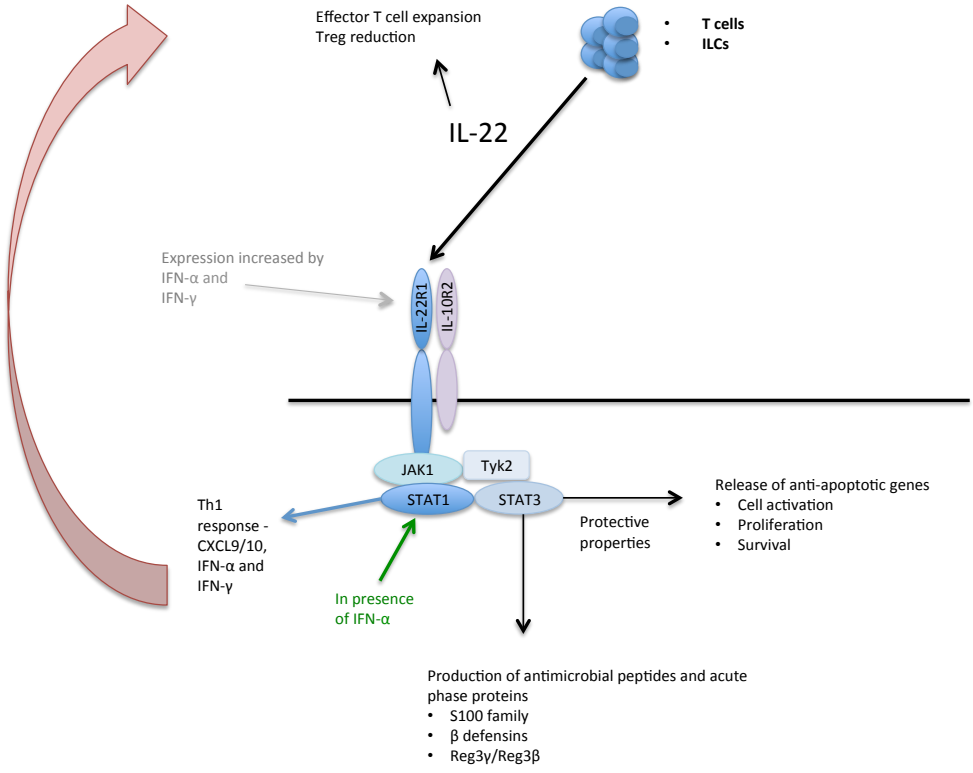

Figure 1.3 A simplified representation of the IL-22-IL-22RA axis, adapted from Sabat et al (Sabat et al., 2014).

1.4.4 Pro-inflammatory role

One of the models to best demonstrate the link between IL-22, ILCs and inflammation is psoriasis. Elevated levels of IL-22 expression in the skin of psoriasis and atopic dermatitis patients compared to healthy controls were demonstrated by Wolk and colleagues in 2004 (Wolk et al., 2004). The number of NKp44+ ILC3s has been shown to be increased in both the blood and skin of patients with psoriasis. In addition, a single case in this study was followed after treatment with an anti-TNF monoclonal antibody. A reduction in the number of NKp44+ ILCs correlated with a reduction in the Psoriasis Area Severity Index (PASI) (Villanova et al., 2014). This work was taken one step further *in vitro* by Teunissen and colleagues (Teunissen et al., 2014), who demonstrate not only an increase in the number of NCR+ ILC3s in the skin and blood of patients with psoriasis, but also that NCR- ILC3s will become NCR+ under the influence of IL-1β and IL-23 (both known to play a role in psoriasis) and that the NCR+ cells are IL-22 producing. Psoriasis is interesting as an example of the pathogenic role of IL-22, because the lesions are the result of over activity of the normal proliferative function of IL-22.

1.4.5 Regulatory role

The role of IL-22 in inflammatory bowel disease (IBD) has been studied in a number of mouse models. In DSS induced colitis, the administration of an IL-22 neutralizing antibody resulted in increased colonic inflammation and greater destruction of the epithelium. In a T cell transfer model, T cells from an IL-22$^{-/-}$ mouse caused more severe colitis than wild type T cells. In addition, it has been shown that the administration of recombinant IL-22 (rIL-22) resulted in recovery of intestinal inflammation (Li et al., 2014). These results suggest a protective role for IL-22 in IBD. It has been proposed, however, in a DSS model of colitis associated colon cancer, using an anti-IL-22 antibody that although IL-22 does have a protective effect in the acute period, it has a negative impact in the recovery period (Huber et al., 2012).

Similarly, a protective role for IL-22 has been shown in a murine model of hepatitis. Concanavalin-A (Con A) was given to induce a transaminitis with associated necrosis. Hepatic damage was more severe when mice were given an IL-22 neutralising antibody and ameliorated when they were pre-treated with rIL-22 (Radaeva et al., 2004). The same effect was seen with rIL-22 in alcohol induced liver injury in mice (Ki et al., 2010). When STAT3 was deleted in the hepatocytes, this protective effect from IL-22 was lost.

1.4.6 IL-22 in allogeneic stem cell transplant

The role of IL-22 in HSCT remains unclear. A murine model has demonstrated the protective role of IL-22 for the transplant recipient. Initially an IL-22 neutralising antibody was used in an MHC mismatched transplant model, resulting in a significant increase in GVHD related mortality. Subsequently, using an IL-22 knockout MHC mismatched model, it was demonstrated the knockout mouse could be used as the donor with no effect on GVHD incidence (and a delay in GVHD mortality, with combined donor marrow and T cell IL-22 deficiency). When the IL-22$^{-/-}$ mouse was the transplant recipient, however, there was a significant increase in GVHD mortality. The effect was particularly prominent in the gastrointestinal tract, where (radio-resistant) ILC-produced IL-22 was key in reducing damage to intestinal stem cells, resulting in reduced morbidity and mortality from gut GVHD. GVHD itself, however, resulted in a reduction of ILC numbers, and of IL-22. Interestingly, no difference was seen in skin pathology in *Il22*$^{-/-}$ recipients. It is important to subsequent discussion to note that levels of IL-22 in the gastrointestinal tract rose in response to conditioning (Hanash et al., 2012).

It was subsequently demonstrated that administration of recombinant IL-22 (starting at Day 7 post HSCT), again in a murine model, resulted in reduced GVHD pathology in the gut and liver (but not skin) at three weeks post-transplant (Mertelsmann et al., 2013).
In contrast to this, two papers looking at the role of donor produced IL-22 in murine GVHD demonstrate in a MiHA model that mice receiving T cells from IL-22 knockout donors have reduced GVHD related mortality and a reduced histological grade of GVHD in the skin and gut compared to mice receiving wild type T cells (Couturier et al., 2013, Lamarthee et al., 2016a).

1.5 Hypothesis:

We propose that, as in the mouse model (Hanash et al., 2012), ILC3 derived IL-22 protects target organs from GVHD by protecting the recipient's tissues from conditioning induced damage, resulting in reduced T cell activation. The role of IL-22 in established GVHD may, however, be pro-inflammatory, and if so, the point of change is not well established. The aim of this project is to further clarify the role of ILCs and IL-22 in a human transplant setting, and so to develop a clearer understanding of their potential therapeutic use.

Overall aims of the project:

Aim 1: to define innate lymphoid cell homeostasis in human haematopoietic stem cell transplantation

Aim 2: to analyse the IL-22 axis in human graft versus host disease

Aim 3: to test the ability of IL-22/ILC3 to prevent GVHD in a preclinical in vitro model

Chapter 2 Innate lymphoid cell homeostasis post allogeneic stem cell transplant

2.1 Introduction

2.1.1 ILC function to maintain health

Primarily resident at epithelial surfaces, ILCs have a variety of functions to maintain health. They have been shown to have a role containing natural commensal bacteria within the gut (Sonnenberg et al., 2012). In a *Rag1*$^{-/-}$ mouse model, the group demonstrate that depletion of ILCs resulted in dissemination of commensal bacteria, with resulting systemic inflammation. Of particular interest to this project, signs of systemic inflammation were also produced if *Rag1*$^{-/-}$ mice (without ILC depletion) were treated with an IL-22 blocking antibody. Anatomical containment and reduction in inflammation could be restored by the administration of recombinant IL-22.

Further evidence of the importance of ILC-produced IL-22 in the gut was shown in a reporter mouse model (Sawa et al., 2011). The authors show that ILCs (both NCR+ and LTi), are the major producers of intestinal IL-22 and that unlike T cells, they do so constitutively. Furthermore, there was evidence of direct feedback between the microbiota and ILCs. Expression of IL-25 by epithelial cells, induced by the microbiota, reduced IL-22 production. The authors propose this is a mechanism by which equilibrium is maintained between the microbiota and the immune system. In addition, ILC3s (and to a lesser extent ILC2s) have been shown to express MHC II (Hepworth et al., 2013). In a mouse model, depletion of ILCs using an anti-CD90.2+ monoclonal antibody resulted in increased, dysregulated CD4+ T cells, not seen in IL-22, IL-17A, and IL-23 deficient mice, suggesting an alternative mechanism. When MHC II was deleted from ILCs alone, mice developed intestinal inflammation, with loss of normal architecture and increased numbers of IFN-γ, IL-17 and TNF-α producing CD4+ cells in the colon compared to controls. This inflammation was prevented if the mice were treated continuously with antibiotics. The authors propose that antigen presentation by ILCs, without the necessary co-stimulation, negatively regulates CD4+ T cells. In a subsequent publication (Hepworth et al., 2015), MHC II+ CCR6+ ILC3s were found to induce apoptotic cell death and deletion of activated commensal specific T cells, so maintaining intestinal homeostasis. The authors comment that this process is similar to negative selection in the thymus, and propose the use of the term ‘intestinal selection’. Of interest to the subsequent discussion of IL-7, one of the hypotheses tested in vitro was that the high levels of the IL-2R and IL-7R expressed by the ILCs may mean that the cell death

induced is a combination of the apoptotic pathway, with reduction in availability of pro-survival cytokines. They were, however, able to reduce cell death with the addition of recombinant IL-2 but not IL-7.

A single report in humans undergoing HSCT for severe combined immunodeficiency (SCID) with IL-2 receptor *(IL2R)* or *Janus kinase-3 (JAK3)* mutations has questioned whether ILCs are redundant in humans with a functional adaptive immune system (Vely et al., 2016). These patients lack ILCs in addition to T cells. With low intensity conditioning, the protocols resulted in T cells of donor origin, while other lineages remained of host origin. They therefore continued to lack ILCs in the post-transplant setting. In follow-up extending to a maximum of 39 years, these patients did not see an increase in infection or inflammation when compared to patients transplanted for comparable immunodeficiencies. It should be noted that the majority of this work was performed on peripheral blood.

2.1.2 ILC involvement in pathology

ILCs have been shown to be pathological in a number of situations. An increased frequency of IL-22 producing ILC3s has been shown in the skin and blood of patients with psoriasis (Teunissen et al., 2014, Villanova et al., 2014). Interestingly, the number of ILC3s was found to correlate with the severity of the disease (as determined by the psoriasis area severity index – PASI), and a reduction in circulating ILC3s was closely associated with a clinical response to an anti-TNF agent.

Disordered composition of ILCs has been demonstrated in the intestines of patients with Crohn's disease compared to controls (with colorectal cancer) (Bernink et al., 2013). Patients with Crohn's disease had a higher percentage of ILC1s and lower percentage of Nkp44+ ILC3s. These ILC1s were shown to express *IFNG*, known to be increased in the lamina propria in patients with Crohn's disease. Plasticity between these ILC subsets has been discussed previously (1.3.3). Total ILC percentage of leucocytes was not significantly different.

2.1.3 IL-7 and its role in SCT

First described in a culture system in 1988, where it was shown to stimulate lymphoid progenitors (Namen et al., 1988), IL-7 is member of the common **γ** chain family (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21)(Lin and Leonard, 2017), its receptor being comprised of the IL-7α subunit (CD127) and the IL-2R**γ** chain (CD132, the common γ chain receptor). Mutations

in the IL-2Rγ chain result clinically in X-linked SCID (Leonard, 1996). IL-7 is known to be critical for lymphocyte development, with IL-$7^{-/-}$ mice showing similar abnormalities of lymphocytes to mice lacking the IL-2Rγ (von Freeden-Jeffry et al., 1995, DiSanto et al., 1995).

In 2001 it was proposed that IL-7 might be involved in T cell homeostasis (Fry et al., 2001). They demonstrated an inverse correlation between IL-7 and CD4 count in two cohorts, paediatric patients with HIV infection and patients undergoing chemotherapy. In both groups there was an increase in IL-7 concentration when the CD4 count was low, and a fall following CD4 recovery. In the paediatric patients, some correlation was also seen between IL-7 and CD8 cells, but this was absent in the post chemotherapy cohort. No correlation was found with the other cytokines tested. Based on these findings, the authors propose two possible mechanisms of IL-7 homeostasis. IL-7 may be produced at a constant rate, but when T cell numbers fall, the reduction in IL-7R results in increased IL-7 availability, or a reduction in T cells results in increased IL-7 production. Further evidence linking IL-7 concentration with lymphocyte homeostasis is seen in a study looking at serum IL-7 levels peri-HSCT in a paediatric setting. Patients were undergoing transplantation for SCID, thalassaemia, aplastic anaemia or acute leukaemia. The pre-HSCT IL-7 concentration was inversely correlated with the absolute lymphocyte count, and during transplant, in the acute leukaemia and SCID patients the IL-7 concentration rises as the lymphocyte count falls, and then falls as the lymphocyte count recovers (Bolotin et al., 1999). The authors argue in favour of a reduction in IL-7 binding in the lymphopenic setting. Fry et al., however, have a third population in their study, patients with idiopathic CD4 lymphopoenia. In this cohort, no correlation is seen between CD4 count and serum IL-7 levels. The authors argue that this makes the first model less likely.

2.1.4 IL-7 as therapy post HSCT

Given the known function of IL-7, it is perhaps not surprising that groups have attempted to improve immune reconstitution post HSCT using recombinant IL-7. In 2002, Sinha et al. (Sinha et al., 2002) demonstrated improved thymopoiesis in a syngeneic and T cell depleted mouse model, when IL-7 was administered daily for 28 days, commencing on the day of stem cell return. In this T replete setting, however, IL-7 administration resulted in the development of clinical GVHD at a lower T cell dose, and increased histological changes in GVHD target organs. In these animals there was no improvement in thymic function, presumed to be due to the toxic effect of GVHD on the thymus.

Endogenous IL-7 has also been linked to GVHD. In a study of 31 patients, receiving identical conditioning with fludarabine and cyclophosphamide, higher serum IL-7 concentration at Day 7 and Day 14 were associated with subsequent aGVHD development (Dean et al., 2008).

2.1.5 ILCs and the role of IL-7

As previously discussed, IL-7 plays a critical role in T cell reconstitution post HSCT, however, the mechanism that governs IL-7 homeostasis has remained somewhat elusive. In 2017, (Martin et al., 2017) a series of murine models were used to investigate IL-7 homeostasis. Initially using bone marrow chimeric mice that only expressed IL-7 in stromal or haematopoietic cells, it was determined that stromal cells are key to IL-7 production in this model, with only a small proportion of IL-7 being haematopoietic cell derived. This concept was then developed, through the production of chimeras where IL-7R was expressed in either haematopoietic or stromal cells. Here the relative cellular importance was reversed, with donor T cell proliferation being affected by the expression of the IL-7R on haematopoietic but not stromal cells. Using observations from the literature that IL-7 concentration in Rag-deficient mice is not as high as that seen in $Il7r^{-/-}$ mice, they hypothesise that cells other than T cells must be using the available IL-7. Using a CD90.2 antibody to deplete ILCs in $Rag1^{-/-}$ recipients, increased T cell proliferation was demonstrated when compared to $Il7^{-/-}$ recipients treated with the same antibody. It was further demonstrated that ILCs (defined as $CD90^{+}$ $NK1.1^{-}$ cells) were more efficient than T cells at utilising IL-7, and did not down-regulate IL-7R to the same extent as T cells when cultured with IL-7. Furthermore, when donor T cells were transplanted into mice that had undergone ILC depletion, they showed increased proliferation. This effect was lost in $Il7^{-/-}$ hosts.

Evidence for reduced T cell proliferation in the presence of increased IL-7 competition had been shown in an earlier paper (Munitic et al., 2004). Here, a transgenic mouse was produced, where the T cells expressed IL-7R throughout their lifetime (CD4+ CD8+ thymocytes do not usually have detectable levels of IL-7R). The transgenic mice showed evidence of thymic hypoplasia, with increased apoptosis of CD4- CD8- cells, and an overall reduction in their number. The authors propose that the increased competition for IL-7 in effect “starves” the CD4- CD8- cells, resulting in the reduction in their number.

2.1.6 ILCs and CD52 expression

When this research began, to my knowledge, no published data were available regarding the expression of CD52 by ILCs. Increasing interest in the use of Alemtuzumab for the treatment of multiple sclerosis, combined with an interest in the role of ILCs in the disease, means that there are now published data showing CD52 expression by ILCs (Gross et al., 2016). The paper explores the effect of treatment with Alemtuzumab on circulating innate immune cells. CD52 expression is seen on ILCs, although at a lower level than on CD4+ T cells (in treatment naïve patients with multiple sclerosis).

2.1.7 Graft sources in allogeneic SCT

Current in vitro manipulation (such as CD34 selection or αβ T cell depletion) has increased the number of potential graft types given to the recipient, but stem cells are still harvested from three sources: bone marrow, mobilised peripheral blood stem cells or cord blood. Different graft sources are known to have different compositions of T cells, monocytes and NK cells (Korbling and Anderlini, 2001, Servais et al., 2017). These known differences do not, however, consistently translate into a difference in aGVHD risk (Ringden et al., 2002, Hahn et al., 2008, Lazaryan et al., 2016), although it is often difficult to separate the effect of the graft source from other factors such as conditioning regimen.

2.1.8 Questions for Chapter 2

Chapter 2 explores ILC homeostasis in human HSCT. The specific questions addressed are detailed below.

1. What is the effect of transplant conditioning therapy, including T cell depletion, on ILCs?

2. What is the effect of lymphopenia on the expansion of ILCs post-transplant?

3. What is the response of IL-7 to conditioning regimens and subsequent lymphopenia?

4. Does ILC reconstitution impact on the development of GVHD?

5. What is the ILC content of different stem cell sources?

Aims:

1. To use flow cytometry to assess ILC homeostasis in human HSCT
   Is this affected by T cell depletion?
   Is there an association with GVHD?

2. To use ELISA to explore IL-7 homeostasis during HSCT

3. To evaluate the ILC content of different stem cell sources

2.2 Methods for Chapter Two

2.2.1 Sample collection

All samples used in this chapter were collected under the 'Improving Outcomes in HSCT' project. Ethical approval was granted by the NRES Committee North East – Newcastle & North Tyneside 2 (14/NE/1136). This non-interventional study was designed to meet a number of objectives in adult HSCT, including improving our understanding of the cellular immune reaction in GVHD and developing new strategies for GVHD prediction. Patient blood samples (both EDTA and serum) were taken with routine clinical samples. Both patients and healthy controls gave informed consent.

The majority of work using patient samples in this thesis was performed on two cohorts of patients who underwent HSCT at the Northern Centre for Bone Marrow Transplantation between 2015 and 2019 (Cohort A and Cohort B), the full details of which are shown in Appendix 1. Where specific subsets of these patients, or additional samples have been used, relevant clinical information is provided with the results. Patients in Cohort A were recruited sequentially by date of transplant, but once six patients were recruited to each T cell depletion group, that group closed. Patients in Cohort B were recruited sequentially.

Samples from graft sources: Bone marrow and peripheral blood stem cell (PBSC) samples were performed on fresh samples, using excess material from clinical harvests. Cord blood samples had been previously frozen, and were defrosted as below. It is worth noting, that this mirrors the clinical use of these graft sources, where both bone marrow and mobilised PBSCs are routinely donated and infused fresh, while cord blood will have been frozen and defrosted prior to infusion.

2.2.2 Media and buffers

Details of media and buffers used in this chapter are provided in Appendix 2.

2.2.3 Clinical data

Clinical data were taken from the BMT Clinical Database, laboratory systems, and where data were missing, from patient notes.

2.2.4 Cell processing - PBMCs

Peripheral blood mononuclear cells (PBMCs) were extracted from fresh blood (taken in EDTA) using the following method. Blood was diluted 1:1 with D-PBS and then layered on

15mls Lymphoprep™ density-gradient media (Alere Technologies). The sample was then centrifuged at room temperature (800g for 15 mins). PBMCs were aspirated, washed in D-PBS (centrifuged at 500g for 5mins) and then underwent a further wash to remove platelets (300g for 7 minutes). The pellet was re-suspended in 1ml of red blood cell lysis buffer (Sigma-Aldrich) and incubated in the dark for 10 minutes. The cells were washed again in D-PBS. Total cell count was estimated using a 1:1 dilution with trypan blue exclusion dye (Invitrogen™, Thermo Fisher Scientific) on an Improved Neubauer Haemocytometer (Weber Scientific International).

Cells from mobilised PBSCs and bone marrow were collected fresh at the time of donation and washed. Cryopreserved cord blood samples were defrosted into warm RF10, and washed twice. All cells were counted as above, and re-suspended in 50µl flow buffer.

2.2.5 Cell preparation

4µl anti-mouse IgG (Sigma-Aldrich) was added to the sample and incubated for 4 minutes. Antibodies were then added as per the panel and incubated at 4°C in the dark for 30 minutes. The cells were washed in flow buffer (500g for 5 minutes) and re-suspended in 200µl flow buffer. 4,6-diamidino-2-phenylindole (DAPI; Partec) was used to exclude dead cells.

2.2.6 Trucounts – cell processing

Peripheral blood samples, collected in EDTA, were inverted a minimum of 20 times to ensure thorough mixing. 200µl of whole blood was mixed with the Trucount antibody panel (details in Appendix 3) in a Trucount™ tube (BD Biosciences), and stained for 20 minutes in the dark at room temperature. The sample was then lysed with 900ul of RBC lysis buffer.

2.2.7 Gating strategy (Trucounts)

In this panel, I was interested in accurate CD3 and total ILC counts. In order to include the additional markers, this panel does not enable sub-setting of ILCs. An example of the Trucount gating strategy is shown in Figure 2.1. Details of the panel and antibodies used are shown in Appendix 3.

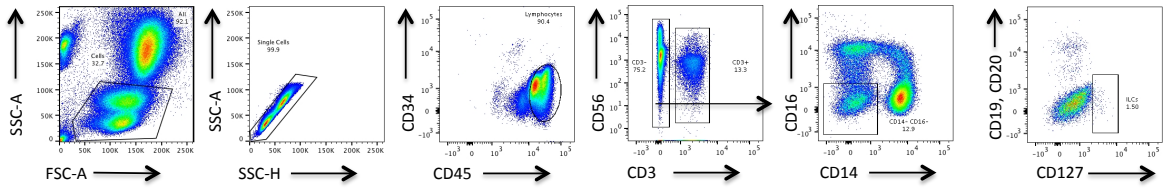

Figure 2.1 An example of the gating strategy used for the Trucount samples. 200µl of whole blood was mixed with the Trucount antibody panel (CD3, CD4, CD8, CD14, CD16, CD19, CD20, CD34, CD45, CD56, CD127, HLA-DR)

2.2.8 Flow cytometry

Flow cytometry was performed using a BD LSRFortessa^{TM} X-20. Data were analysed using FlowJo versions 10.1 and 10.5.3.

2.2.9 Cell sorting

Cell sorting was performed using a BD FACSARIA^{TM} Fusion. The sorted populations (CD3+ and ILCs) were sent to the Northern Molecular Genetics Service for chimerism analysis, as informative markers for each patient had already been determined for clinical use.

2.2.10 Serum samples

Serum samples were centrifuged at 500g for 5 minutes. Serum was stored in aliquots of 1ml at -80^{0}C.

2.2.11 Serum IL-7 quantification

IL-7 quantification was performed using a human IL-7 Quantikine HS enzyme-linked immunosorbent assay (ELISA) Kit (R&D systems). The assay was performed according to the manufacturers instructions. Briefly, a dilution series was produced using the human IL-7 standard provided, to produce standards, which were tested in duplicate. 200µl of standard or sample was placed in each well of a 96 well plate and incubated for 14-20 hours. Based on the results of a preliminary experiment, samples were diluted 1:10 with calibrator diluent, to ensure results were within the range of the standard curve. Samples were tested in singlet. Each well was washed six times with wash buffer and 200µl of human IL-7 conjugate was added to each well. The plate was then incubated for a further two hours at room temperature. Each well was then washed a further six times, after which 50µl of substrate solution was added per well. The plate was incubated for a further 45 minutes at room temperature, after which 50µl of amplifier solution was added per well. The plate was incubated for a further 45

minutes, after which 50µl of stop solution was added to each well. The optical density was measured using a Multiskan Ascent®plate reader (Multiskan Ascent, Thermo Labsystems, Thermo Fisher Scientific), set at 690nm and 492nm, and data were analysed using Ascent® software.

To calculate the values, the readings at 690nm were subtracted from those at 492nm. All values were log-transformed and non-linear regression was performed using GraphPad Prism (version 7). Final values were multiplied by ten, to account for the initial dilution. The minimum detectable value of the assay was 0.1pg/mL.

Serum IL-22 quantification was performed using a human IL-22 Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D systems). The assay was performed according to the manufacturers instructions. Full details are given in 3.2.4.

2.3 Results

2.3.1 CD52 expression by ILCs

As discussed, at the time of starting this project, published data were not available for CD52 expression by ILCs. As Alemtuzumab is given in approximately 60% of the adult allogeneic stem cell transplants performed at the Northern Centre for Bone Marrow Transplantation, prior to looking at ILC homeostasis in stem cell transplantation, I first wished to establish whether ILCs expressed CD52. Figure 2.2 shows the results from experiments using four healthy donors. Part a) shows the standard gating strategy, discussed further in 2.3.3, for CD52 versus (top) an isotype control (below). Part b) demonstrates that CD52 expression was seen on the combined ILC population of all four donors, when compared to an isotype control (grey), and c) that CD52 was expressed by all three subsets. I was therefore satisfied that ILCs expressed CD52.

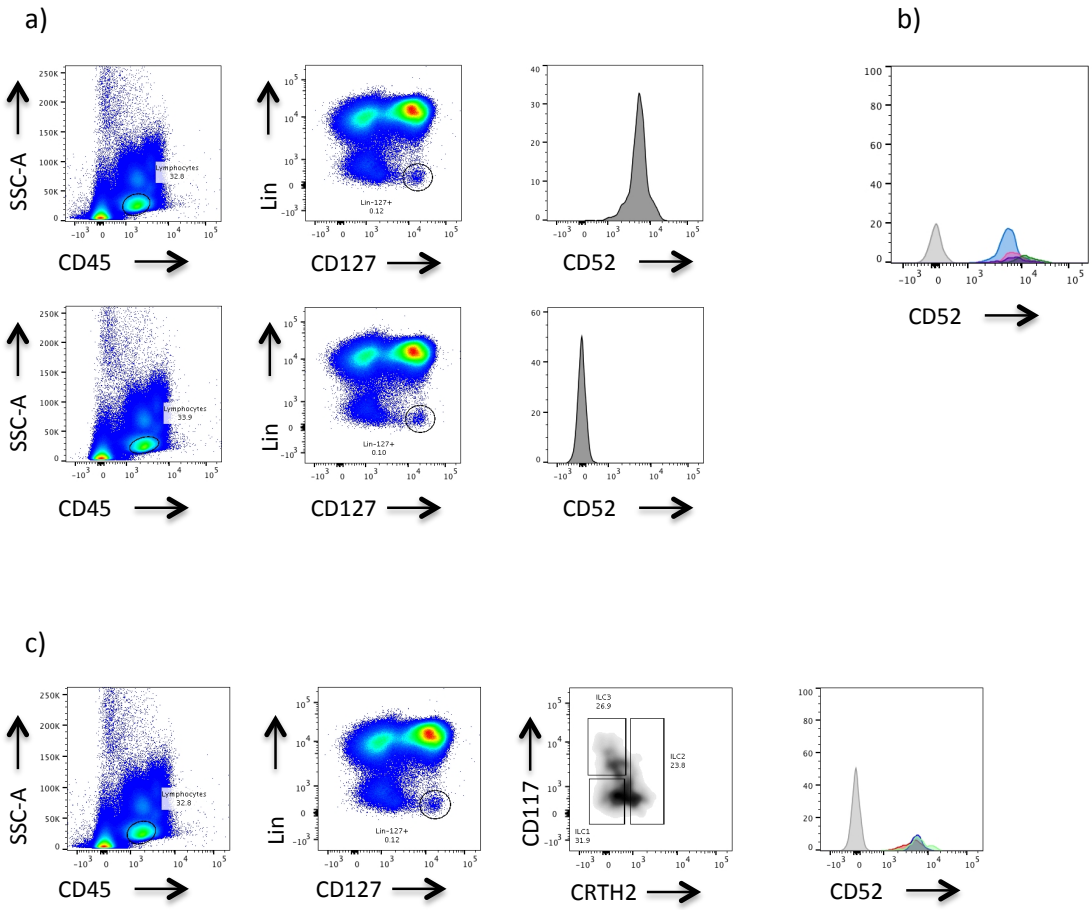

Figure 2.2 a) CD52 expression on combined ILC population (top) vs isotype control (below), b) CD52 expression demonstrated on whole ILC population (four healthy controls compared to an isotype control in grey), c) CD52 expression demonstrated on all ILC subsets (ILC1 green, ILC2 blue, ILC3 red, isotype control grey).

2.3.2 Patient Characteristics

In order to investigate ILC homeostasis, peripheral blood samples were collected from patients undergoing HSCT. Samples were collected from two separate cohorts. In Cohort A, patients were recruited into four groups, with six patients in each, based on the T cell depleting agent he or she received (or none). Four standard regimens are used in our institution: T cell replete, ATG, 30mg Alemtuzumab or 60mg Alemtuzumab. One additional patient was recruited into the 60mg Alemtuzumab group, due to one early death in that group. Samples for this group were collected at the following time points:

Admission	Day 0	Day 7	Day 14	Day 21	Day 28	Day 56	Day 100

10mls of peripheral blood was requested at each time point, and a total of 156 samples were received. PBMCs were isolated from these samples and analysed using panel ILCA. A summary of the patient characteristics is shown below and more detailed information is shown in Appendix 1 (Cohort A). As the choice of T cell depletion is dependent on conditioning regimen, disease and donor, there are obvious mismatches between the groups. Our standard practice is to use the Seattle protocol for patients with myeloma, ATG as part of a FLAMSA protocol, 30mg Alemtuzumab with the majority of sibling donors and 60mg for matched unrelated donors. The first adult transplant using a haploidentical donor in our institution was performed in 2015.

For validation purposes, and to allow for the collection of additional serum samples, a second cohort of transplant patients was collected. Due to time constraints, Cohort B consists of sequential patients who gave consent to be part of the study. Blood was analysed using the Trucount method, using the panel shown in Figure 2.1. Samples were collected at Day 14, Day 28, Day 56 and Day 100, with a total of 73 samples received.

The two cohorts were subsequently combined for analysis (2.3.5). A summary of patient characteristics is shown in Table 2.1.

	T replete	ATG	Campath 30	Campath 60
N	11	8	9	17
Sex	M=9, F=2	M=2, F=6	M=5, F=4	M=10, F=7
Conditioning	Seattle=5 RIC Haplo=5 MAC Haplo=1	Flu Bu=3 FLAMSA Bu=1 FLAMSA TBI=4	Flu Mel=7 LEAM=1 Cy TBI=1	Flu Bu=4 Flu Mel=11 Flu Cy=1 Cy TBI=1
Disease	MM=5 AML/MDS=4 HD=1 DLBCL=1	AML/MDS=6 MF=1 CML=1	AML=3 ALL=2 CLL=2 FL=1 Burkitt lymphoma =1	AML/MDS=13 ALL=1 HD=1 CVID=1 SAA=1
Donor	UD=4 MSD=1 Haplo=6	UD=5 MSD=3	UD=2 MSD=7	UD=16 MSD=1
aGVHD II-IV	Y=4 N=7	N=8	Y=2 N=7	Y=4 N=13

Table 2.1 Patient characteristics for Cohorts A & B, split by method of T cell depletion

2.3.3 Panels ILCA and ILCB

Both panel ILCA and ILCB were run using isolated PBMCs. An initial panel to allow ILC isolation and subtyping (ILCA) was designed using a combination of isotype controls and fluorescence minus one panels (examples are shown in Appendix 4). Relevant antigen expression was taken from the literature, drawing especially on the following publications: Munneke et al (Munneke et al., 2014), Hazenberg and Spits (Hazenberg and Spits, 2014), Teunissen et al (Teunissen et al., 2014).

The gating strategy involved isolation of live single cells. Lymphocytes were then gated using CD45 expression, and from this gate ILCs were isolated as lineage negative, CD127+, where the lineage gate included CD3, CD14, CD16, CD19, CD20, CD34, CD123. Subdivision into ILC1, ILC2 and ILC3 was then possible using CD117 and CRTH2. An example of the gating strategy is shown in Figure 2.3. Cell sorting was performed using panel ILCB. The gating

strategy was similar, but an additional gate was added to allow sorting of CD3+ cells separately. An example of the panel used for cell sorting is shown in Figure 2.4. A list of panels and antibodies used in is shown in Appendix 3.

One of the difficulties of assessing ILCs using flow cytometry, is that they are primarily defined by the absence, rather than expression of key markers. As our knowledge of ILCs increases, so expression of new markers is demonstrated in certain situations. For example the dendritic cell marker CD11c is commonly included in the lineage gate to exclude these cells, but CD11c expression has been demonstrated on IL-22 producing NKp46+ cells in a mouse model of *C. rodentium*-induced colitis (Satoh-Takayama et al., 2008). Given the inflammatory state post HSCT, I have chosen to limit the markers included in my lineage gate. This reduces the likelihood of losing the cells I am interested in, but increases the potential for contamination within the gates. For this reason secondary analysis was performed using t-distributed stochastic neighbour embedding (t-SNE), to investigate whether the cells of interest formed a cluster, without manual gating (Figure 2.5b).

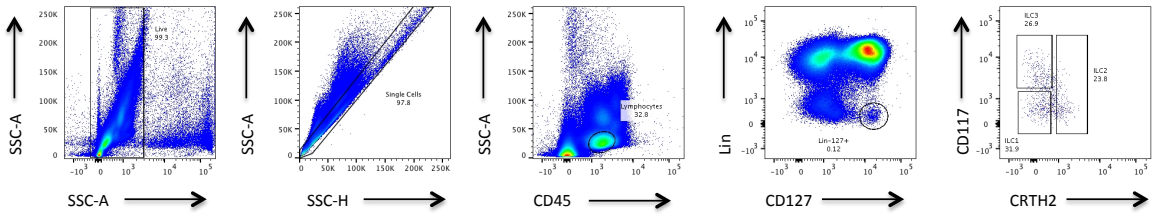

Figure 2.3 Example of gating strategy ILCA. Live single cells were gated using CD45 to isolate the lymphocyte population. ILCs were then gated as Lineage negative (Lineage markers CD3, CD14, CD16, CD19, CD20, CD34, CD123), CD127+. Subdivision into ILC1, ILC2 and ILC3 was then performed using CD117 and CRTH2.

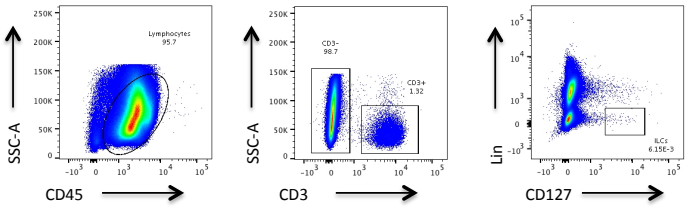

Figure 2.4 The cell sorting strategy (ILCB) altered from ILCA after the lymphocyte gate, in that CD3 was gated separately, to allow the CD3+ population and the ILC population to be sorted.

2.3.4 Back gating

By double staining the cells for the key populations, I was able to demonstrate where key populations were located within my gates. T cells and B cells showed the highest expression of lineage markers, and were split primarily by CD127 expression, with T cells (light blue) being positive and B cells (dark green) negative. CD14 positive cells (dark blue) were a relatively homogeneous population with intermediate expression of lineage markers. Expression of CD56 (yellow) and HLA DR (pale green) was more heterogeneous (Figure 2.5).

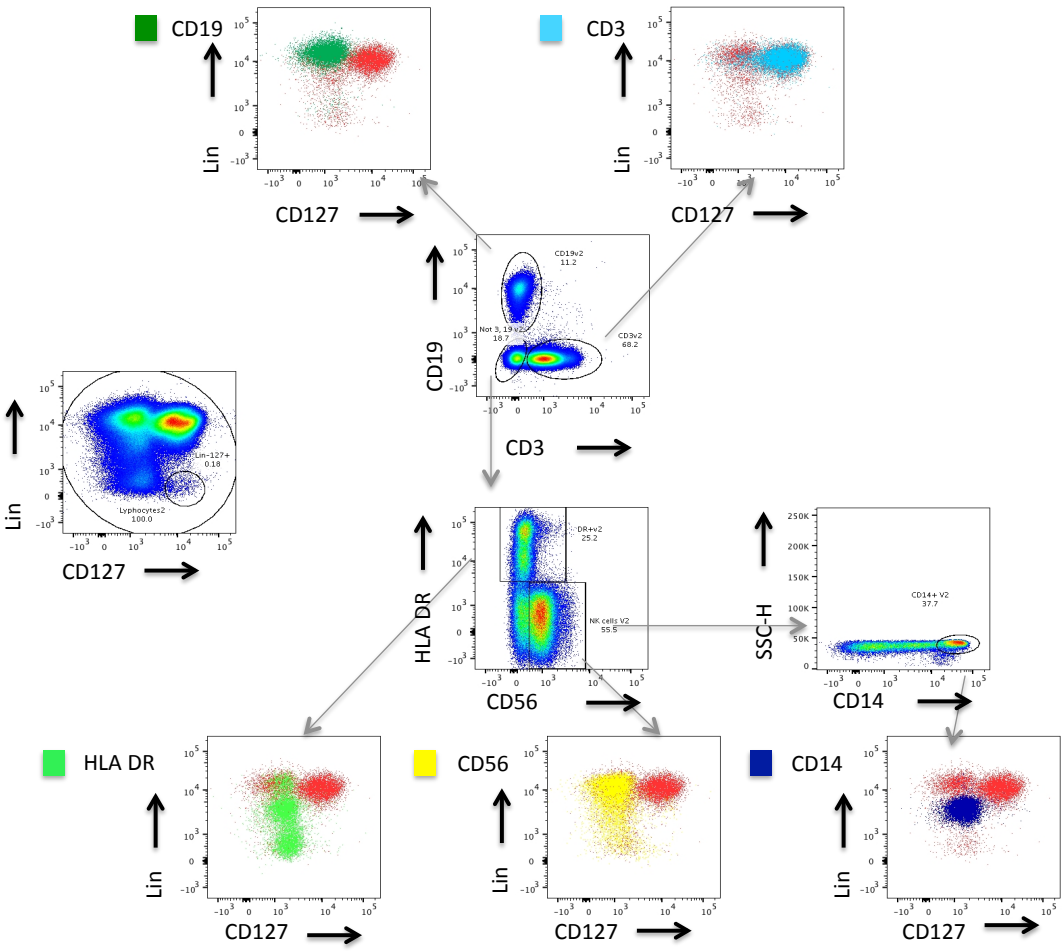

Figure 2.5a Back-gating of panel ILCA to demonstrate where the major populations are located. The CD3+ population is shown in light blue, the CD19+ population in dark green, CD56+ in yellow, CD14+ in dark blue and HLA DR+ in light green.

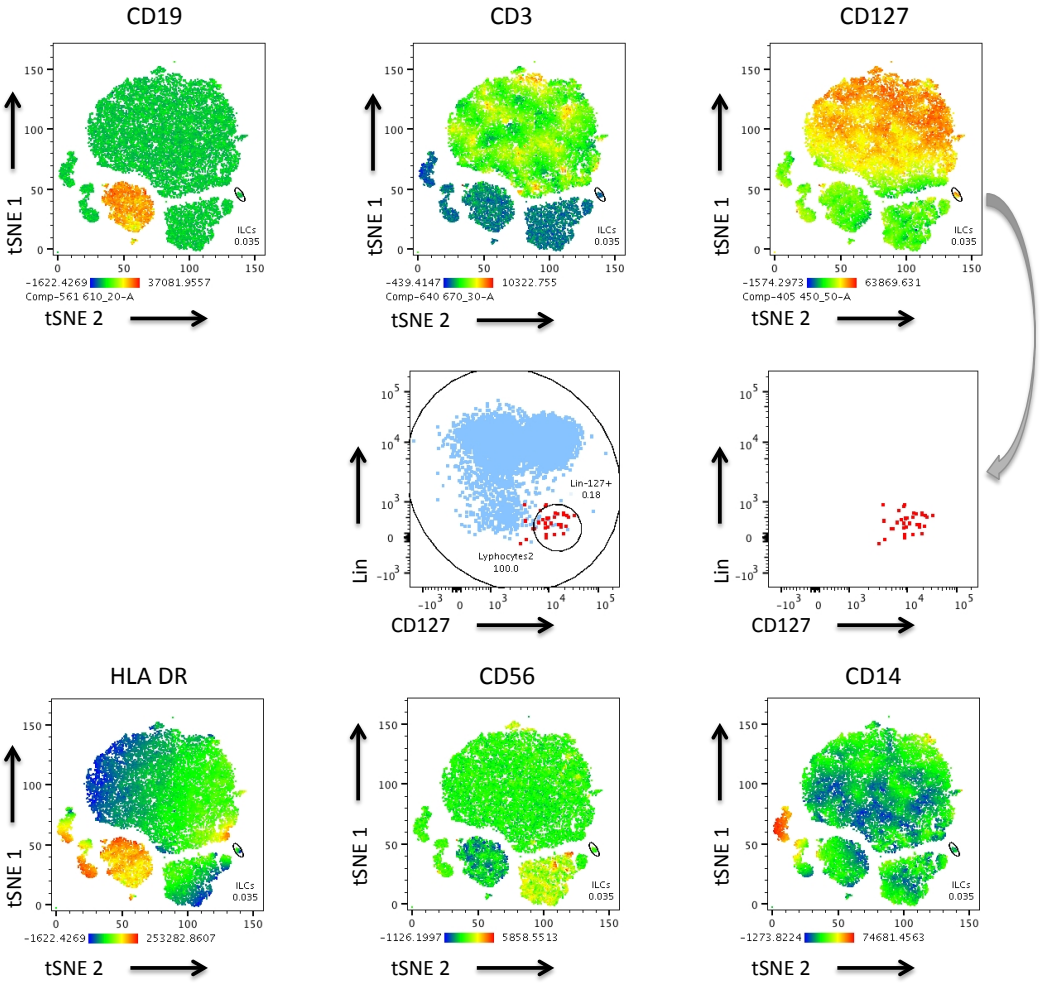

Figure 2.5b The same data illustrated by t-SNE. ILCs form a single cluster (top panel), which is Lin-, CD127+.

2.3.5 Combining Cohort A and Cohort B

To maximise the number of patients available for analysis and therefore improve the statistical power of the study, it was decided to combine the two cohorts if possible. Given the different techniques used, it was first necessary to assess correlation between the results obtained. In Cohort A, flow cytometry was performed on isolated peripheral blood mononuclear cells. Absolute counts are therefore derived, using the cell number in the lymphocyte gate and the lymphocyte count from testing performed in the NHS laboratory on the same day. In Cohort B, analysis was performed using the Trucount method, so it was not necessary to subsequently derive absolute counts. To assess the degree of correlation, CD3 and ILC counts for Cohort B were derived, using the relative proportions of the lymphocyte gate and the NHS laboratory count, in the same way as they had been calculated in Cohort A. These results were then compared to the 'absolute' count given by the Trucount method. The correlation between the two is shown in Figure 2.6.

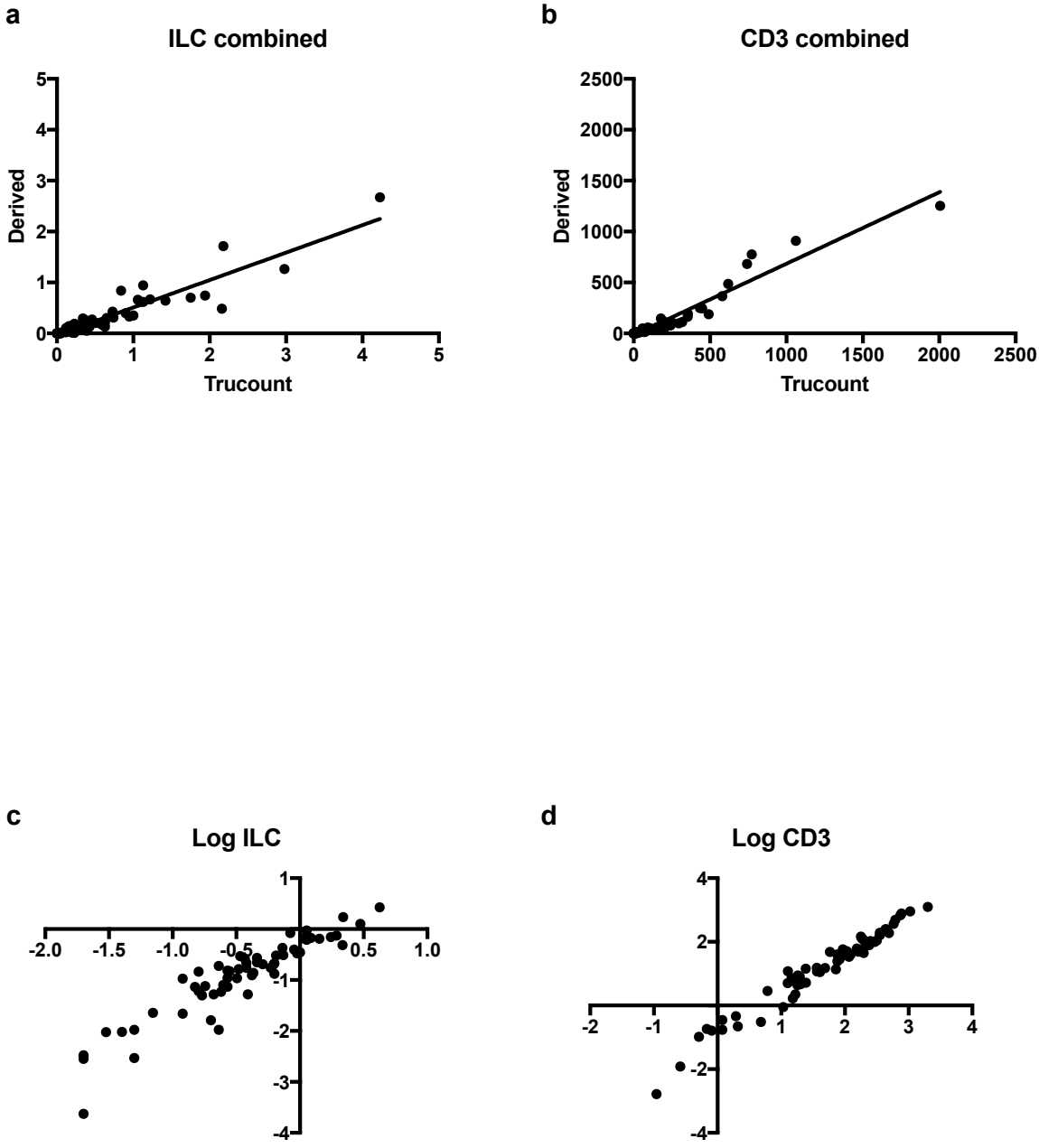

Figure 2.6 Graphs showing the correlation between the two methods of calculating absolute cell count. The Spearman rank correlation coefficient in part (a) is 0.95, and in part (b) is 0.97.

Given the degree of correlation, it was decided to combine the results of the two cohorts for subsequent analysis, and to use the laboratory derived method for all samples. As the values from the Trucount method were consistently slightly higher, I considered adjusting the derived results using the gradient of the line. Given, however, that I was only able to calculate the gradient for Cohort B, I felt to do so was potentially introducing a further source of error.

It is, however, possible, that all the results shown below are slightly lower than the true values.

I had initially intended to investigate individual subsets of ILCs. My results from Cohort A, however, showed so few ILCs, especially at the early time points in which I was most interested, that accurate subsetting was not possible. Cohort B did not contain the antigens to allow subsetting. The results below are therefore for total ILCs.

2.3.6 ILC homeostasis in HSCT

Looking initially at all the patients combined, both ILCs and CD3+ cells show a similar pattern, with a nadir reached at Day 0 (Figure 2.7). Early recovery is seen by Day 14, but for both cell types this is more marked by Day 28. Neither has reached the levels seen at admission by 100 days post-transplant. Significant intra-sample variability is seen, which is probably due to a number of factors, including method of T cell depletion (discussed further below), underlying disease and treatment received prior to transplantation, complications suffered post transplantation, and treatment received for these, including GVHD and viral reactivation, also further discussed below.

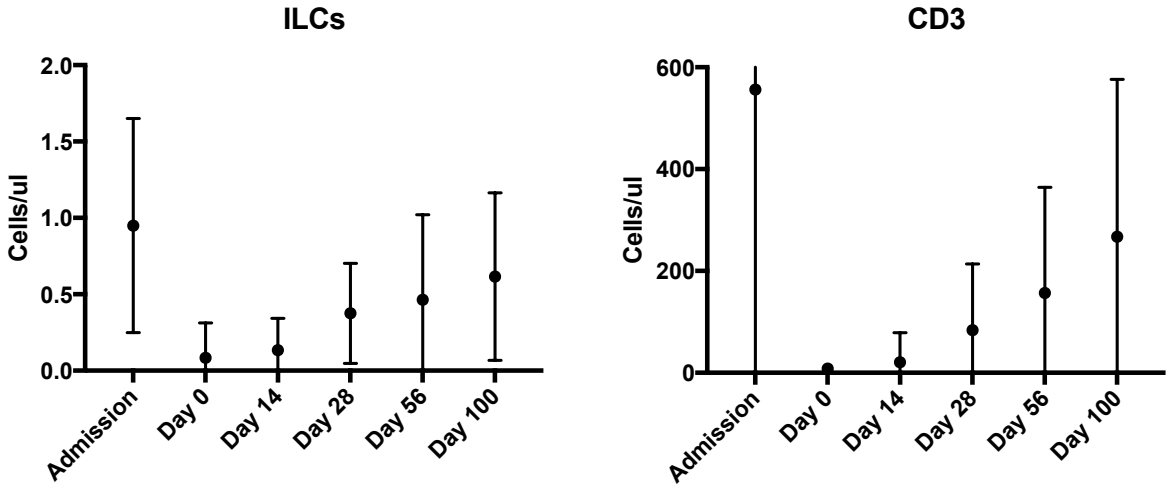

Figure 2.7 Graphs showing ILC and CD3 homeostasis from admission to 100 days post-transplant, with a nadir reached at D0 (mean with standard deviation). Neither cell type has recovered to admission levels by D100. Results from all 45 patients described in table 2.1 are shown.

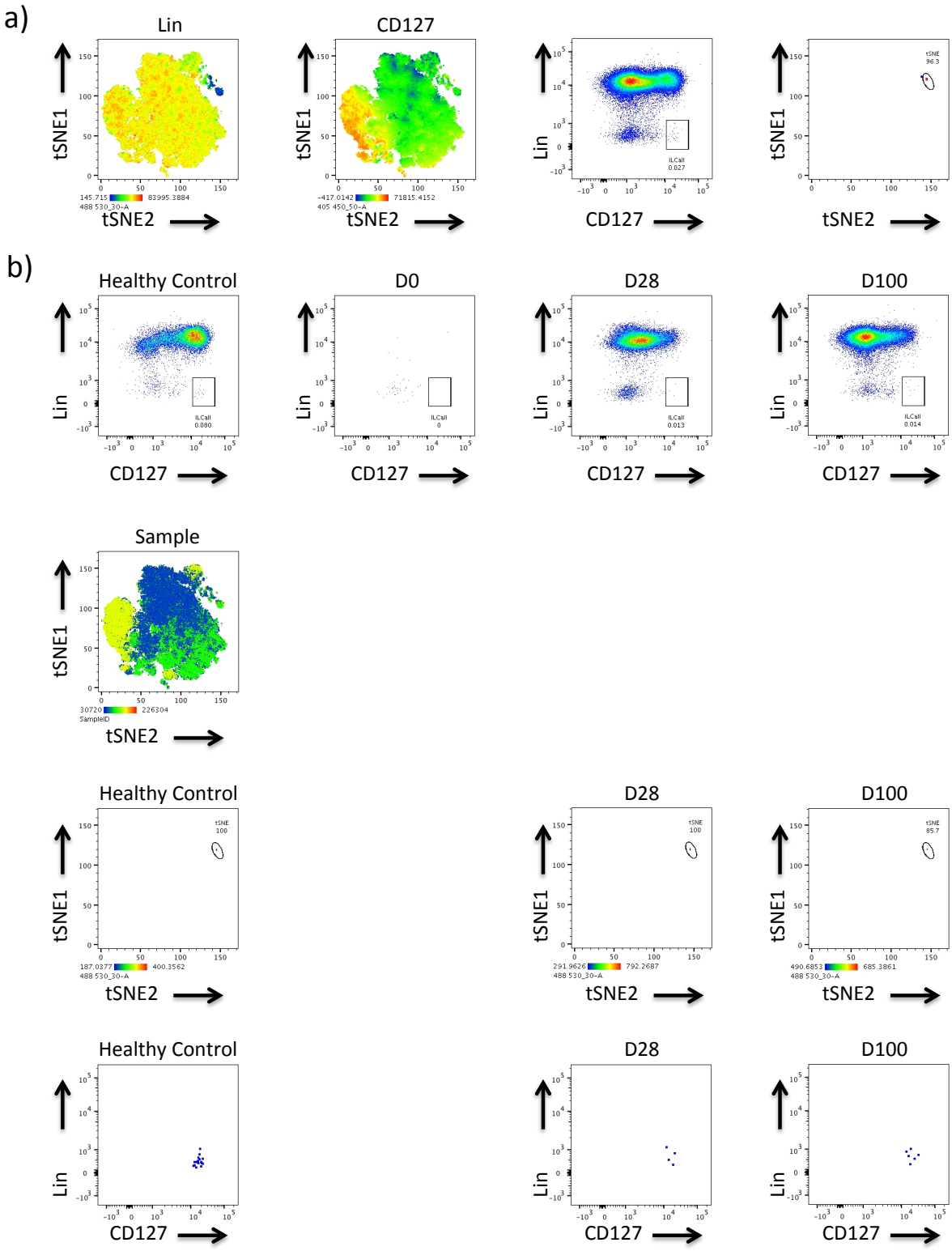

Figure 2.8 t-SNE plots of ILC reconstitution during the first 100 days. a) The top panel illustrates that the Lin- CD127+ cells form a cluster. b) The four columns show that this remains the case at the four time points (no ILCs at Day 0). Data shown represents one healthy control and one reconstituting patient at each time-point shown. At each time point illustrated, the ILC 'cluster' is located in the Lin- CD127+ quadrant, consistent with the known ILC population.

In order to confirm the identity of the small number of cells falling in the lineage- versus CD127+ gate, I performed a tSNE analysis of a healthy control sample concatenated with post-transplant samples. I identified the cluster corresponding to ILC in the tSNE map and confirmed that the cells in the lin- CD127 region, identified by conventional gating in the post-transplant samples, clustered together in the ILC region of the tSNE plot. This lends support to their identity as it takes into account all of the parameters in the experiment. A healthy control was combined with a Day 0, Day 28 and Day 100 sample (events were downsampled in proportion to the lymphocyte count of the sample and concatenated). Figure 2.8 shows the samples combined in the top row, and lymphocyte reconstitution over time in part (b). Clustering of the Lin- CD127+ gate is shown in the penultimate row, and the final row shows that this cluster falls in the appropriate Lin- CD127+ zone. This provides additional evidence that the appropriate cells fall in my ILC gates by standard flow cytometry methods.

2.3.7 ILC reconstitution and the impact of Alemtuzumab

Patients were subsequently divided into two groups, those who did and those who did not receive Alemtuzumab as part of their conditioning therapy (Figure 2.9). Given that I had previously demonstrated the presence of CD52 expression on ILCs, it was interesting to observe the more rapid ILC recovery in those patients who did not receive Alemtuzumab. This difference was significant at Day 28 and Day 56 post-transplant. Not surprisingly, this difference was also observed in the T cell population, where the difference was statistically significant at all time points from Day 14 onwards.

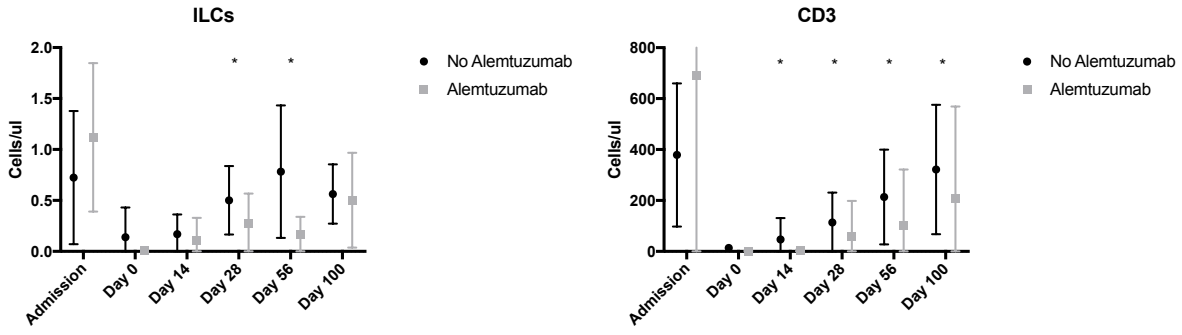

Figure 2.9 Graphs showing ILC and T cell recovery for all 45 patients according to use of Alemtuzumab. Statistically significant differences were seen for ILCs at Day 28 (p=0.04) and Day 56 (p=0.0001). For CD3+ cells, statistically significant differences were seen at Day 14 (p=0.04), Day 28 (p=0.002), Day 56 (p=0.002) and Day 100 (p=0.03) (Mann-Whitney).

ILCs are predominantly found at mucosal surfaces, so circulating ILCs may not be representative of total ILC numbers. It is, therefore, not possible to know whether these findings reflect the response of the total pool of ILCs to Alemtuzumab, or simply those found in the peripheral blood. The early kinetics are, however, interesting, suggesting that where the regimen does not include Alemtuzumab, some ILCs may survive conditioning therapy. Given this possibility, it was important to assess the percentage donor/recipient chimerism.

2.3.8 Chimerism

As previously discussed, there is an apparent mismatch between humans and murine models in relation to time to reach full donor chimerism, although tissue sources have varied. Day 28 chimerism was successfully analysed on three patient samples, with two fractions being sorted for each patient, a CD3+ subset and an ILC subset (Figure 2.10). In patients who had received T cell depletion, full donor chimerism was reached in both subsets by Day 28. In the patient receiving a T replete graft (A8139), T cells were 96% donor and ILCs 91% donor at Day 28. This would suggest that even if some ILCs do survive conditioning, within the first month post-transplant the majority of these have been replaced (in the peripheral blood) with cells of donor origin. Attempts were made to assess chimerism at earlier time points, but it was not possible to obtain sufficient cells for analysis.

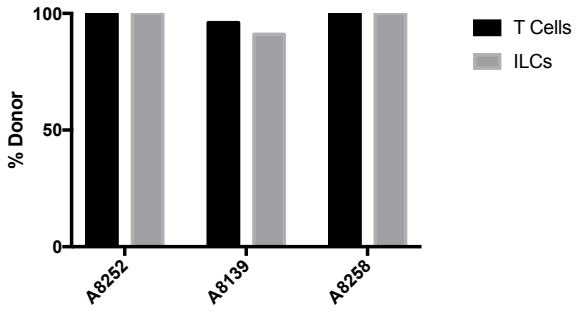

Figure 2.10 Graph showing percentage donor chimerism achieved at Day 28. In the two patients who had received T cell depletion, full donor chimerism was reached in both subsets by Day 28. In the patient receiving a T replete graft, with minimal conditioning chemotherapy (A8139), T cells were 96% donor and ILCs 91% donor at Day 28.

2.3.9 ILC reconstitution and aGVHD

Patients were retrospectively divided into two groups: aGVHD Grade 0-I and Grade II-IV, using the modified Glucksberg criteria (Glucksberg et al., 1974, Przepiorka et al., 1995).

Grade I GVHD was considered with Grade 0 because of the diagnostic difficulties of Grade I (especially Stage 1 skin) GVHD. For the purposes of this study, the classical definition of acute, i.e. occurring within 100 days of transplant, was used.

Of the 45 patients studied, ten developed Grade II-IV aGVHD. Of these, nine developed maximum Grade II (seven skin Stage 3, and two gut Stage 1, skin Stage 1) and one Grade III (gut Stage 2, skin Stage 2). Four of these were biopsy proven.

Cell recovery was then considered according to whether or not the patient developed aGVHD Grade II-IV or not (Figure 2.11). Perhaps surprisingly, no statistically significant differences were seen at any time point for either cell type. Potential confounding factors are considered in more detail below.

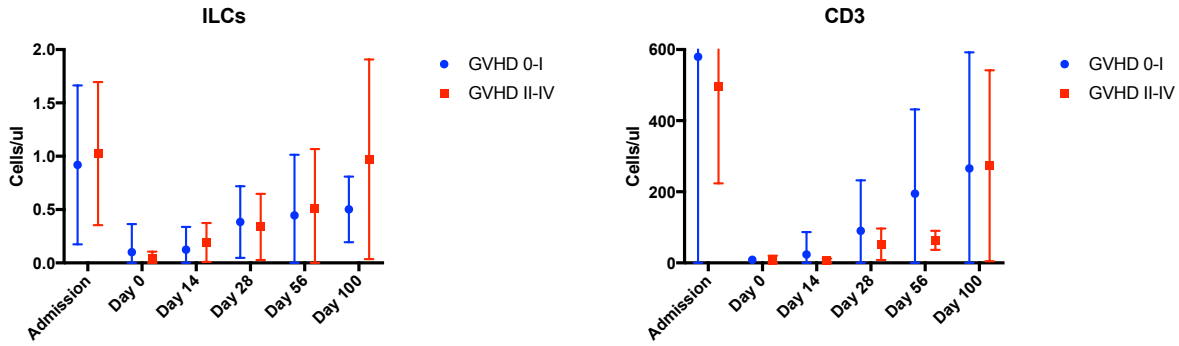

Figure 2.11 Graph showing ILC and CD3+ recovery for all 45 patients. The data were analysed according to whether or not the patient developed aGVHD Grade II-IV. No significant differences in reconstitution between the groups were seen at any time point for either cell type (Mann-Whitney).

2.3.10 Potential confounding factors

Potential confounding factors that might have impacted on recovery, or masked an effect of aGVHD were considered. Ciclosporine level at Day 28 did not correlate with either ILC or CD3 count at that point (r=0.06 and r=0.05 respectively) (Figure 2.12).

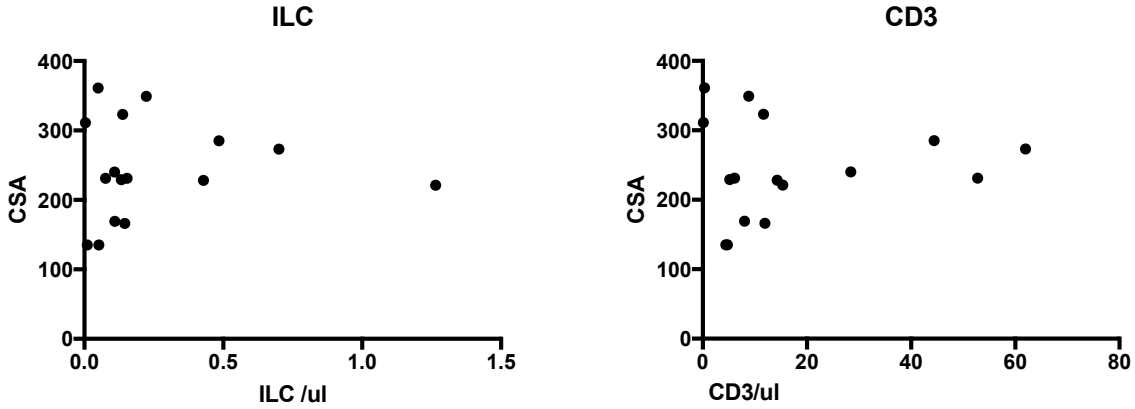

Figure 2.12 Ciclosporine levels at D28 were analysed to elucidate if this had an impact on ILC or CD3 reconstitution. No correlation was seen between ciclosporine level at Day 28 and ILC recovery (r=0.06) or CD3 recovery (r=0.05) (Spearman rank correlation).

The presence or absence of viral reactivation did, however, appear to have a significant effect on reconstitution. This was best illustrated by looking at the ratio of CD3 to ILC count. I would propose that this is because while either individual value can be affected by degree of engraftment, T cell depletion or graft composition, the ratio to some extent compensates for this. Figure 2.13 shows CD3/ILC ratio at Day 28 for patients who developed aGVHD Grade II-IV, who had reactivation of CMV or EBV to a level where pre-emptive therapy would be considered by Day 28 (one exception had a respiratory virus at Day 28) or neither. Only one of the patients with GVHD also had viral reactivation (with a CD3/ILC ratio of 57.22). Median time to onset of aGVHD for the group shown was 36 days post-transplant. These results suggest viral reactivation may mask differences in immune reconstitution related to GVHD. Amongst those patients who developed viral reactivation, there was a trend towards an increased CD3/ILC ratio in those who had not received Alemtuzumab (data not shown).

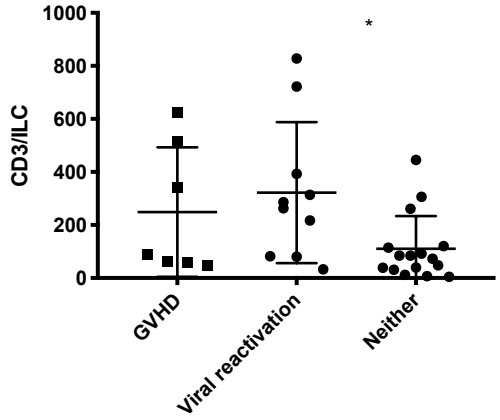

Figure 2.13 The impact of viral reactivation post transplant on cell recovery was investigated as another potential confounding factor. A comparison of CD3:ILC recovery for patients who developed Grade II-IV aGVHD, viral reactivation or neither is shown. A statistically significant difference is seen between those with viral reactivation vs neither p=0.02 (Mann Whitney). The differences between the other groups were not significant.

2.3.11 ILC recovery and IL-22

As this project was interested in the role of ILCs and IL-22, I explored the relationship between ILC recovery at Day 28 and serum IL-22 concentration (Figure 2.14). Red dots represent patients who already have aGVHD at this point, blue will go on to develop aGVHD, black never develop aGVHD.

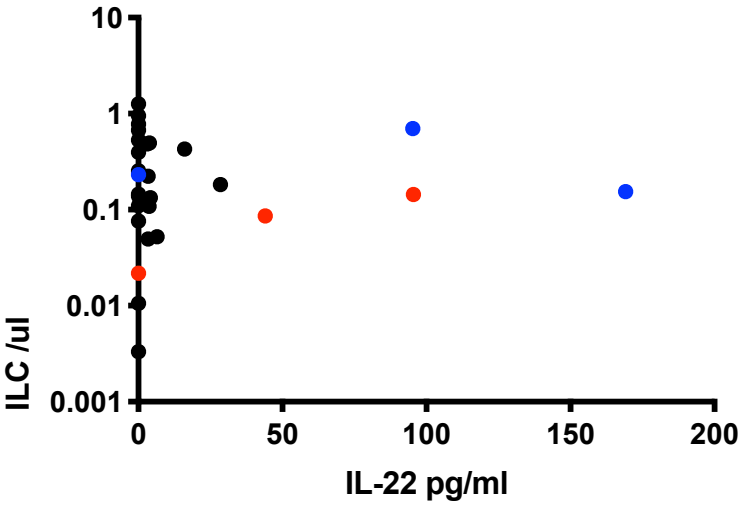

Figure 2.14 Relationship between ILC recovery and serum IL-22 at Day 28. Red dots represent patients who already have Grade II-IV aGVHD at this point, blue will go on to develop aGVHD Grade II-IV. No correlation between ILC recovery and serum IL-22 concentration was seen. High serum IL-22 levels may be associated with aGVHD, but further data are required.

As discussed above, data shown is for total ILCs, not ILC3s, but the data shown does not suggest any correlation between ILC recovery and serum IL-22 concentration. There is, however, a suggestion of elevated serum IL-22 in patients who have, or will develop, aGVHD. This is discussed further in Chapter 3.

2.3.12 IL-7 homeostasis post HSCT

Given the difference seen in reconstitution between patients who did and did not receive Alemtuzumab and known ILC dependence on IL-7, I investigated the impact of differing T cell depletion on serum IL-7 concentration post HSCT. The majority of the first phase of this work was performed by Edward Osmund, a BSc student, under my supervision.

In order to try to minimise heterogeneity between the patient groups, and to enable the work to be performed within the timeframe his project allowed, 16 patients transplanted between August 2014 and September 2016 were retrospectively selected, four in each group, based on method of T cell depletion (further clinical details are shown in Table 2.2). Serum IL-7 concentration was measured at five time-points: admission, Day 0, Day 14, Day 28 and Day 100 post-transplant, using ELISA as described above. Two patients were subsequently excluded from analysis (one due to a serum IL-7 concentration above the upper limit of the standard serial dilution, despite 1:10 sample dilution as described, the second due to a splenectomy shortly prior to HSCT).

	T replete	Alemtuzumab 60	Alemtuzumab 30	ATG
Disease	Myeloma (4)	AML (4)	AML (4)	AML (1) MPD (3)
Conditioning	Seattle (4)	Flu Mel (3) Flu Bu (1)	Flu Mel (3) Flu Bu (1)	Flu Bu (3) FLAMSA TBI (1)
Donor	MUD (2) Sib (2)	MUD (4)	MUD (1) Sib (3)	MUD (3) Sib (1)
Stem cell source	PBSC (4)	PBSC (4)	PBSC (4)	PBSC (4)

Table 2.2 Patient Characteristics, IL-7 homeostasis cohort

If one accepts the hypothesis proposed by Fry et al. (Fry et al., 2001) that IL-7 concentration will rise in response to induced lymphopenia, it would be anticipated that serum IL-7 concentration will rise following conditioning, and that this will be more pronounced the more intensive the lymphodepletion. Our data would support this theory – the greatest increase in serum IL-7 concentration was seen in the Alemtuzumab 60mg group, and the least in the patients receiving a T replete transplant (Figure 2.15).

The normal range quoted by the manufacturer for serum IL-7 is 0.267-9.80pg/ml (mean 2.51pg/ml) calculated from 32 healthy volunteers. The mean serum IL-7 concentration for all patients on admission was just above the quoted normal range (9.9pg/ml), with admission concentration highest in the Alemtuzumab 60mg group (17.8pg/ml). Given that the patients in this group all had AML, this is likely to represent response to the lymphopenia induced by the cycle of chemotherapy given prior to admission, although the Alemtuzumab 30mg group, also comprising of four patients with AML, have a mean IL-7 concentration of 4.0pg/ml on admission.

The peak in mean IL-7 concentration is seen at Day 14 for all three groups receiving T cell depletion, after which it begins to return towards baseline, although the mean concentration for all three T cell depleted groups at Day 100 is still above that measured at the time of admission. The maximum IL-7 concentration is seen in at Day 14 in the Alemtuzumab 60mg group, with a mean concentration of 90.7pg/ml, approximately nine times the upper limit of the quoted normal range.
The T replete group show a very similar rise to that seen in the ATG group between admission and Day 0. Rather than continuing to increase, this concentration then remains fairly constant until Day 28, and is returning towards baseline by Day 100.

Changes in lymphocyte count represent an almost inverse relationship to IL-7 concentration in relation to T cell depletion (Figure 2.15), although with a slight time lag such that by the time peak IL-7 levels are reached, lymphocyte recovery is beginning. The nadir was reached at Day 0, with lymphocyte recovery beginning by Day 14, with more rapid recovery seen initially in those patients who did not receive Alemtuzumab (and whose serum IL-7 concentration was lower). Many potential confounding factors may also impact T cell recovery, including degree of lymphodepletion resulting not just from T cell depleting agents, but also from additional conditioning agents, CD3 content of the graft, viral reactivation and graft versus host disease (and its treatment).

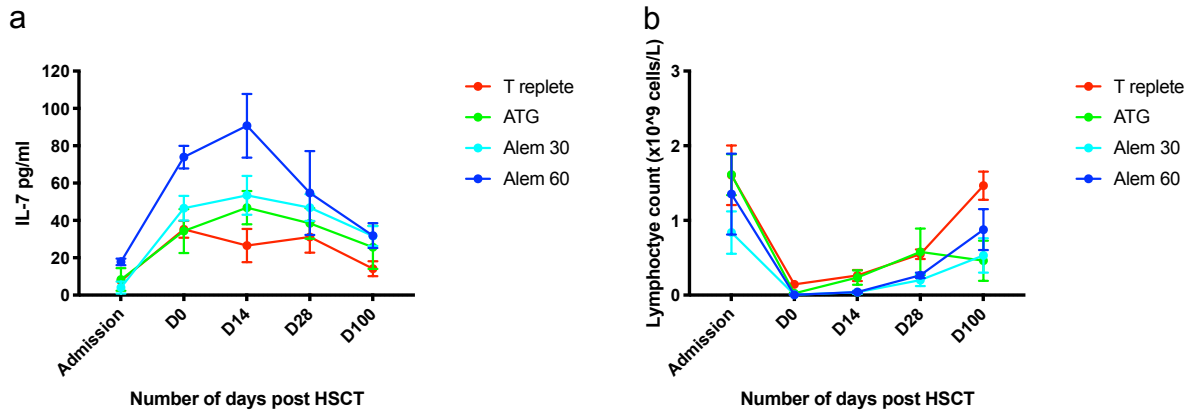

Figure 2.15 a) IL-7 concentration post HSCT for patients described in table 2.2, grouped by method of T cell depletion. Results of 14 patients shown. b) Total lymphocyte count at respective time-points (both graphs show mean with SEM). An inverse relationship between serum IL-7 concentration and lymphocyte recovery is shown.

2.3.13 IL-7, ILCs and GVHD

I subsequently collected serum samples for IL-7 measurement from patients in Cohort B, to enable me to assess the impact of IL-7 on ILC recovery.

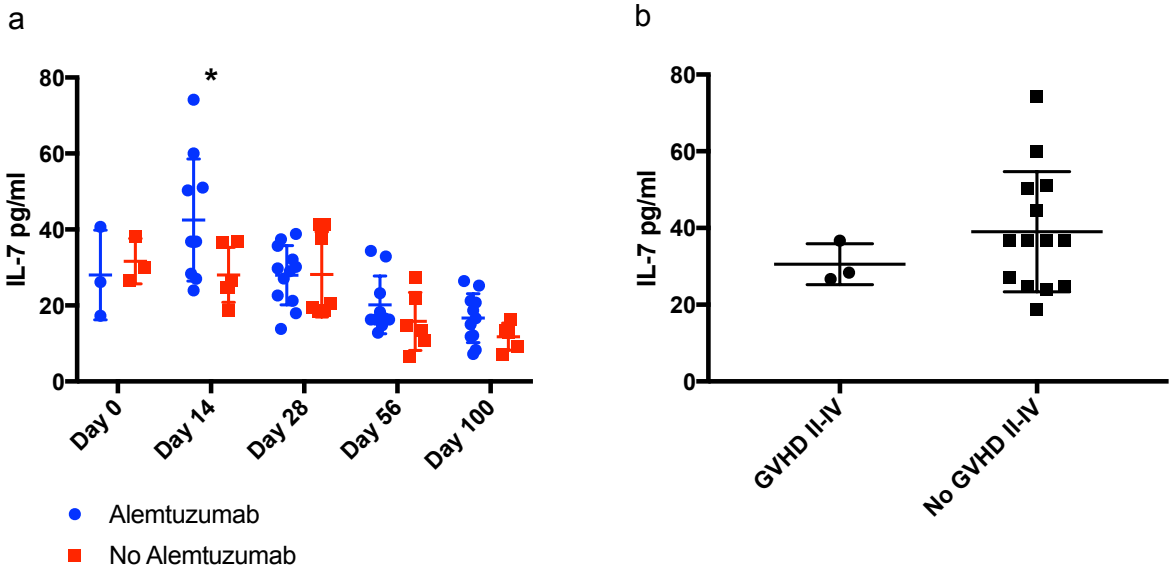

Figure 2.16 Data shown are for patients in Cohort B a) Peak IL-7 is reached at Day 14, with a significant difference between those who did and did not receive Alemtuzumab (p=0.04, Mann-Whitney). b) No significant difference in IL-7 concentration is seen at Day 14 for those who will and will not subsequently develop aGVHD.

As with the previous cohort, peak serum IL-7 concentration was reached at Day 14 (Figure 2.16), with a significant difference between those who did and did not receive Alemtuzumab (p=0.04, Mann-Whitney). In contrast to previously published data (Dean et al., 2008), no significant difference in IL-7 concentration was seen at Day 14 between those patients who subsequently would or would not develop aGVHD (p=0.45, Mann-Whitney). I would propose that any differences in IL-7 concentration related to aGVHD in my cohort has potentially been masked by the difference seen with Alemtuzumab (none of the patients in the Dean study received T cell depletion).

It has been suggested (Martin et al., 2017) that ILCs are better at utilising IL-7 than T cells. A potential relationship between IL-7 concentration and total ILC recovery was therefore investigated (Figure 2.17).

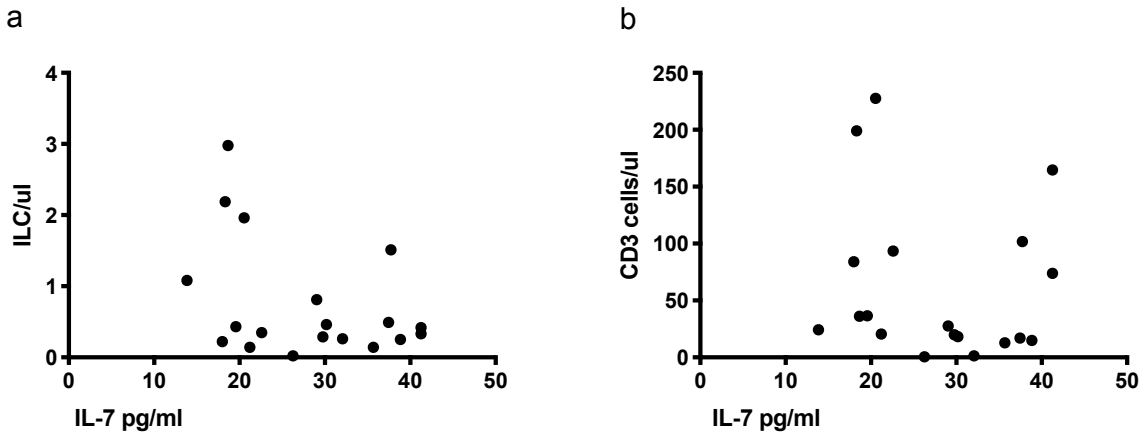

Figure 2.17 Serum IL-7 concentration and cell recovery at Day 28 is shown. No correlation was seen between serum IL-7 concentration and either ILC (a) or CD3 (b) recovery (data shown is for patients in Cohort B).

No correlation was seen at Day 28 between serum IL-7 concentration and either ILC or CD3 recovery. The potential for an exponential (one phase decay) relationship was explored, but was not found to apply. A difference in count recovery between patients who did and did not receive Alemtuzumab had been demonstrated for the whole cohort at Day 28 (Figure 2.9), but by Day 28 there was no statistically significant difference in serum IL-7 concentration between these two groups. At Day 14, where the difference in IL-7 concentration was observed, count recovery was not sufficient for meaningful comparison.

2.3.14 ILCs in donor sources

Flow cytometry was performed using panel ILCA. Samples from six different donors of bone marrow, mobilised PBSCs and cord blood were analysed. ILCs were considered as a percentage of live cells and as a percentage of total lymphocytes (Figure 2.18). Numbers are small, but, although there appears to be a trend towards an increased ILC content in cord blood, no statistically significant differences were seen between donor sources (Kruskall-Wallis, p=0.58 for live cells and p=0.28 for lymphocytes).

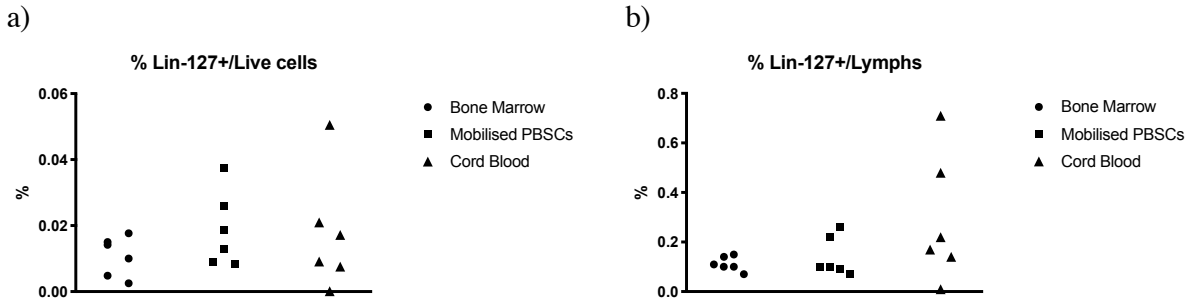

Figure 2.18 a) shows the ILC content of different graph sources in relation to live cells, and b) in relation to total lymphocytes. Although there is a trend towards increased ILCs in cord blood, this did not reach statistical significance.

2.4 Discussion for Chapter Two

This chapter explored ILC homeostasis in human HSCT. Samples were collected from 45 patients who underwent HSCT at our institution, and analysed using two methods of flow cytometry. The two cohorts were then combined for the final analysis. The major benefit of this strategy was to increase the total number of patients available for the statistical analysis, but it also had the advantage of the data being collected using two different methods. There is, however, potential for error in the calculations, due to the use of laboratory counts to derive absolute counts. In addition, due to the different panel used for the Trucount method, I was unable to perform ILC subsetting for these patients. Given the small number of circulating ILCs, especially in the early post-transplant period, however, I had already found I was unable to reliably subset the ILCs at the early time-points post-transplant. The results therefore were taken for the whole ILC cohort. I consider this the major weakness in this analysis, especially as differential rates of recovery between subsets have previously been demonstrated in humans (Munneke et al., 2014).

I have, however, been able to make some interesting and novel observations on the impact of T cell depletion on ILCs. Total circulating ILCs were reduced by transplant conditioning, with a nadir at Day 0. There was significant inter-patient variability, but early recovery was seen by Day 14. By Day 100, the mean count had not reached pre transplant levels.

I have demonstrated CD52 expression by ILCs, in keeping with the finding of Gross et al (Gross et al., 2016). It was therefore, perhaps not surprising that a difference was seen in both ILC and T cell recovery in patients who received Alemtuzumab versus those who did not. As ILCs are predominantly found at the body's barrier surfaces, it is not possible from this work to determine whether Alemtuzumab acts to deplete ILCs in all compartments, or simply in the peripheral blood.

Although no statistically significant difference between those patients who received Alemtuzumab and those who did not was seen at Day 0 and Day 14, it is interesting that the mean ILC count for patients who did not receive Alemtuzumab did not fall to 0. This may simply reflect different kinetics between patients receiving ATG, and those receiving T replete grafts or errors in detection, but it is worth noting that of the patients in whom chimerism was assessed at Day 28, only the patient receiving a T replete graft had not reached complete donor chimerism in both the ILC and CD3 compartments. This raises the possibility

that some recipient ILCs have survived the conditioning regimen. ILC resistance to radiotherapy has previously been demonstrated in a murine model (Hanash et al., 2012). The Seattle (T replete) regimen contains minimal chemotherapy (90mg/m^2 Fludarabine, in addition to the 200cGy TBI), when compared to the majority of other conditioning protocols used in our institution, so it may be that similar resistance is being seen. This observation warrants further investigation in humans. Given the difficulty of investigating a small population of cells in patients who have undergone treatment to further reduce cell numbers, especially where these cells are largely defined by absence of expression of antigens, an alternative method of investigation, such as mass cytometry (CyTOF) might be preferable.

The main focus of this project was the potential impact of ILCs on the development of GVHD. When looking at ILC recovery between patients who did or did not develop Grade II-IV aGVHD, no statistically significant difference was seen. A number of possibilities exist to explain this lack of difference. ILC reconstitution (or at least the ILC population as a whole) may not be a factor in the development of aGVHD. It may be that they do play a role, but that it is ILCs in the tissues and not in the blood that are important. It may be that they do play a role, but that the difference in numbers is masked by a confounding factor. It is worth noting that no statistically significant difference was seen in the T cell compartment either, although the importance of T cells in aGVHD is well documented. I therefore considered potential confounding factors. As discussed, a difference in reconstitution between patients who did, and did not receive Alemtuzumab was observed. In addition, a statistically significant difference was shown in CD3 to ILC ratio between patients who did and did not develop viral reactivation. It is therefore possible that any difference in reconstitution that might impact on aGVHD is masked by differences seen as a result of T cell depletion and viral reactivation. The heterogeneity of transplant patients makes it very difficult to avoid potentially confounding factors.

As this project was interested in a link between ILCs, IL-22 and aGVHD, potential correlation between ILC recovery and serum IL-22 was investigated. Again, this is taken with the caveat that this is total ILC recovery. There is, however, no evidence that ILC recovery correlates with serum IL-22 concentration in this cohort. Of note, the three patients who have already developed GVHD at the time-point illustrated all have low ILC counts, raising the possibility that GVHD itself reduces the number of ILCs. This has been observed in a mouse model (Hanash et al., 2012), where recipient IL-22+ ILCs were significantly reduced in GVHD. It was also interesting to note that the four patients with the highest serum IL-22 concentration

all had, or subsequently developed, aGVHD. The role of IL-22 in GVHD is discussed further in subsequent chapters.

The difference in rate of count recovery observed between patients who did and did not receive Alemtuzumab led to further investigation of the role of IL-7 in HSCT. It was interesting to document the mirror effect of lymphodepletion and serum IL-7. Although I remain unable to determine which of the proposed mechanisms of IL-7 homeostasis is correct (increased production in response to lymphopenia, or reduced consumption during lymphopenia), the correlation with different methods of T cell depletion is consistent with the importance of haematopoietic cells in regulating serum IL-7 concentration (Martin et al., 2017). I have not here demonstrated an interaction between serum IL-7 and GVHD.

Finally, we had hypothesised that the ILC composition between graft sources might explain some of the difference seen in GVHD potential not explained by the differing T cell content of different graft sources. Although there was a trend toward an increased ILC content in cord blood observed, I have not found a statistically significant difference in the ILC content of the graft sources measured.

The main strengths of this work include the relatively large number of patients involved, and that regular follow-up samples were obtained. Flow cytometry has been performed using two different methods, with comparable results. The major weakness is that I have been unable to perform ILC subset analysis. This is in part due to the small numbers of circulating ILCs, especially in the early post-transplant period, and also due to the difficulty in isolating cells by flow cytometry that are primarily defined by the absence rather than presence of antigens.

Chapter 3 The function of IL-22 in graft versus host disease

3.1 Introduction

3.1.1 IL-22R expression

In addition to the constitutive expression of IL-22R described in 1.4.1, several groups have demonstrated the potential for up-regulation of IL-22R *in vitro*, in HaCaT cell lines in response to IFN-γ (Wolk et al., 2004), in hepatocytes in murine models in response to concanavalin (Radaeva et al., 2004), or LPS (Tachiiri et al., 2003) and in human keratinocytes in a living skin equivalent model in response to IFN-α (Tohyama et al., 2012). It has also been shown that in UVB-irradiated primary human keratinocytes, rIL-22 increases the production of pro-inflammatory cytokines (IL-1α, IL-6 and IL-18) (Kim et al., 2017). The same study demonstrated an increase in IL-22R expression in the skin of mice and humans irradiated with UVB and thus suggests that skin inflammation due to UVB damage is partially due to the increased responsiveness to IL-22 of keratinocytes.

3.1.2 STAT1 versus STAT3 response and the role of IFNa

An increase in plasmacytoid dendritic cells (pDCs), professional Type 1 IFN producing cells, has been demonstrated in the gut (Bossard et al., 2012) and skin (Malard et al., 2013) of patients with aGVHD. IFN-α has been shown to increase IL-22R expression, as discussed above, but also to increase the signal transducer and activator of transcription factor-1 (STAT1) activating function of IL-22 (Bachmann et al., 2013). Bachmann and colleagues have shown in DLD-1 colon epithelial/carcinoma cells that pre-incubation with IFN-α resulted in increased STAT1 activation, while having only a minimal effect on STAT3. This increase in phosphorylated signal transducer and activator of transcription factor-1 (pStat1) was confirmed in primary keratinocytes. It was subsequently demonstrated that this increase in pStat1 translated into a significant increase in the mRNA expression of chemokines CXCL-9 and CXCL-10 when DLD-1 colon epithelial/carcinoma were pre-incubated with IFN-α and then stimulated with IL-22. IL-22 alone did not induce CXCL-10. Data on CXCL-10 mRNA production correlated well with protein production. CXCL9, CXCL10 and CXCL11 are the ligands of the receptor CXCR3, expressed mainly by activated or memory CD4+ and CD8+ T cells. This axis is known to be upregulated in inflammation (Flier et al., 2001, Schroepf et al., 2010, Marshall et al., 2017). Thus in the presence of IFN-α, IL-22 may contribute to the maintenance of a Th1 signal. pDCs have also been shown to increase differentiation towards a Th22 phenotype *in vitro* (Duhen et al., 2009). A combination of the mechanisms described

above has the potential to create a positive feedback loop, sustaining an inflammatory response.

3.1.3 IL-22 deficient T cells and SCT

The potentially detrimental role of donor derived IL-22 was briefly alluded to in Chapter 1. Two papers using murine models have explored the impact of donor-derived IL-22 on GVHD. Couturier *et al* (Couturier et al., 2013) report reduced aGVHD mortality and a reduction in histopathological grade of GVHD in the skin and colon of mice receiving IL-22$^{-/-}$ T cells when compared to wild type, with the gut scores when the IL-22$^{-/-}$ donors were used being comparable to syngeneic T cells. In addition they show that recipients of IL-22$^{-/-}$ cells have an increased proportion of CD25+ Foxp3+ Tregs at Day 6 post HSCT when compared to wild type recipients (with no difference in the Treg content of the graft). In a series of experiments using CD25-depleted T cells in both IL-22$^{-/-}$ and wild type donors they demonstrate that, in the absence of IL-22, both the conversion of naïve CD4+ CD25- T cells into Tregs and the expansion of existing Tregs appear to be more effective (the mechanism, is unclear – Tregs do not express IL-22R). Looking at the inflammatory response in the first week post HSCT, the recipients of IL-22$^{-/-}$ T cells have a reduction in plasma levels of TNF-α, IFN-γ and MCP-1 (monocyte chemoattractant protein 1). Looking at specific target organs, in the small intestine there was a reduction in IFN-γ, and the chemokines CXCL9, CXCL10 and CXCL11. In the skin there was a reduction in expression of β-defensin 3 and calthelicidin. These results suggest (donor) IL-22 deficiency may protect against GVHD through both reduction in recruitment of effector cells and an increase in Tregs. Importantly, they were also able to demonstrate that the GVL effect was preserved. The authors state that they did not observe any non T cell source of IL-22, but they are not able to exclude the presence of radio-resistant ILCs.

Further work (Lamarthee et al., 2016a) showed that at seven days post-transplant, pSTAT-1 was higher in the gut of recipients of wild type than IL-22$^{-/-}$ T cells. In vitro they demonstrate, like Bachmann *et al* (Bachmann et al., 2013), that cxcl10 mRNA is significantly induced in the presence of IFN-α and IL-22, when compared to either alone. This effect was lost in the presence of the JAK1/2 inhibitor Ruxolitinib. In a minor HA mismatched mouse model, with IFNAR$^{-/-}$ (IFN receptor deficient) recipients and wild type and IL22$^{-/-}$ donors, it was shown that only a combination of IFNAR$^{-/-}$ and IL22$^{-/-}$ donor T cells resulted in a statistically significant reduction in intestinal barrier destruction (as shown in a FITC-dextran model). These mice also had reduced circulating levels of IFN-α and IFN-γ. In a group of patients,

with and without GVHD, there was increased pSTAT1 and CXCL10 in the gut biopsies of those with acute GVHD, suggesting the previous findings are also relevant in humans. The model proposed by the authors is that pDCs secrete IFN, and activated donor T cells produce IL-22. These activate STAT-1, resulting in increased CXCL10 secretion from the intestinal epithelial cells. In turn this induces increased infiltration by Th1 cells, thus increasing GVHD.

3.1.4 Cytokine polymorphisms

The Hardy-Weinberg equilibrium, described separately by Hardy and Weinberg in 1908, states that, "in the absence of migration, mutation, natural selection, and assertive mating, genotype frequencies at any locus are a simple function of allele frequencies" (Wigginton et al., 2005). Cytokine polymorphisms present a way of indirectly investigating the involvement of a particular cytokine on the development of a specific condition. A number of polymorphisms have already been demonstrated to impact on the development of GVHD (Dickinson and Holler, 2008, Goussetis et al., 2011, Masetti et al., 2015, Broen et al., 2011, Middleton et al., 1998).

3.1.5 IL-22 polymorphism in disease

Polymorphisms of IL-22 have been shown to associate with a variety of malignant, inflammatory and infective conditions, including ulcerative colitis (Chi et al., 2014), gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Liao et al., 2014), non-small cell lung cancer (NSCLC) (Liu et al., 2014), gastric cancer (Qin et al., 2015) and hepatitis C (Hennig et al., 2007). Some groups have gone further, to try to establish a functional reason for this difference. In the study on gastric MALT lymphoma, for example, PBMCs from patients with ten versus two risk alleles were co-cultured with *H. pylori* (a known risk factor for gastric MALT lymphoma). Difference in IL-22 production was not significantly different, although there was a trend towards lower production with the increased number of risk alleles. In the Liu study of 324 individuals, using a case-control design, a CT genotype at rs2227484 (a nearby variant of rs2227485) showed an odds ratio of 1.9 for NSCLC when compared to a CC genotype ($p=0.038$). Plasma levels of IL-22 were subsequently measured. Plasma IL-22 was significantly increased in patients with NSCLC compared to healthy controls, and also for patients with advanced compared to early stage disease. When the IL-22 levels were compared by genotype, no statistically significant difference was seen between genotypes in the control group, but higher plasma IL-22 levels were observed for the CT compared to CC genotype for patients with NSCLC.

3.1.6 Normal range for IL-22

No standardised reference range for serum IL-22 in healthy individuals has been established (details of control values given with the ELISA used in this project are discussed in section 3.2.4). Different groups have published healthy control values for comparison with the group under investigation. These published ranges vary quite widely; Median 4.6pg/ml (range 4.6-4.6pg/ml) (de Oliveira et al., 2015), 12.58 +/- 12.59pg/ml (Meephansan et al., 2011), 20+/-13pg/ml (Ruggeri et al., 2014) and mean 67.45pg/ml (da Rocha et al., 2012). Common to all of these studies, however, is an increase in serum IL-22 in the study group (rheumatoid arthritis, psoriasis and auto-immune thyroiditis).

3.1.7 Questions for Chapter 3

Having proposed that ILC derived IL-22 protects target organs from GVHD, this chapter aims to further analyse the IL-22 axis in GVHD. Specific questions addressed are listed below.

1. How does transplant conditioning therapy (full and reduced intensity) impact on IL-22 expression?

2. Is there evidence for a change in IL-22 expression in GVHD?

3. Are ILCs responsible for the IL-22 signature in cutaneous GVHD?

4. Is expression of the IL-22R altered during GVHD?

5. Does IL-22 gene polymorphism impact on incidence or outcome of GVHD?

Aims:

1. To use a combination of gene expression data and ELISA to explore the impact of conditioning therapy on IL-22 expression in skin and serum

2. To use the same methods to explore IL-22 expression in GVHD

3. Using flow cytometry, to investigate the ILC population in the skin in GVHD, and to determine whether these cells are the source of IL-22

4. Using immunohistochemistry, to explore expression of the IL-22 receptor in GVHD

5. To explore the impact of a single nucleotide polymorphism for the IL-22 gene on the development of GVHD

3.2 Methods for Chapter three

3.2.1 Sample collection

Skin samples in this chapter came from two sources. Healthy skin used was surplus tissue obtained from plastic surgery, primarily breast and abdominal surgery. Ethical approval for samples from healthy donors was granted by the NRES Committee North East – Newcastle & North Tyneside 1 (17/NE/0361). Where skin samples were from patients undergoing HSCT, shave biopsies were taken either specifically for research purposes, or, where the patient had a rash suggestive of GVHD, material excess to that needed for diagnostic purposes was taken from clinical samples. Ethical approval for patient samples was granted by the NRES Committee North East – Newcastle & North Tyneside 2 (14/NE/1136).

Serum sample collection and storage is described in 2.2.1 and 2.2.10.

DNA samples were obtained from the EuroTransplantBank repository. All patients consented for sample collection and molecular testing. The project was approved by the Newcastle and North Tyneside 1 Research Ethics Committee, and the Ethics Committee of the University Hospital, Regensburg, Germany. Correlative clinical data were obtained from the EuroTransplantBank database (www.eurotransplantbank.org).

3.2.2 Media and buffers

Details of media and buffers used in this chapter are provided in Appendix 2.

3.2.3 Nanostring

NanoString nCounter allows the detection of mRNA gene expression without the need for pre-amplification, thereby removing potential pre-amplification bias. Genes of interest are multiplexed with fluorescently labelled probes, and can then be read by a computerised optical lens. The panel used in this study was the 579 probe NanoString Human Immunology V2^{TM} code set.

Skin biopsies were lysed in RLT lysis solution (RLT lysis buffer, Qiagen) with 1% 2-mercaptoethanol (Sigma Life Science), and then homogenised using a 21g needle. Samples were stored at -80^{0}C.

For the full intensity and GVHD samples, 150ng of RNA was used, with samples made up to 10µl using nuclease free water. 250ng of RNA, made up in 5µl of nuclease free water was

used for the reduced intensity samples. Hybridised mRNA was captured using a NanoString cartridge and Prep Station. The cartridge was screened using a NanoString Digital Analyser at 550 fields of view.

Raw NanoString data was collected and normalised using the Nanostring nSolver v3.0 software. Analysis was then performed using nSolver Advanced Analysis, allowing for differentially expressed genes to be revealed. P-values generated by a two-tailed test were adjusted using the Benjamini-Hochberg false-discovery rate (FDR). Quality control (QC) removed genes that did not pass this check, allowing for generation of volcano plots using the 'ggplot' package in RStudio. Work up to, and inclusive of, the generation of volcano plots was performed by A Resteu, with the assistance of L Sayer (an MSc student in our laboratory). Further interpretation of the results was performed by myself.

3.2.4 IL-22 quantification

IL-22 quantification was performed using a human IL-22 Quantikine enzyme-linked immunosorbent assay (ELISA) Kit (R&D systems). The assay was performed according to the manufacturers instructions. Briefly, a dilution series was produced using the human IL-22 standard provided. 100µl of standard or control were placed in each well of a 96 well plate and incubated for two hours at room temperature. Each well was washed four times with wash buffer and 200µl of human IL-22 conjugate was added to each well. The plate was then incubated for a further two hours at room temperature. Each well was then washed a further four times, after which 200µl of substrate solution was added per well. The plate was incubated for a further 30 minutes in the dark at room temperature. 50µl of stop solution was then added per well, and the optical density was measured using a microplate reader (Multiskan Ascent, Thermo Labsystems, Thermo Fisher Scientific), set at 540nm and 450nm. To calculate the values, the readings at 540nm were subtracted from those at 450nm. All values were log transformed, and non-linear regression was performed using GraphPad Prism (version 7).

The mean minimum detectable dose of the assay is reported to be 2.7pg/ml. The product datasheet reports 53 serum IL-22 concentrations. Of these only 4% are detectable, with a mean of detectable value of 35.7pg/ml.

3.2.5 Mononuclear cell isolation from skin biopsies

Skin shave biopsies were cut into fragments measuring approximately 1mm x1mm. These were placed in RF-10 with 1:100 collagenase and incubated overnight at 37^0C in a humidified, 5% CO_2, 95% air incubator. After 16-18 hours, the sample was dissociated using a pipette, filtered through a 100µl filter and washed with PBS. Total cell count was estimated using a 1:1 dilution with trypan blue exclusion dye (Invitrogen) on an Improved Neubauer Haemocytometer (Weber Scientific International).

3.2.6 Flow cytometry

For extracellular staining, cells were processed as above, then re-suspended in 50µl flow buffer. 4µl anti-mouse IgG (Sigma Aldrich) was added to the sample and incubated for 4 minutes. Antibodies were then added as per panels ILCA and ILCB, as described in 2.2.6. and incubated at 4^oC in the dark for 30 minutes. The cells were washed in flow buffer (500g for 5 minutes) and re-suspended in 200µl flow buffer. 4,6-diamidino-2-phenylindole (DAPI, Partec) was used to exclude dead cells.

For intracellular staining, cells were processed as above, then placed in 1ml of RF-10, in a flat-bottomed 24 well plate, at a concentration of $1x10^6$/ml (stimulated, unstimulated, isotype controls). Starting from a published protocol (Withers et al., 2016), and titrating concentrations and duration of incubation, stimulation was with 25ng/ml PMA and 1000ng/ml Ionomycin (Sigma Aldrich) for four hours, with 10µl /ml Brefeldin A (Sigma Aldrich) added at one hour. Cells were then transferred to a 96 well V bottomed plate, washed twice with PBS and re-suspended in 100µl zombie acqua (BioLegend®)/well. After staining for 20 minutes in the dark, cells were washed with RF-10 and re-suspended in surface stains/flow buffer. Cells were stained for 30 minutes in the dark at 4^0C. Cells were then washed, and incubated for 20 minutes at 40^0C in Fixperm (BD, Biosciences), after which they were washed twice and re-suspended in intra-cellular stains/Perm buffer (BD, Biosciences). Cells were stained for 30 minutes in the dark at 4^0C. Cells were washed twice with Perm Buffer, re-suspended in flow buffer and transferred to FACS tubes (BD, Biosciences).

3.2.7 Immunohistochemistry

IL-22 receptor staining was performed by the Molecular Pathology Node, Newcastle upon Tyne Hospitals NHS Foundation Trust. Staining for the antibody had previously been optimised within the department. The antibody used was IL-22 R alpha 1 Antibody, NBP2-38496, Novus Biologicals, at a concentration of 1:750. Staining was performed on a

Discovery IHC machine (Ventana). Positive cells were counted by two blinded, independent assessors. Images were taken with an Axio Imager microscope (Zeiss).

3.2.8 SNP selection and processing

A literature review identified IL-22 single nucleotide polymorphisms (SNPs) that had been previously shown to be associated with malignancy, infection and auto-immune disease. Ten common polymorphisms of IL-22 are known, of which eight are within a block having high linkage disequilibrium (Weger et al., 2009). One SNP within this block (rs2227485), which has also been linked to ulcerative colitis (Chi et al., 2014), gastric mucosa-associated lymphoid tissue lymphoma (Liao et al., 2014) and papillary thyroid cancer (Eun et al., 2013) was identified as a potential candidate for investigation (Figure 3.1). Located in the promoter region (Eun et al., 2013, Hu et al., 2014), the allele distribution in the population is almost equal, with a minor allele frequency of 0.4846 (dbSNP, 1000 genomes). Published functional studies have shown the TT genotype of this SNP to correlate with high levels of IL-22 (De Luca et al., 2013).

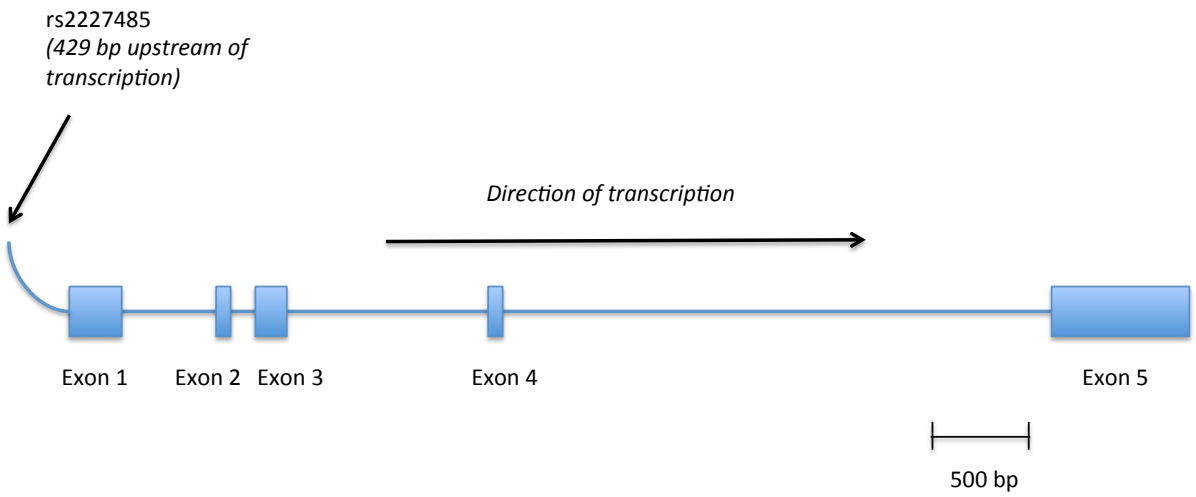

Figure 3.1 A graphical representation of the IL-22 gene. The SNP rs2227485 is located in the promoter region, 429 base pairs upstream of the transcription start site.

Donor and recipient DNA samples from two transplant centres that use the EuroTransplantBank repository (Northern Centre for Bone Marrow Transplantation, UK and University Hospital Regensburg, Germany) were outsourced to LGC Genomics Ltd for SNP typing at rs2227485. The company use KASP^{TM} genotyping, using competitive allele-specific PCR, which allows bi-allelic scoring of SNPs at pre-determined loci. The overall clinical

aGVHD grade was diagnosed by the patients' clinicians, in accordance with the modified Glucksberg criteria (Glucksberg et al., 1974, Przepiorka et al., 1995).

3.2.9 SNP Study, statistical analysis

Kaplan-Meier survival curves were plotted, and the differential survival between the CCTT group and other genotypes was compared using the log-rank test.
The impact of the chimeric genotype on (i) incidence of death due to GVHD, and (ii) incidence of non-relapse mortality, was assessed using the semiparametric proportional hazards regression model proposed by Fine and Gray (Fine and Gray, 1999) via a competing risks analysis as implemented by Scrucca et al., (Scrucca et al., 2007, Scrucca et al., 2010) which allows for multivariate assessment of clinical covariates in the model. The competing risk for (i) death due to GVHD is death due to relapse or other non-relapse, non-GVHD causes; and the competing risk for (ii) non-relapse mortality is relapse.

The impact of the chimeric genotype on incidence of aGVHD (Grade II-IV) was assessed by fitting a logistic regression model. Simple univariate statistical analyses were used to screen potential clinical factors for inclusion in the regression model. A p-value of 0.2 was used for the χ^2 tests, and the following factors were identified as possible confounding variables: underlying disease (leukaemia or other), stem cell source (bone marrow or peripheral blood), Centre (Newcastle or Regensburg), donor match (matched unrelated or other), whether the patient had a "female to male" transplant or not, whether donor and/or recipient were CMV positive or not, time of transplant (before or after 2005), conditioning intensity (reduced intensity or not), and whether the transplant was T-cell replete or depleted. A stepwise procedure minimising the Bayesian Information Criteria (BIC) (Schwarz, 1978), and checking that all terms were significant in the model, was used to select the best regression model.

Statistical software R, version 3.5.1 (http://www.r-project.org/) and JMP®, Version 13 (SAS Institute Inc., Cary, NC, 2016) were used for the computations. The R function *CumIncidence* (based on package *cmprsk*) and package *crr* were used to estimate cumulative incidence curves.

The statistical analysis for the SNP study was performed by C Lendrem, with input from myself.

3.3 Results

3.3.1 IL-22 induction in conditioning

It has been proposed that the mechanism by which recipient IL-22 is protective in SCT is through its induction during transplant conditioning, lessening target organ damage, thus reducing activation of APCs and so limiting phase one of the three phase model of GVHD. This theory of protective IL-22, however, is based on the findings of a mouse model, where transplant conditioning was performed with irradiation alone, to which ILCs have been shown to be resistant, and where recipient ILCs persist at three months post-transplant. In addition, the differences noted in pathology were observed in the gut and liver, but not the skin (Hanash et al., 2012). To my knowledge, the response of IL-22 in humans post-transplant conditioning has not been documented, but it has previously been shown that circulating human ILC3s are primarily of donor origin by seven weeks post-transplant (Munneke et al., 2014). I therefore investigated the response of IL-22 to conditioning in humans undergoing full or reduced intensity conditioning.

3.3.2 Full intensity

Paired skin biopsies were taken on admission and Day 0 from six patients undergoing SCT for acute lymphoblastic leukaemia. All received conditioning with cyclophosphamide, total body irradiation and Alemtuzumab (either 30mg or 60mg). Further clinical details are shown in Table 3.1. One admission sample was excluded from analysis after not passing QC measures. Differential gene expression between the two time-points was analysed using NanoString technology. 348 genes showed differential expression between admission and Day 0, illustrated in the volcano plot in Figure 3.2. Those with a positive fold change showed increased expression in the Day 0 skin when compared to the admission sample. The genes with a Benjamini-Hochberg adjusted p value of less than 0.05 are shown in black. Neither IL-22 nor IL-22R are shown, as they did not meet the QC measures of the nCounter advanced analysis module. Genes that do not achieve a minimum number of counts in at least half of the samples are filtered out by the software and omitted from the differential expression analysis. This was the case for IL-22 in all of the following analyses. The genes showing the greatest up-regulation, CUL9 (log2 fold change (FC)=1.88), BAX (log2 FC=1.8), TNFRSF10 (log2 FC=1.89) and CDKN1A (log2 FC=1.54) have all been previously linked to DNA damage and cell death (Li and Xiong, 2017, Oltvai et al., 1993, Sheikh et al., 1999, Hussain et al., 2017). Down-regulation of the IL-2Rβ may simply reflect the reduction in leucocytes seen at the end of conditioning. There is, perhaps, less up-regulation of inflammatory cytokines

than might be expected. It would be interesting to compare these findings in the skin with other target organs of aGVHD.

Serum IL-22 concentration was also compared for four patients undergoing full intensity conditioning on day of admission and Day 0. Three patients received cyclophosphamide and TBI (with or without Alemtuzumab) and one TBI alone (myeloablative haploidentical protocol). IL-22 concentration was measured using ELISA, as detailed in 3.2.4. No significant difference was seen between pre and post conditioning samples ($p= 0.875$, Wilcoxon signed rank test), with IL-22 concentration on day of admission ranging from undetectable to 12.8pg/ml, and on Day 0 from undetectable to 14.4pg/ml, with a median value of 7.0pg/ml and 7.8pg/ml respectively (Figure 3.3). The reason why the levels at both admission and Day 0 are slightly higher in the full intensity than the reduced intensity group is not clear, but all the values in both groups fall within the range of levels I recorded in healthy donors.

	RIC	MAC	GVHD	Controls
n	6	6	23	6
Conditioning	Flu Bu A (4) Flu Mel A (2)	Cy TBI A (6)	Flu Mel A (11) Flu Bu A (7) Cy TBI A (3) Flu Cy TBI (1) Seattle (1)	NA
Disease	AML (4) ALL (1) HD (1)	ALL (6)	AML/MDS (13) ALL (3) HD (1) NHL (4) Other (2)	NA
Donor	MUD (4) Sib (2)	MUD (3) Sib (3)	MUD (18) Sib (4) Haplo (1)	NA
GVHD	NA	NA	Gd 2+ (18)	NA

Table 3.1 Patient characteristics of samples included in gene expression study

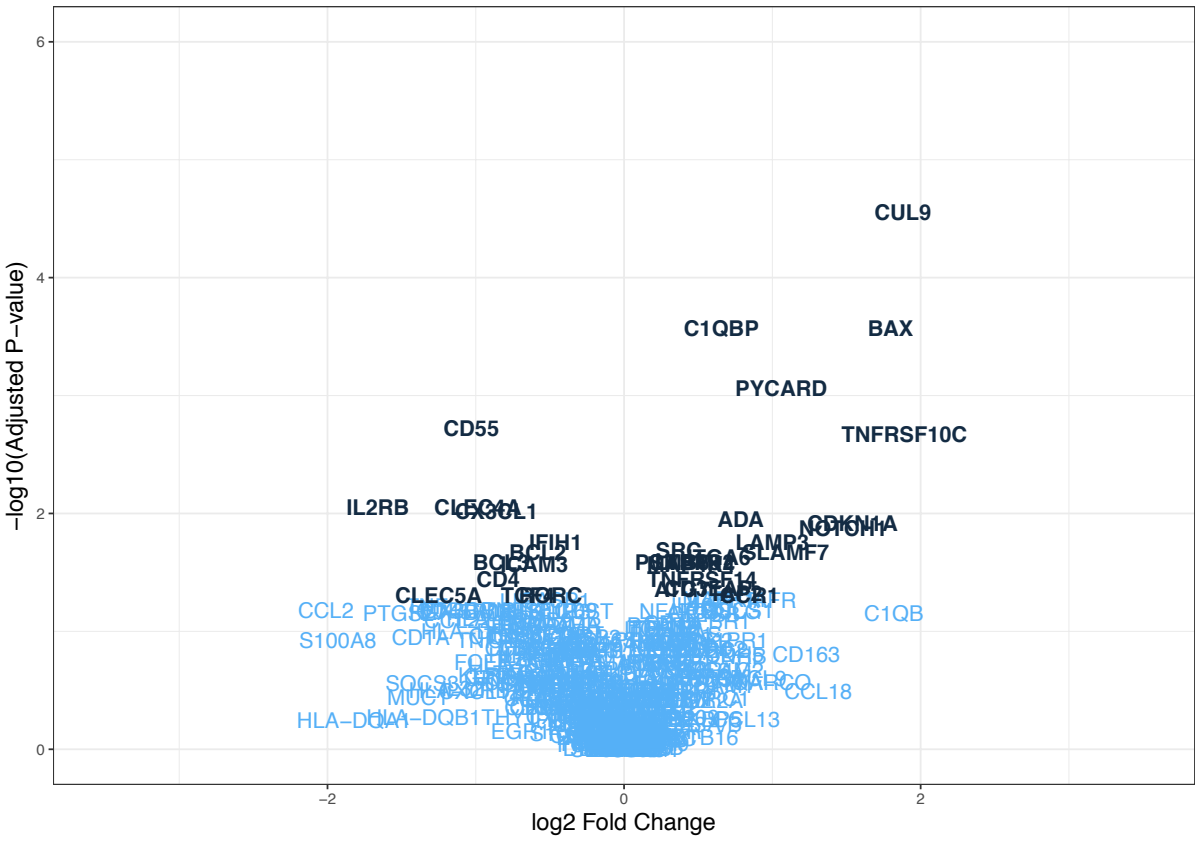

Figure 3.2 Genes differentially expressed between admission and Day 0 from skin biopsies of six patients receiving full intensity conditioning. Genes showing an increased fold change were up-regulated post conditioning. Those shown in black showed have an adjusted p-value of <0.05. The genes showing the greatest up-regulation have been previously linked to DNA damage and cell death.

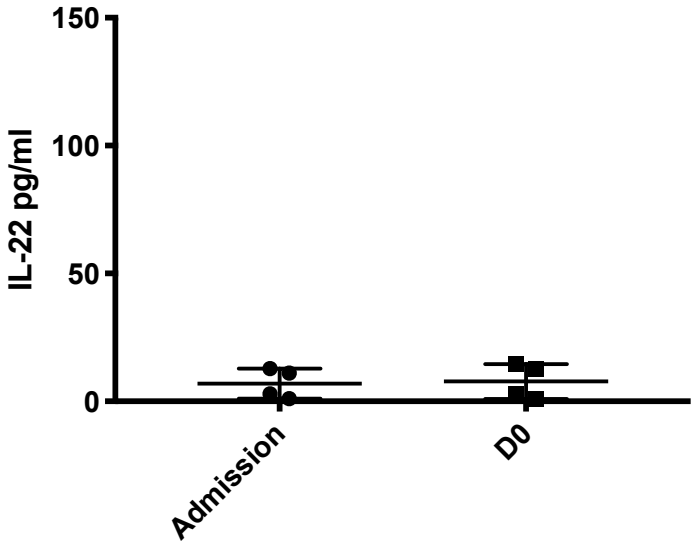

Figure 3.3 No statistically significant change was seen in the serum IL-22 concentration between admission and Day 0 for patients receiving full intensity conditioning.

3.3.3 Reduced intensity

Using the same techniques, paired skin biopsies were taken from six patients undergoing reduced intensity conditioning with Fludarabine, either Melphalan or Busulphan and Alemtuzumab. Indication for SCT was acute myeloid leukaemia (5), acute lymphoblastic leukaemia (1) and Hodgkin lymphoma (1). Two admission samples were excluded from the analysis as they did not pass the QC measures. 381 genes were found to be differentially expressed, when comparing admission samples to Day 0. The IL-22R had a log2 fold change of 0.165, but with a p value of 0.996. The most interesting finding was the lack of a statistically significant difference in expression of any gene following reduced intensity conditioning (Figure 3.4). Potential explanations for this include sample size or timing of the biopsy (it is possible, for example, that the maximal impact of conditioning is seen at a later time point), but absence of a 'damage' signal calls into question the applicability of the conditioning induced tissue damage as the first step in the development of aGVHD in reduced intensity conditioning.

Paired serum samples from five patients undergoing reduced intensity conditioning (further details in Appendix 1) were also tested for IL-22 concentration on admission and Day 0. Admission values ranged from undetectable to 3.9pg/ml, and Day 0 from undetectable to 2.6pg/ml ($p=>0.99$, Wilcoxon signed rank test). The median value was undetectable on both occasions (Figure 3.5).

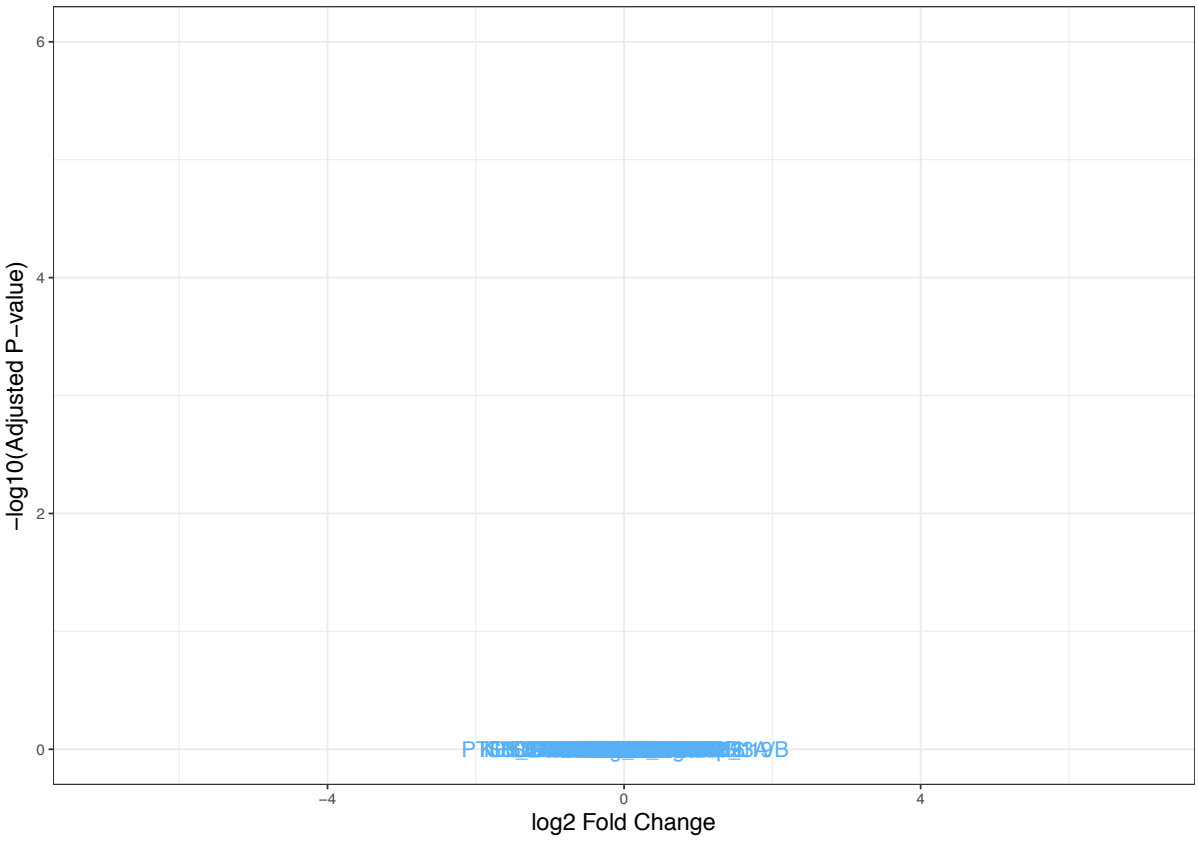

Figure 3.4 Genes differentially expressed between admission and Day 0 from skin biopsies of six patients receiving reduced intensity conditioning. Genes showing an increased fold change were upregulated post conditioning. Interestingly, no statistically significant differences were seen.

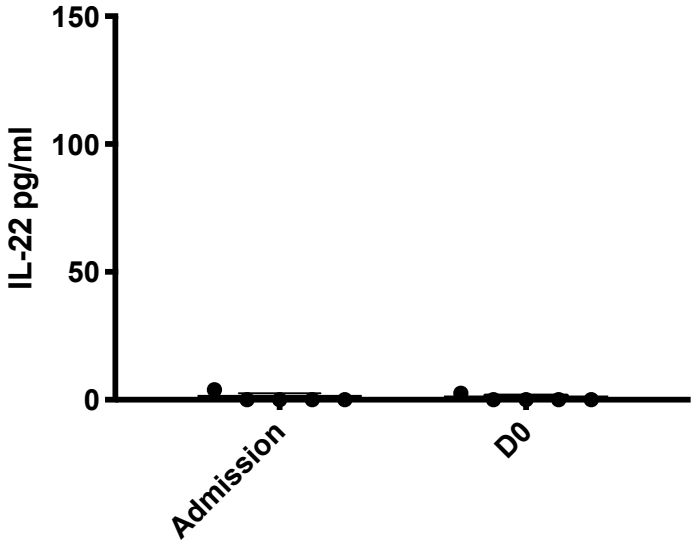

Figure 3.5 No statistically significant change was seen in the serum IL-22 concentration between admission and Day 0 for patients receiving reduced intensity conditioning.

3.3.4 IL-22 induction in GVHD

Having found no evidence of IL-22 induction during conditioning, I next investigated whether IL-22 levels were elevated in GVHD. 35 skin biopsies were initially analysed, 22 with GVHD meeting the histopathological criteria for Grade 2+ ('Grade 2+'), seven performed as possible GVHD, but not histopathologically confirmed ('rash') and six healthy controls ('HC'). Four of the patient biopsies were removed for clinical reasons (onset too late for acute GVHD, and not post DLI) and two samples did not meet QC requirements. Clinical details for the samples in the final analysis are shown in Table 3.1. Samples were analysed twice, comparing 'Grade 2+' (n=18) versus 'rash' (n=5), and 'Grade 2' versus 'HC'.

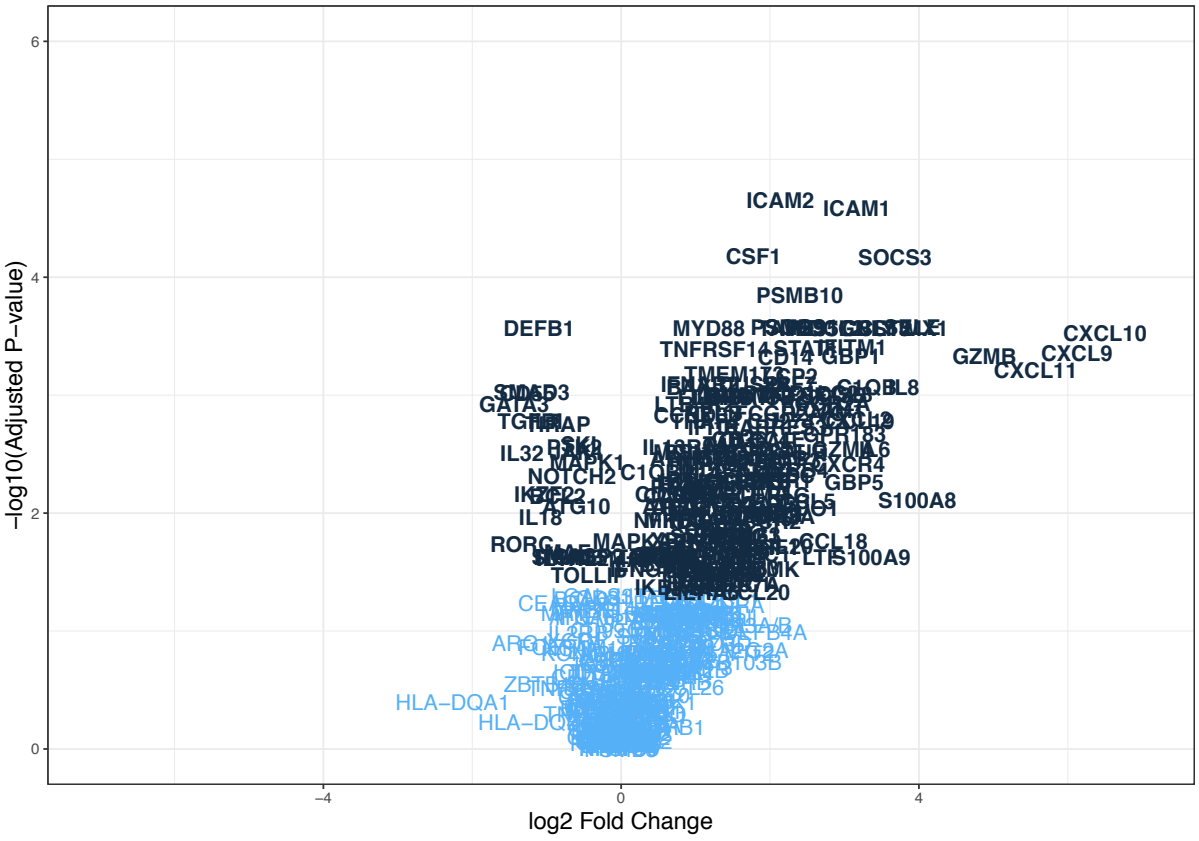

Figure 3.6 Volcano plot showing differential gene expression between GVHD (Grade II-IV) and healthy controls (skin biopsies). Genes shown in black showed a statistically significant change in expression (adjusted p-value of <0.05). The greatest up-regulation was seen in ligands of the chemokine receptor CXCR3.

In the comparison of GVHD versus healthy controls (Figure 3.6), the greatest up-regulation in the GVHD samples is seen in CXCL10 (Log2 FC=6.45), CXCL9 (Log2 FC=5.98) and CXCL11 (Log2 FC=5.52), all ligands of the chemokine receptor CXCR3, and known to be

induced by STAT1 (Kanda and Watanabe, 2007). These findings are consistent with the increase in STAT1 signalling seen in the gut of patients with aGVHD (Lamarthee et al., 2016a), and in this experiment a 2.51 Log2 FC was seen in STAT1 (p=0.0004). It is not possible to tell from this analysis what is driving the STAT1 pathway, but there is greater increase in IL22RA2 (Log2 FC=1.81, p=0.01), and IL-10RA (Log2 FC=1.16, p=0.01) than in the interferon receptors IFNAR1 (Log2 FC=-0.376, p=0.08), IFNAR2 (Log2 FC=1.06, p=0.0008) and IFNGR1 (Log2 FC=0.385, p=0.03).

The three genes with the most significant difference in expression, and a Log2 fold change of greater than two were ICAM (intercellular adhesion molecule) 1, ICAM2 and SOCS3 (suppressor of cytokine signalling 3). ICAM 1 has been previously shown to be upregulated in the gut, liver and skin in a murine model of GVHD (with a non-significant small increase of ICAM 2 in the skin only) (Eyrich et al., 2005). Given its importance in T cell activation, and interaction between leucocytes and endothelial cells, this is perhaps not surprising. The SOCS genes, however, are involved in negative feedback of cytokine signalling. The increase in SOCS3 seen here, therefore, seems a logical response to the cytokine production seen in GVHD. A previous study using qRT-PCR, however, showed a reduction in SOCS3 in the mononuclear cells of patients with aGVHD (and post HSCT without GVHD) when compared to healthy donors (Lee et al., 2013).

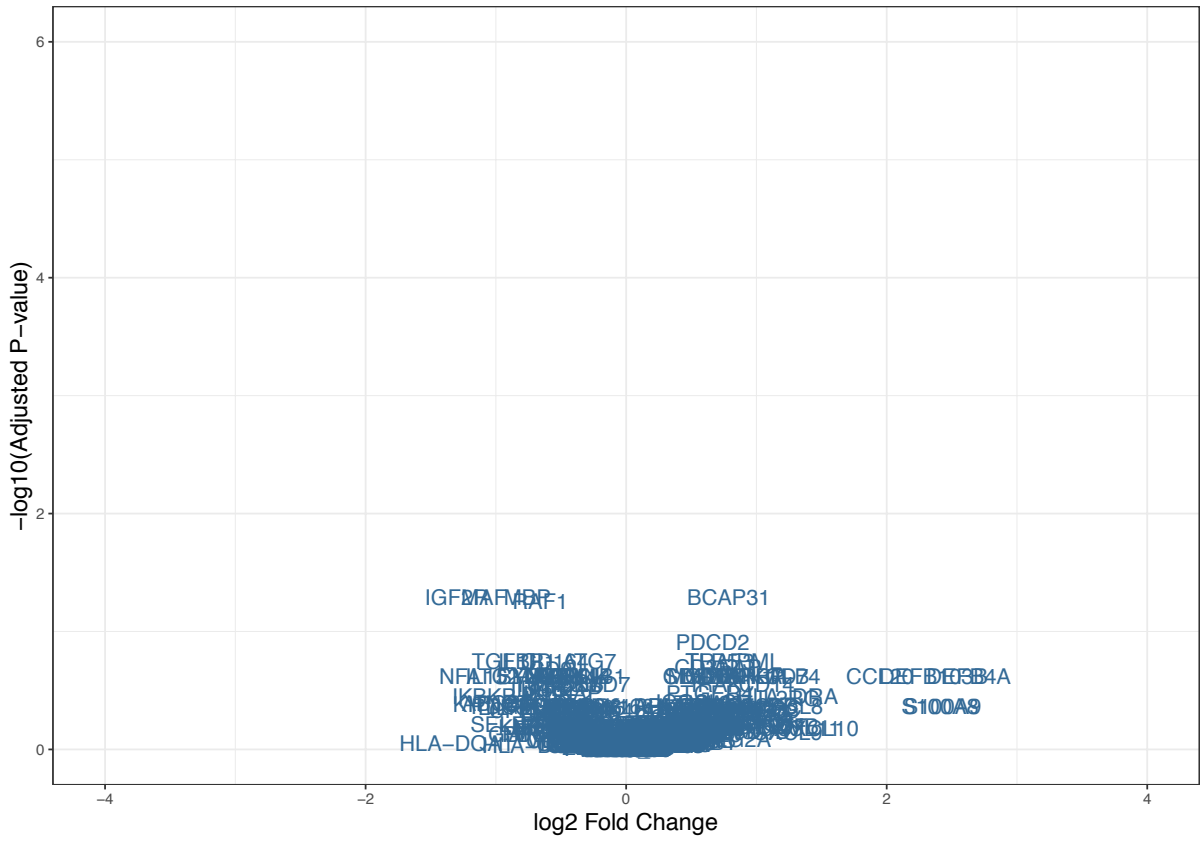

Figure 3.7 Volcano plot showing differential gene expression between GVHD (Grade II-IV) and GVHD Grade 0 (skin biopsies). Less differential gene expression is seen than when the comparison is healthy controls, with none reaching statistical significance.

As would be anticipated, there is less differential gene expression when comparing GVHD with other inflamed skin samples (Figure 3.7) than when the comparison group is healthy controls. The antimicrobial peptide DEFB4A (beta-defensin 4A) has the greatest fold increase in GVHD (Log2 FC 2.63), followed by the calcium binding proteins S100A8 and S100A9, although these do not reach statistical significance. There is slight down regulation of IL22RA, although this does not reach statistical significance (Log2 FC -0.34, p=0.88).

In the serum, a significant increase (p=0.0054, Kruskal-Wallis) in IL-22 concentration was seen in patients with GVHD, when compared both to healthy and post-transplant controls (Figure 3.8).

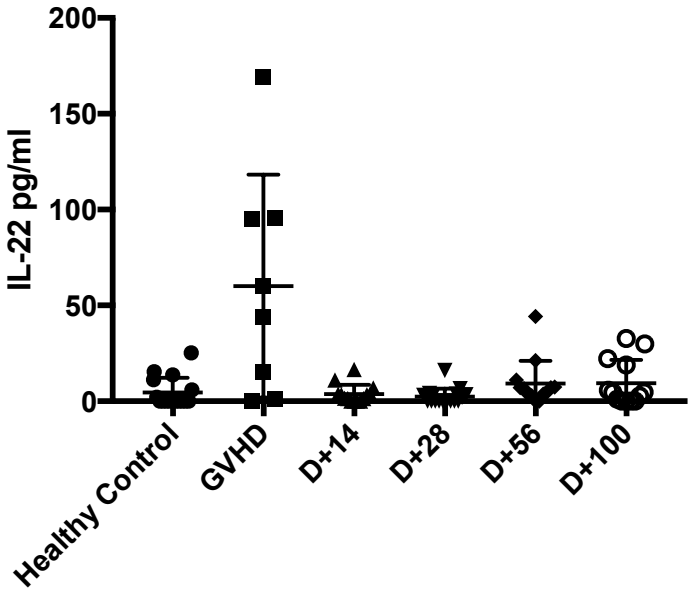

Figure 3.8 Serum IL-22 levels in healthy controls versus patients with GVHD, versus post-transplant controls (at Day 14, 28, 56 and 100). A significant increase in IL-22 concentration is seen in the serum of patients with GVHD (p=0.0054, Kruskal-Wallis).

The presence of increased serum IL-22 in patients with GVHD still does not answer the question as to whether the elevated IL-22 is contributing to the GVHD, or is part of the body's mechanism for defence and repair. For Cohort B, serum samples had been collected at all time points in addition to the EDTA samples, allowing the timeline to be analysed. Figure 3.9 illustrates serum IL-22 concentration over time for the three patients who developed Grade II-IV GVHD, compared to the mean of the remaining 17 patients in the cohort. One Day 100 serum has been excluded from the group, as a significant outlier. In all three patients with GVHD a rise in IL-22 concentration is seen. It is interesting to note that in two of the three patients who developed GVHD, serum IL-22 concentration appears to have peaked, prior to the development of clinically apparent GVHD. None of the three patients had either CMV or EBV reactivation within the first 100 days post-transplant.

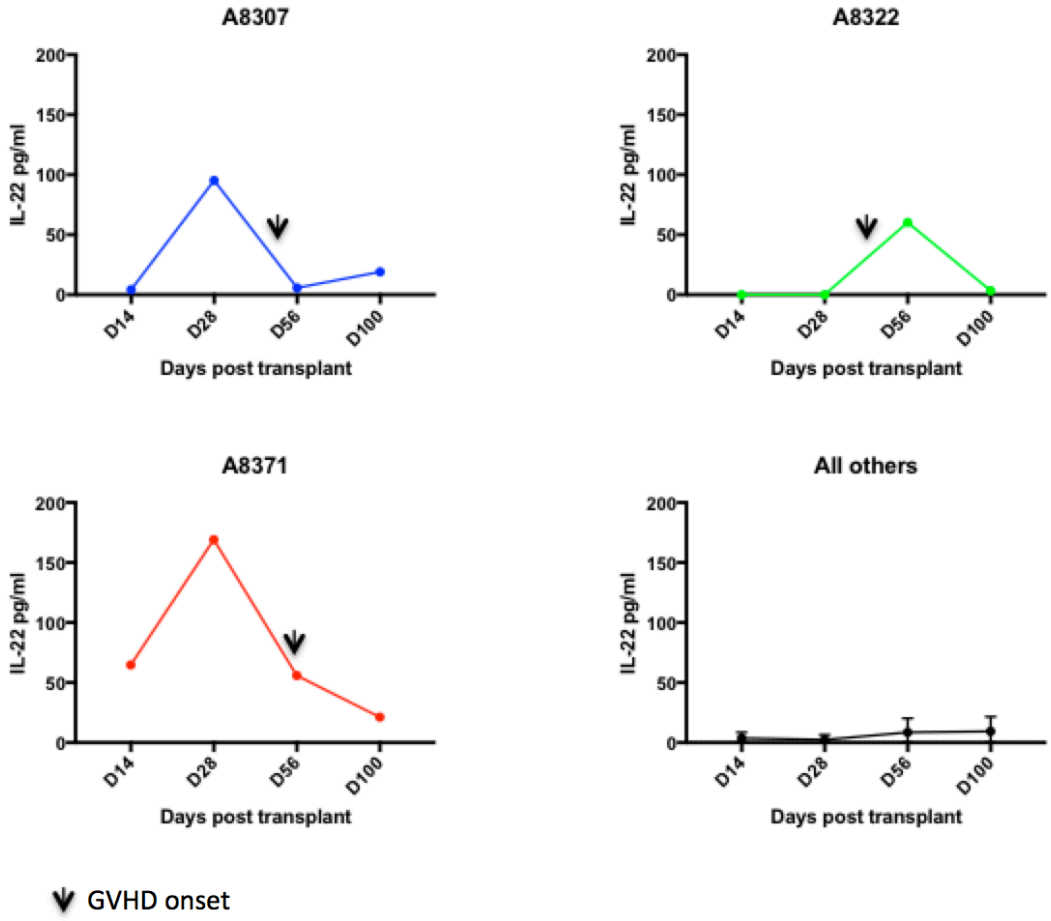

Figure 3.9 Timelines for the three patients who developed GVHD in Cohort B, compared to the mean of the other 17 patients (error bars show SD). In all three patients, a rise in serum IL-22 concentration is seen. In two of the three patients, this rise predates the development of clinically apparent GVHD.

3.3.5 Are ILCs responsible for IL-22 production in GVHD?

Published data show an increase in CD4+, IL-22 producing cells in the skin of patients with aGVHD compared to healthy controls, in addition to increased IL-22 mRNA expression in lesional biopsies (Bruggen et al., 2014). Whether the downstream effect of IL-22 is pro-inflammatory, or intended to reduce damage, is not however established. In addition, rapid reconstitution of NCR+ ILC3, cells able to produce IL-22, in the peripheral blood of patients post SCT correlated with a reduction in the risk of GVHD development (Munneke et al., 2014). This study looked only in the peripheral blood. I therefore wished to investigate whether ILCs were increased in the lesional skin of patients with GVHD, and whether they were producing IL-22.

Three flow cytometry panels were used, described in 3.2.6, two with extracellular markers only (ILCA and ILCB), the third an intracellular panel, allowing staining for IL-4, IL-17, IL-22 and IFN-γ. Looking first at the extracellular panels, healthy control samples were compared to lesional samples that were subsequently histopathologically confirmed as GVHD. In addition, biopsies taken for GVHD diagnosis that were not diagnostic of GVHD were used as post-transplant controls. A total of 12 samples were analysed for extracellular markers only. Whether expressed as a proportion of lymphocytes (data not shown) or as a proportion of live cells (Figure 3.10), there was no evidence for an increase in the Lin-127+ ILC population in GVHD, when compared either to control or post-transplant non-GVHD samples (p=0.68, Kruskal-Wallis).

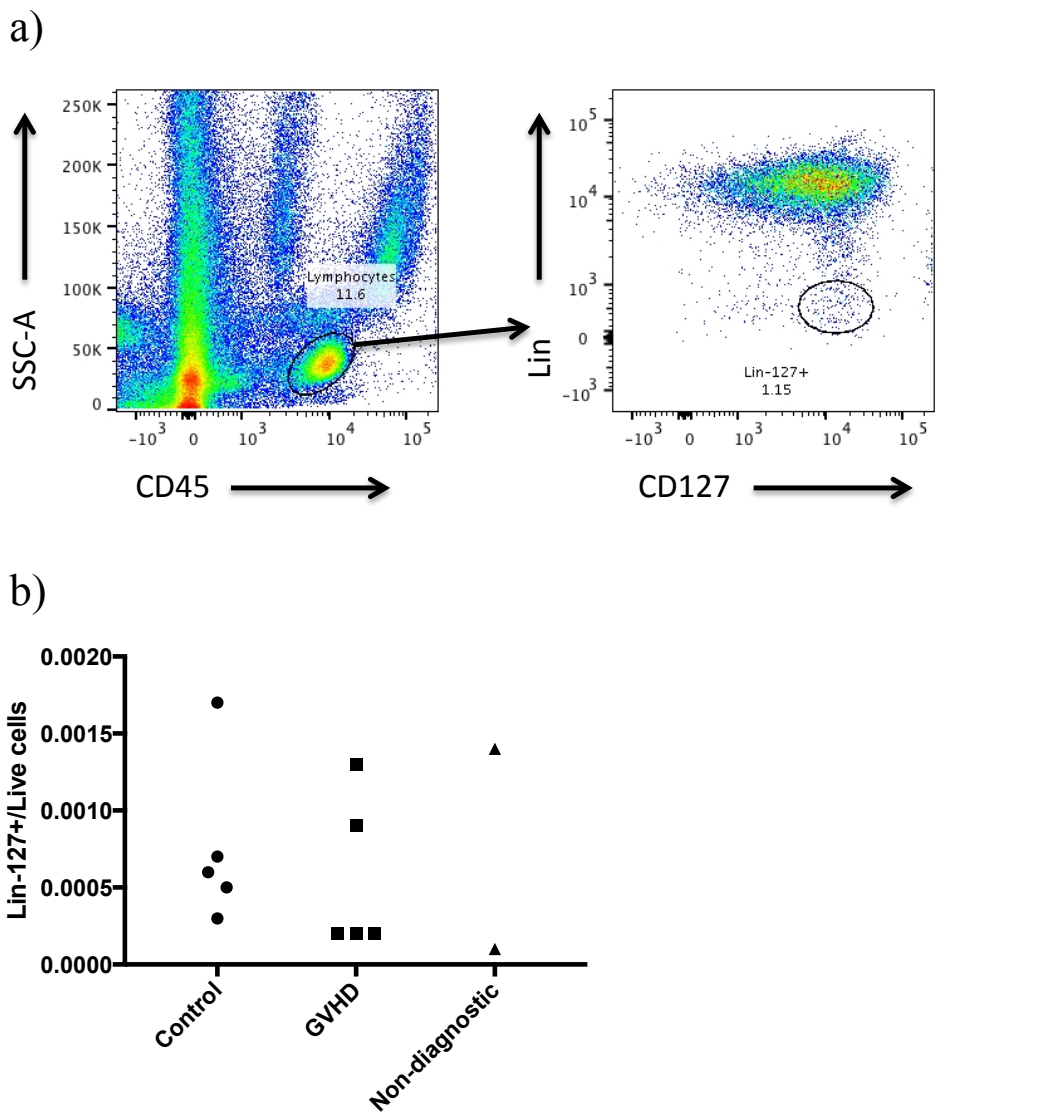

Figure 3.10 a) Example of gating strategy for ILCs from skin biopsies. b) ILCs isolated from skin biopsies from healthy controls, patients with GVHD, and non-diagnostic samples (gated on live singlets), as a proportion of live cells. No significant difference is seen between the groups (p=0.68, Kruskal-Wallis).

Simultaneous work looked to identify which cells were producing IL-22. Phorbolmyristate and ionomycin (Sigma Aldrich) stimulation, was used to investigate cytokine production by T cells and ILCs from healthy skin. IL-17 production by both subsets was demonstrated, in addition to IL-22 production by T cells, but not by ILCSs (Figure 3.11).

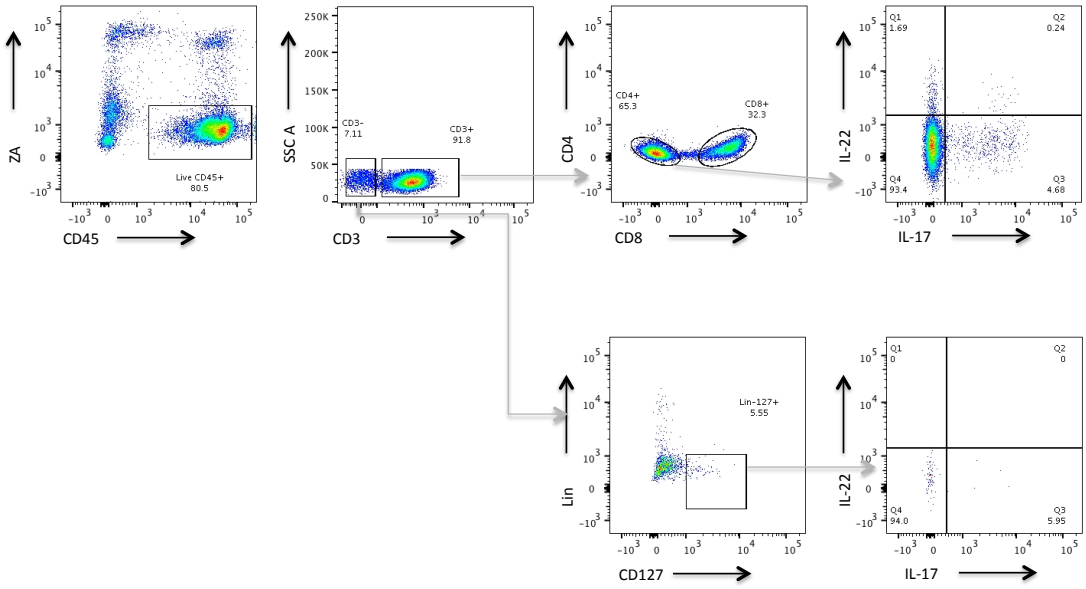

Figure 3.11 An example of the gating strategy used (gated on live single cells) for the intracellular staining protocol. IL-22+ and IL-17+ T cells can be seen. A small number of IL-17+ ILCs, but no IL-22+ cells, are seen.

When isolating cells from the much smaller fragments of excess material from diagnostic skin biopsies, the starting number of events was far fewer than from healthy skin. Figure 3.10 has illustrated that there was no evidence for an increase in Lin-127+ cells in the skin of patients with GVHD. I have, therefore, not been able to isolate sufficient Lin-127+ cells from pathological samples to comment on IL-22 production by ILCs in GVHD.

3.3.6 The IL-22 receptor in GVHD

The role of IL-22R up-regulation in UVB induced skin damage has been discussed in 3.1.1. Given the apparent increase in IL-22 observed in GVHD, the NanoString results, and the previously discussed ability of inflammatory signals to up-regulate expression of the IL-22R, I further investigated IL-22R expression in GVHD skin biopsies.

15 samples were stained for the IL-22R, with examples shown in Figure 3.12. Five had a histopathological GVHD grade of 2 (three classical acute, two on withdrawal of

immunosuppression), five biopsies were taken where the patient had a rash suggestive of GVHD, but had a histopathological grade of 0 (although two were subsequently felt clinically to evolve to GVHD) and five were healthy controls. Biopsies were scored as the number of positive cells out of 100 (Figure 3.13).

Minimal variation was seen amongst the healthy controls. While significant variation was seen amongst both pathological groups, there was an increase in IL-22R expression for pathological samples in comparison to the healthy controls, with the median percentage of positive cells being six in the control group and 28 in the GVHD group ($p=0.03$, Mann-Whitney U). The two patients who were subsequently felt to evolve to GVHD had scores of 11 and 28. The relatively greater increase seen for IL-22R staining, when compared to the results of the gene expression data probably relates to exactly what is being examined. When looking at the IL-22R staining, the proportion of positive cells along the dermal-epidermal junction only was considered. The gene expression data was taken from digested whole skin shaves.

a) b)

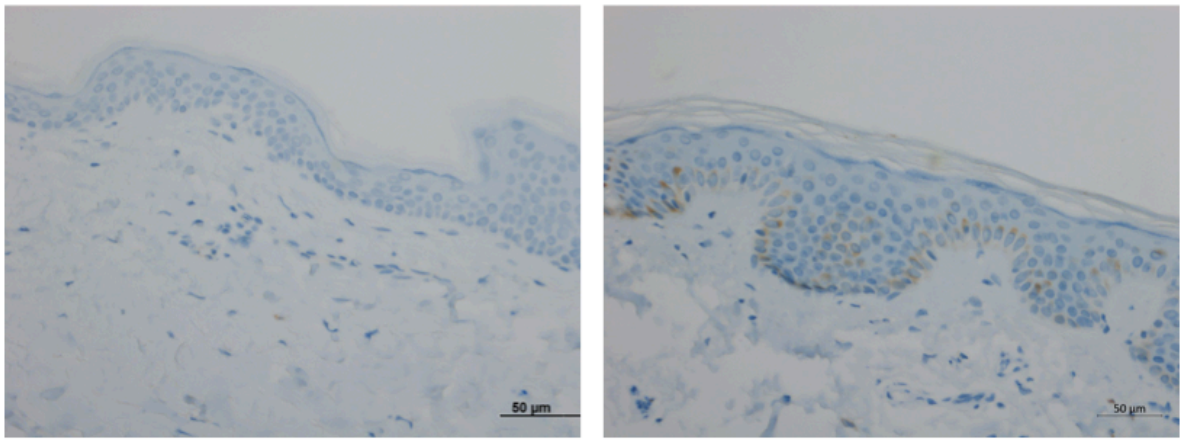

Figure 3.12 Examples of IL-22R staining in a) healthy control, b) GVHD. A clear increase in IL-22R staining along the dermal-epidermal junction is seen in the GVHD sample.

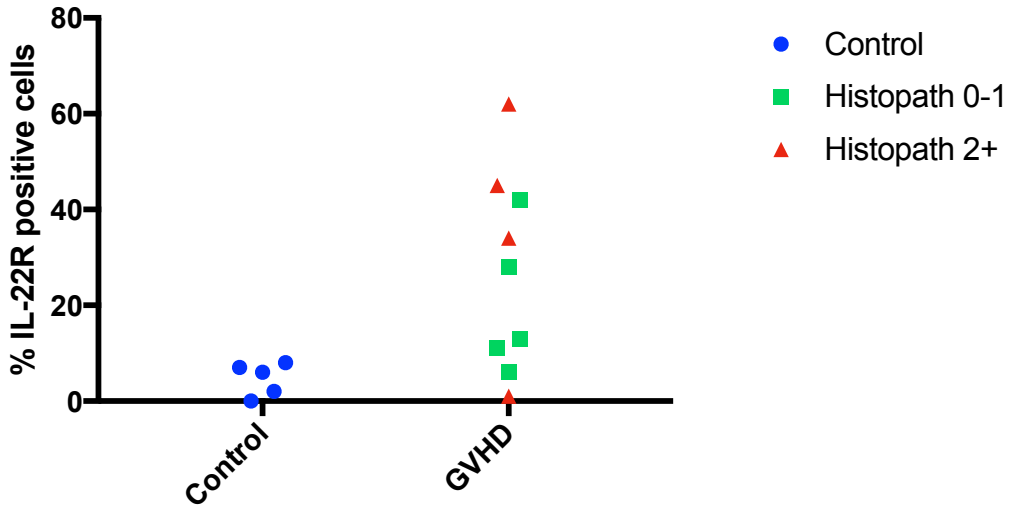

Figure 3.13 % of IL-22R positive cells along the dermal-epidermal junction. An increased level of IL-22R expression is seen in the pathological specimens, compared to the healthy controls. This difference reached statistical significance (p=0.03, Mann-Whitney U).

3.3.7 Impact of IL-22 polymorphism on GVHD risk

As has previously been discussed, murine models have shown that the role of IL-22 in GVHD differs depending on whether the IL-22 is produced by the donor or recipient. A summary of these findings is shown in Table 3.2. While IL-22 production by the recipient appears necessary to reduce GVHD, the reverse is true of the donor.

Paper	Transplant	Donor	Recipient	Findings
Hanash et al Immunity (2012)	MHC – mismatched, BM and T cells	WT	WT	IL-22 neutralising antibody results in increased GVHD mortality
		IL-22-/-	WT	No impact
		WT	IL-22 -/-	Increased GVHD mortality
	MiHA model	WT	IL-22-/-	Increased GVHD mortality, and increased organ pathology in liver/ intestine (not skin)
	MiHA model (chimera)	IL-22-/-	WT	Delay in GVHD mortality
Mertelsmann et al Blood (2013)	MiHA	WT	WT	Daily rIL-22, decreased GVHD pathology in liver and intestine (not skin)
Couturier et al Leukemia (2013)	C57BL/6 (B6) to BALB/c	IL-22-/-	WT	Decreased GVHD mortality and score. Reduced histopathological score in skin and intestine
Lamarthee et al Mucosal Immunol (2016)	B6 to BALB/c	IL-22 -/-	WT	Decreased GVHD mortality and scores
	MiHA	IL-22 -/-	WT or Type 1 IFN receptor -/-	Reduced weight loss and histopathological score with IL-22 -/- donor (only statistically significant reduction in GVHD mortality when **also** IFNAR-/- recipient

Table 3.2 A summary of the impact of IL-22 on GVHD in murine models

In addition, it has been shown that the TT genotype at rs2227485 correlates with increased IL-22 production in vaginal fluid in humans (De Luca et al., 2013), Figure 3.14. It was therefore hypothesised that a transplant from a TT donor into a CC recipient would result in increased GVHD and reduced survival.

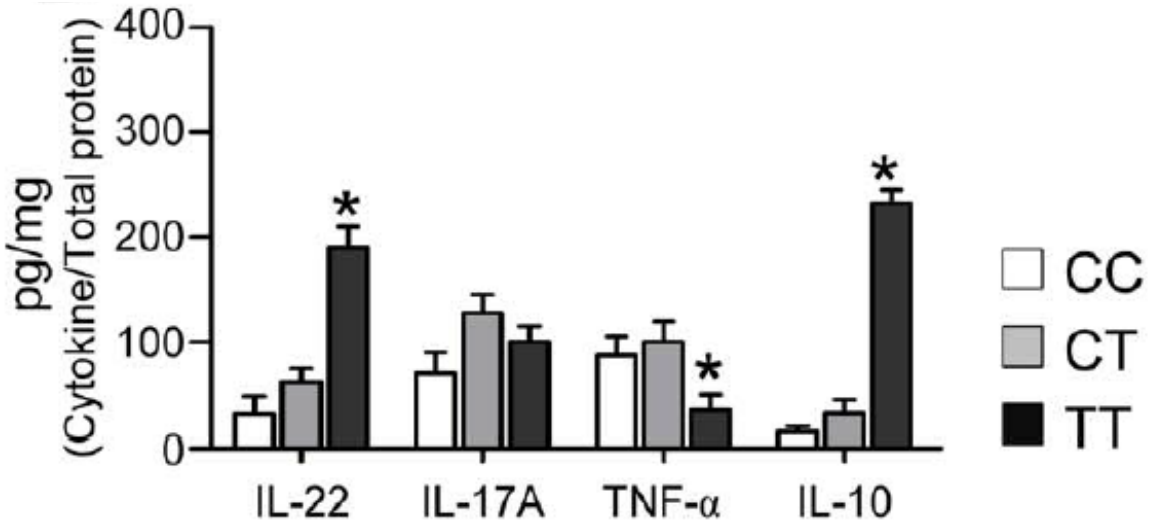

Figure 3.14 Cytokines (pg/mg, cytokine/total proteins (mean values +/- SEM) in the vaginal fluids of women bearing the CC, CT or TT genotypes at rs2227485 in IL22. *Taken from De Luca et al IL-22 and IDO1 Affect Immunity and tolerance to Murine and Human Vaginal Candidiasis, PLOS Pathogens (2013).*

Genotype information at rs2227485 was available for a total of 524 transplants. Patient baseline characteristics are shown in Table 3.3. The mean patient age was 46.3 years. Approximately two thirds of the transplants were T cell depleted and two thirds received reduced intensity conditioning. 57% of grafts were from matched unrelated donors and 76% received mobilised peripheral blood stem cells. 76% of transplants have been performed since 2005.

		Other	CCTT	Total
10 Year Status	Alive	30	0	30
	Dead	219	17	236
	LtF	246	12	258
	Total	495	29	524
T cell depletion	Replete	158	6	164
	Depleted	337	23	360
	Total	495	29	524
Donor	Sib/Other	214	9	223
	MUD	281	20	301
	Total	495	29	524
Transplant Date	Before 2005	124	4	128
	After 2005	371	25	396
	Total	495	29	524
Female to Male	Not F to M	405	23	428
	F to M	89	4	93
	Total	494	27	521
Conditioning	Myeloablative	142	7	149
	RIC	353	22	375
	Total	495	29	524
Stem cell source	PBSC	372	23	395
	Other	122	5	127
	Total	494	28	522
Transplant Centre	Regensburg	290	18	308
	Newcastle	205	11	216
	Total	495	29	524
CMV Status	CMV -/-	180	10	190
	Other	308	18	326
	Total	488	28	516
Disease	Leukaemia	244	15	259
	Other	249	14	263
	Total	493	29	522
Mean Age		46.3 years	46.4 years	

Table 3.3 Patient characteristics, SNP study

3.3.8 Impact of genotype on overall survival

The ten year overall survival of the patients is shown in Figure 3.15. Part (a) shows the entire cohort, and part (b) shows the patients with a CCTT genotype versus all other patients. There is a trend towards reduced overall survival in the CCTT group, but this is not statistically significant. This may be due to the small sample size of the CCTT group.

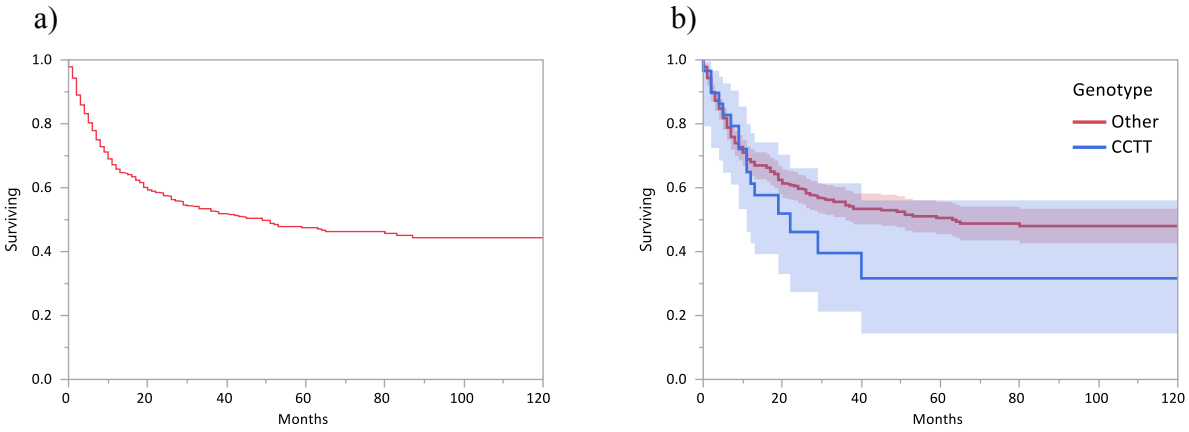

Figure 3.15 10 year overall survival for all patients, and split to show CCTT patients versus all others (95% confidence interval shown). Data suggest reduced overall survival in the CCTT genotype, but this does not reach significance.

3.3.9 Impact of genotype on cumulative incidence of death due to GVHD

Given that the literature would suggest that if excess deaths occur in the CCTT group, these should be due to GVHD, the cause of death was examined. For all patients, during the ten year post-transplant period, death due to GVHD occurs in approximately 11% of all patients, while overall mortality (from all causes) is approximately 52% Figure 3.16 (a). Prior to considering any potential effect from covariates, there appears to be little difference in risk for patients with the CCTT chimeric genotype in comparison to other genotypes, for cause of death other than GVHD (Figure 3.16 (b)). It appears, however, that the CCTT genotype confers a greater risk of death from GVHD than other genotypes. For comparison, the potential effect of risk factors known to impact on development of GVHD, T cell depletion and donor, are shown (Figure 3.16 (c) and (d)). These show minimal impact on risk of death due to GVHD in this analysis.

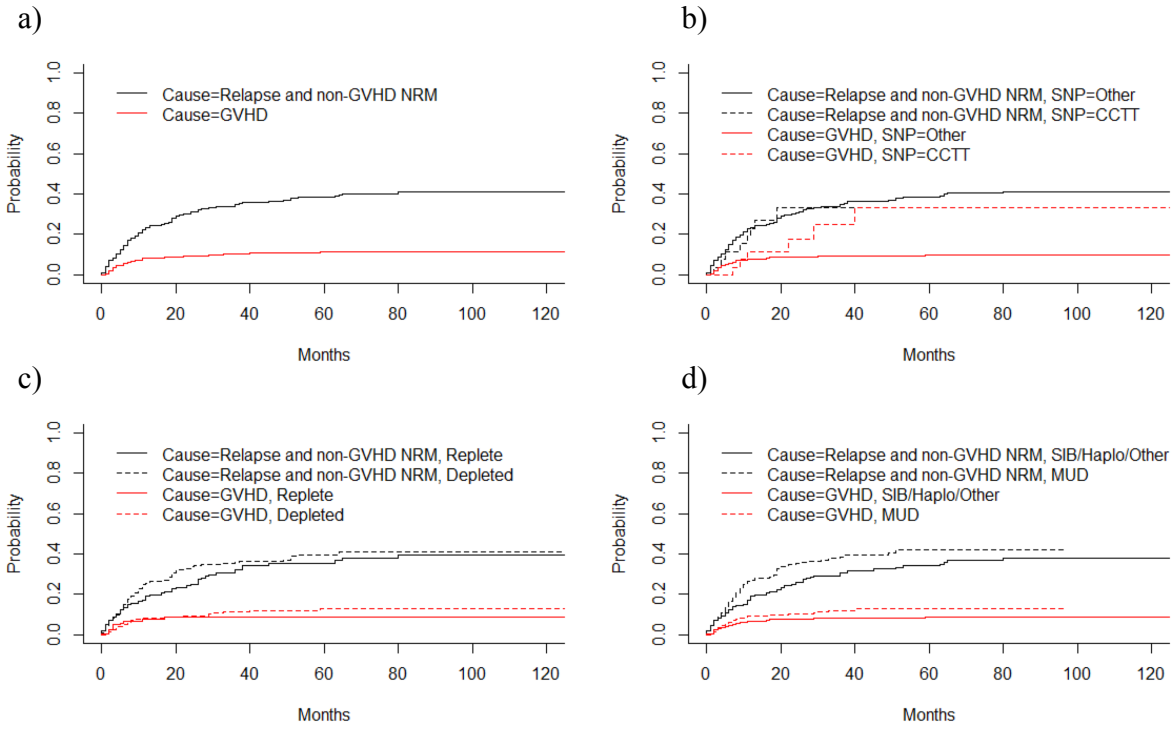

Figure 3.16 Impact of genotype, T cell depletion and donor type on GVHD, and non-GVHD mortality. For the whole cohort approximately 10% of deaths are due to GVHD (a). For the CCTT genotype GVHD deaths rise to approximately 35% (b). Neither T cell depletion (c) nor donor type (d) impact on the proportion of deaths due to GVHD.

To analyse this finding in more detail, a competing risks analysis was undertaken, taking into account CCTT genotype, and the other covariates shown in Table 3.3. The full model fitted includes CCTT and all covariates plus an interaction term for the most significant covariate (RIC, p=0.15) from the initial model (i.e. CCTT * RIC). The model effect p-values from the full model were used to inform a forward/backwards stepwise model selection procedure to find the final model, which minimised the increase in Bayesian Information criterion (delta BIC) over the null model, whilst ensuring that all model terms were significant.

The final model (delta BIC= 2.627) for the effect of CCTT genotype on overall survival, death from GVHD, or death from causes other than GVHD contains only the term for CCTT (p= 0.021). No covariates were significant following the model selection procedure. Patients with a post-transplant chimeric SNP genotype of CCTT have a much greater risk of death from GVHD than the other genotypes (hazard ratio=2.53, 95% confidence interval=(1.149, 5.571). This finding has potential clinical significance. While it is not practical to select donors to avoid a CCTT chimeric genotype, given the other factors that have to be taken into account when selecting a donor, it might be possible to incorporate this risk into the patient's

post-transplant care. This could include slower tapering of immunosuppression, or more aggressive up-front treatment should the patient develop GVHD.

Finally, serum IL-22 concentration for a subset of these patients and donors was measured, to investigate whether the effect of genotype could be seen. The donors were analysed by the three potential genotypes, where samples were available (n=11). As with the other healthy controls, IL-22 was often undetectable, with a mean concentration of 4.4pg/ml. Given this, not surprisingly, no difference could be detected between genotypes.

3.4 Discussion for Chapter Three

The aim of this chapter was to explore the IL-22/IL-22R system in human HSCT. I first examined the effect of transplant conditioning therapy (full and reduced intensity) on IL-22 expression. In contrast to the mouse mode described by Hanash et al (Hanash et al., 2012), I found no evidence of IL-22 induction by reduced or full intensity conditioning in skin (by gene expression analysis) or serum (by ELISA), when looking at paired pre- and post-conditioning samples. There are a number of potential reasons for this. The Hanash study was performed on serum and homogenised intestinal tissue two weeks post-transplant. It is, therefore, possible that the time point I had chosen was too early to see an effect. The reasons for choosing this early time point were in part that it limited other potentially confounding factors, and in part the difficulty of performing skin biopsies on patients (for research purposes only) at a later time, when platelet counts are likely to be lower and the patients feel less well. It is also possible that, although I did not see IL-22 induction in the serum, it would be present in the gut or liver, if it were possible to study these. It may be that the lack of IL-22 induction observed is the result of different conditioning protocols used for mice and humans. This data, however, challenges the concept of conditioning-induced IL-22 providing a protective effect against subsequent GVHD development in humans.

I have, however, shown evidence of elevated serum IL-22 in patients with GVHD when compared to healthy and post-transplant controls. One of the unanswered questions relating to the role of IL-22 in GVHD is whether it is exerting a protective or pathogenic response. This work has not enabled me to conclusively answer this question, however, Figure 3.9 illustrates that of the three patients with Grade II-IV aGVHD, in two the peak serum IL-22 occurs prior to the onset of clinically detectable GVHD. In addition, the gene expression data for healthy controls versus patients with GVHD shows significant upregulation of CXCL9 and CXCL10. I have not measured serum IFN, but my findings are consistent with those of Lamarthée et al, who demonstrated increased CXCL9 and CXCL10 expression in the presence of IL-22 wild type donor T cells, via phosphorylation of STAT 1, when compared to IL-22$^{-/-}$ T cells (Lamarthee et al., 2016a). These findings are suggestive of a pathological role for IL-22 in GVHD.

In addition to the increase in IL-22, I have shown increased IL-22R expression in GVHD, both by gene expression analysis and immunohistochemistry. As has been previously discussed, inflammation is known to increase IL-22R expression in a number of experimental

models. The up-regulation of both IL-22 and IL-22R shown in this chapter may result in a positive feedback loop, where both the production and impact of IL-22 are increased.

Finally, the impact of gene polymorphism for IL-22 has been investigated. These data are in keeping with the findings of previous murine models and data regarding high and low producer phenotypes at this SNP. Where the patient has a 'low IL-22 producer' phenotype, and the donor is a 'high IL-22 producer' there is a greater risk of death from GVHD. Several of the GVHD deaths in the CCTT cohort occur relatively late post-transplant (Figure 3.16 (b)). This finding is consistent with what has previously been shown regarding the importance of donor-derived IL-22 in chronic cutaneous GVHD in a murine model (Gartlan et al., 2017). Unfortunately, serum samples were not available at the relevant time-point for the patients in the CCTT group who experienced late GVHD deaths, as it would have been interesting to investigate whether this associated with an elevated IL-22 concentration at that time. These late deaths raise the possibility that elevated IL-22 is to some extent responsible for driving the GVHD process, thus making it more resistant to treatment. The cohort studied here, and in particular the CCTT genotype group, was relatively small. Given the potential clinical implications of this finding, it would be interesting to repeat this study with a larger cohort.

The major strength of this chapter is that multiple methods have been used to examine the role of the IL-22/IL-22R axis in GVHD. This has in some ways, however, also proved a weakness, in that to be able to perform the work, in the time frame available, it was necessary sometimes to use samples that were already available. This has meant that all the work has not been performed on the same cohort of patients, potentially introducing further variables. Were I to prospectively design this study, it would be beneficial to use the same methods, but to have a single cohort of patients, allowing for greater cross-referencing between findings.

Chapter 4 The effect of IL-22 on an in vitro model of graft versus host disease

4.1 Introduction

The therapeutic options for established GVHD remain limited and the search is on-going for agents that will treat GVHD while preserving the GVL effect (Wolf et al., 2012, Cassady et al., 2018). In this respect, the IL-22/IL-22R system represents an interesting target, as the absence of IL-22 receptors on leucocytes (Wolk et al., 2004) means that treatment would potentially directly target the affected tissues and not the donor lymphocytes.

4.1.1 Targeting the IL-22/IL-22R system

The differing impact of the IL-22/IL-22R system in different situations means that both enhancement and inhibition have been considered as therapeutic interventions. While many conditions would potentially benefit, those where research is currently more advanced are briefly discussed here.

Two Phase I trials, NCT00563524 and NCT01010542, have evaluated the use of the anti-IL-22 monoclonal antibodies ILV-094 and ILV-095 respectively, in the treatment of psoriasis. The first was unpublished, and the second terminated when it failed to meet the primary efficacy endpoint (Tsai and Tsai, 2017).

F-652, a recombinant protein made of human IL-22 and human immunoglobulin G2-Fc, (Generon, (Shanghai) Corp. Ltd.) has been found to be safe and well-tolerated in Phase I trials (Tang et al., 2018), although eczematous lesions were seen at the injection site in a proportion of patients, when it was administered subcutaneously. Some ocular and cutaneous adverse events were reported at the higher intravenous doses tested, but there were no severe adverse events. A dose escalation study is currently recruiting to assess the safety and efficacy of the drug in patients with alcoholic hepatitis (NCT02655510).

4.1.2 IL-22 as treatment for GVHD

As discussed in Chapter 1.4.6, IL-22 given prophylactically in a murine model has already been shown to reduce GVHD morbidity from, and histological severity of, GVHD (Mertelsmann et al., 2013). A Phase II study looking the use of F-652, in patients with grade II-IV lower gastrointestinal graft versus host disease is currently in the recruitment phase (NCT02406651).

4.1.3 The skin explant model

The skin explant model allows *in vitro* production of the histological changes seen in GVHD. Originally described by Vogelsang (Vogelsang et al., 1985), the classical model, where donor and recipient lymphocytes are initially cultured together and then co-cultured with skin from the transplant recipient, was shown to be predictive for the future development of GVHD post-transplant in T replete sibling transplants. In addition to being used clinically for GVHD prediction and modification of prophylaxis (Dickinson et al., 1999, Wang et al., 2006), the skin explant model has been used to dissect, in order to better understand, the biology of both GVHD and GVL. This has included the role of cytotoxic T lymphocytes in a minor histocompatibility mismatched model, demonstrating that minor histocompatibility antigens are a key target in GVHD (Dickinson et al., 2002) and the role of the Fas/Fas-ligand pathway, showing a correlation between higher grades of GVHD and increased epithelial Fas expression (Ruffin et al., 2011). Finally, it has been used to test potential agents that might reduce GVHD, for example IL-10 (Wang et al., 2002).

4.1.4 Pathological GVHD grading

The Lerner classification is used to describe the severity of pathological damage in tissues affected by GVHD and is distinct from clinical grading of GVHD (Lerner et al., 1974).The changes seen in target organs are assigned grades 0-IV. In the gastrointestinal tract, the grading is based on the presence or absence of mucosal glands and 'denudation' of the mucosa. In the liver the key feature is the percentage of abnormal bile ducts, as this was found to be more consistent than changes to the cells of the liver parenchyma. This study used models of cutaneous GVHD only, where the grading is as follows:

Grade 0 Normal skin

Grade I Vacuolisation of epidermal basal cells

Grade II Diffuse vacuolisation of basal cells with dyskeratotic bodies

Grade III Subepidermal cleft formation

Grade IV Complete epidermal separation

4.1.5 Questions for Chapter 4

In this chapter I explored the hypothesis that IL-22 modifies the severity of GVHD using the skin explant model. This was achieved by adding IL-22 to the model, blocking endogenous IL-22 with antibodies and documenting the expression of IL-22R in the model. Specific questions addressed in this chapter are detailed below.

1. Is IL-22 involved in pathological damage due to GVHD?
2. Does IL-22 ameliorate pathological damage due to GVHD?
3. Are the effects of IL-22 concentration dependent?

Aims:

1. To create a reproducible model of GVHD in a skin explant
2. To explore the effect of the addition of differing concentrations of IL-22 to this model
3. To explore the effect of the addition of an IL-22 blocking antibody to this model

4.2 Methods for Chapter Four

4.2.1 Sample collection

Skin and blood samples in this chapter were collected as described in 2.2.1 and 3.2.1.

4.2.2 Media and buffers

Details of media and buffers used in this chapter are provided in Appendix 2.

4.2.3 Mixed lymphocyte reaction

A mixed lymphocyte reaction (MLR) was produced using peripheral blood from healthy donors. PBMCs were isolated from whole blood, collected in EDTA, as previously described. $1x10^6$ PBMCs from two healthy donors were suspended per 1ml of MLR culture medium (with human AB serum). These were cultured in 25 cm^2 flasks (Cellstar) at 37^0 in a humidified, 5% CO_2, 95% air incubator. After five days, the cultures were transferred to a universal container and centrifuged at 500g for 5 minutes. 50% of the supernatant was removed and frozen in 1ml aliquots at -80^0C (MLR D5). 50% of cells were also removed. The remaining cells were re-suspended in the remaining medium and cultured for a further two days. After a total of seven days culture they were transferred to a universal container, centrifuged at 500g for 5 minutes and the supernatant frozen in 1ml aliquots at -80^0C (MLR D7).

4.2.4 The skin explant

When using healthy control skin, 6mm punch biopsies were trimmed of excess dermis and cut into four pieces of equal size. Skin fragments were placed in different test conditions, further details of which are described with the results of each model, and incubated for 72 hours at 37^0C, in a humidified, 5% CO_2, 95% air incubator. Recombinant IL-22 (rIL-22) (ImmunoTools, Friesoythe), was reconstituted as per the manufacturer's instructions, and stored at -80^0C in single use aliquots. The IL-22 monoclonal antibody (IL22JOP, Invitrogen, Thermo Fisher Scientific) was stored at 4^0C.

4.2.5 Immunohistochemistry

After 72 hours, biopsies from the skin explant model were fixed in 10% buffered formalin. Paraffin embedding, microtomy and haemotoxylin and eosin (H&E) staining were performed by the Newcastle Molecular Pathology Node. Embedding and orientation were performed as per the clinical GVHD protocol.

4.2.6 Histopathological grading

Assessment of the GVHD grade of the samples was performed blinded, by an independent assessor, with experience in GVHD grading. Assessment was made using the Lerner grading system, which grades from 0-IV (Lerner et al., 1974).

4.2.7 Luminex

Cytokine analysis of the MLR supernatants was performed using the ProcartaPlex® Multiplex Immunoassay (eBioscience). This uses magnetic bead technology to allow simultaneous detection of multiple protein targets. The assay was performed according to the manufacturer's instructions, using 25µl of each sample. The plate was read using a Luminex 200 and data analysed with ProcartaPlex Analyst version 1.0. Heatmaps were produced in RStudio, by A Resteu.

4.3 Results

4.3.1 Isolated effect of IL-22 on skin integrity in vitro

Given my finding of elevated serum IL-22 in GVHD, I sought first to establish whether IL-22 alone would act in a pro-inflammatory manner when added to a non-inflammatory system. rIL-22 was therefore added to 200µl of skin explant culture medium (Appendix 2) at the following concentrations:

Culture medium alone	30pg/ml IL-22	150pg/ml IL-22	300pg/ml IL-22

This experiment was repeated three times (with three different skin donors). No evidence of rIL-22 induced skin damage was seen at any of the concentrations tested (Figure 4.1 and Figure 4.2).

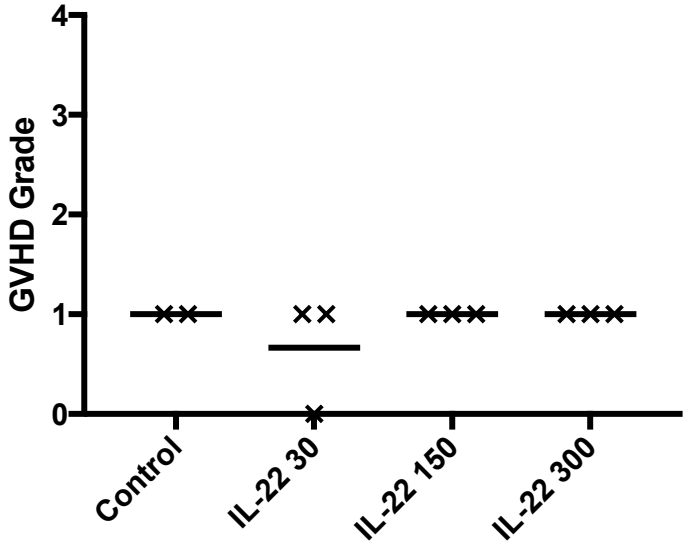

Figure 4.1 Impact of increasing rIL-22 concentration on healthy skin. No evidence of histopathological damage was seen at any of the concentrations tested.

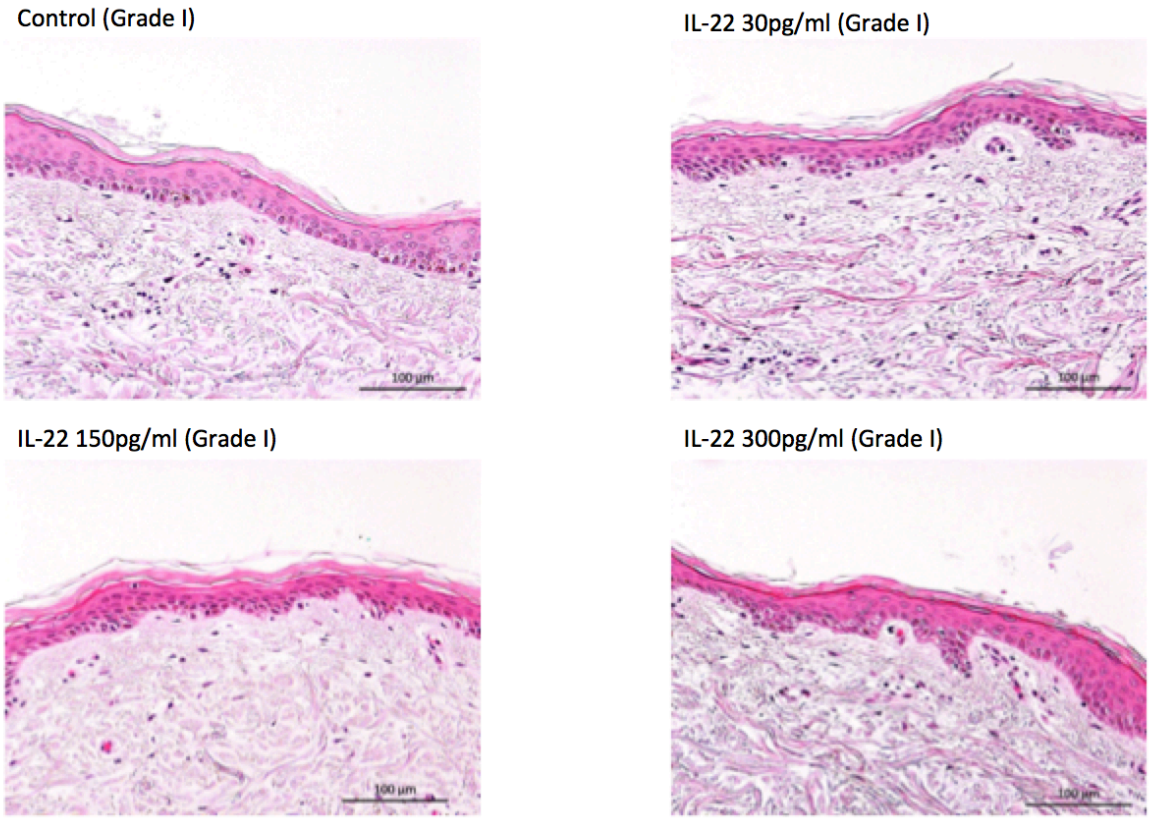

Figure 4.2 Examples of H&E stained sections at increasing concentrations of rIL-22. No difference in GVHD grade is shown at any concentration of IL-22.

4.3.2 Anti IL-22

Having established that rIL-22 alone, even at concentrations almost twice the highest I measured in the serum of patients with GVHD, did not cause histopathological evidence of skin damage, I next investigated the impact of an IL-22 blocking antibody. Four conditions were used for this experiment, again using 200µl of culture medium or MLR supernatant (MLR 2 D5, which had a baseline IL-22 in the middle of the range measured, further details in 4.3.3).

Culture medium alone	MLR supernatant alone	375ng/ml Anti-IL-22 MLR Sup	150pg/ml IL-22 MLR Sup

The concentration of anti-IL-22 required was calculated using the eBioscience Cytokine Neutralization protocol. For human IL-22 neutralisation, it is recommended that 1µg/ml of antibody is used to neutralise 0.2ng/ml of cytokine (http://diyhpl.us/~bryan/irc/protocol-

online/protocol-cache/NU.htm#chart). When the IL-22 present in the MLR supernatant was neutralised, no improvement in, or worsening of, GVHD grade was observed (Figure 4.3).

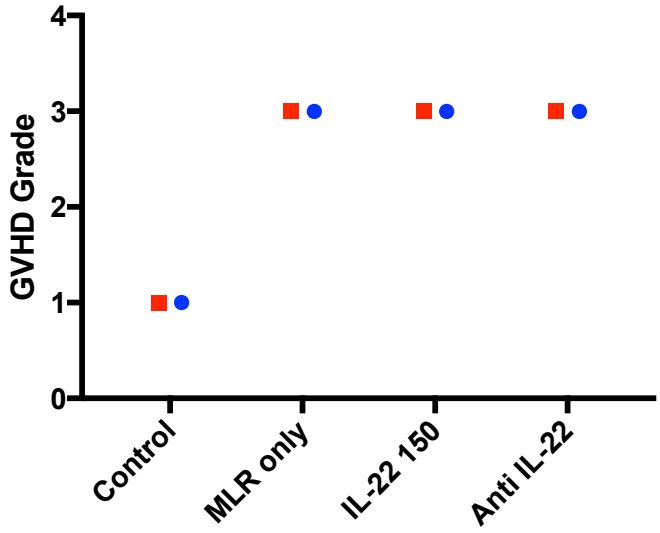

Figure 4.3 Impact of an IL-22 blocking antibody on MLR supernatant induced skin damage. No improvement in GVHD grade was observed when anti IL-22 was used to neutralise the IL-22 found in the MLR supernatant. Performed twice with different skin donors (red and blue).

4.3.3 Ability of IL-22 to ameliorate supernatant-mediated GVHD reactions

I next selected two concentrations of rIL-22 to test in a model where damage was induced using 200µl neat supernatant from an MLR reaction. The 30pg/ml concentration was in the range of the physiological concentration that I measured in the patients, while 150pg/ml was used as a supraphysiological level (only one patient had a serum IL-22 concentration higher than 100pg/ml). The experiment was repeated eight times, using the following conditions:

Culture medium alone	MLR supernatant alone	30pg/ml IL-22 MLR Sup	150pg/ml IL-22 MLR Sup

As previously, skin in culture medium alone showed Grade I damage. The MLR supernatant reliably produced either Grade II or III damage. The addition of 30pg/ml of rIL-22 did not always have an effect, but where it did, this was always to increase the GVHD Grade. The addition of 150pg/ml rIL-22 resulted in resolution to Grade I in 50% of the experiments performed. This response was comparable to that observed when 100µg/ml of methylprednisolone was added to a cellular, HLA mismatched explant model: 36% resolution to Grade I, 21% to Grade II (Figure 4.4, XN Wang, unpublished results).

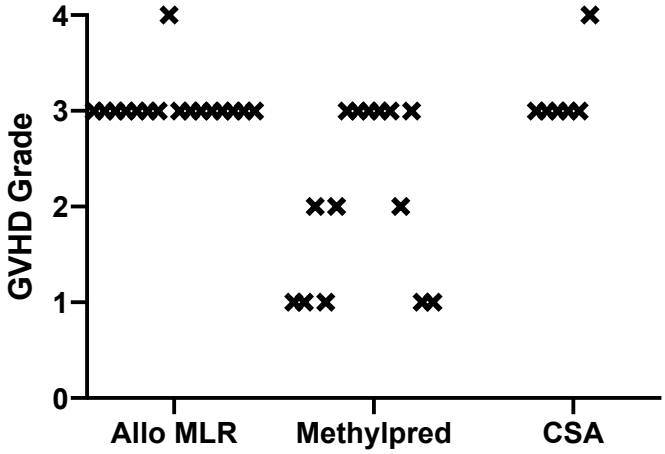

Figure 4.4 Response in GVHD grade to the addition of 100µg/ml of methylprednisolone or cyclosporine A (CSA) to an HLA mismatched skin explant model. Methyprednisolone resulted in 36% resolution to Grade I, 21% to Grade II. No impact was seen with the addition of CSA.

A summary of my results is shown in Figure 4.5. Part (a) shows the result of each individual skin/MLR pair. No improvement is seen with the addition of 30pg/ml, but with the addition of 150pg/ml there was resolution to Grade I in half of the experiments performed. Wilcoxon matched-pairs for IL-22 30 vs IL-22 150, p=0.13. Part (b) shows these results combined (mean values with SEM). Parts (c) and (d) show the results colour-coded by skin and by MLR respectively. These illustrate that different outcomes are observed when different supernatants are paired with the same skin donor, and that the same supernatant can cause different outcomes when paired with a different skin donor. Examples of the H&E stained sections are shown in Figure 4.6. In the example showing MLR supernatant alone, in addition to the basal cell vacuolation that defines Grade II, there is evidence of severe epidermal damage.

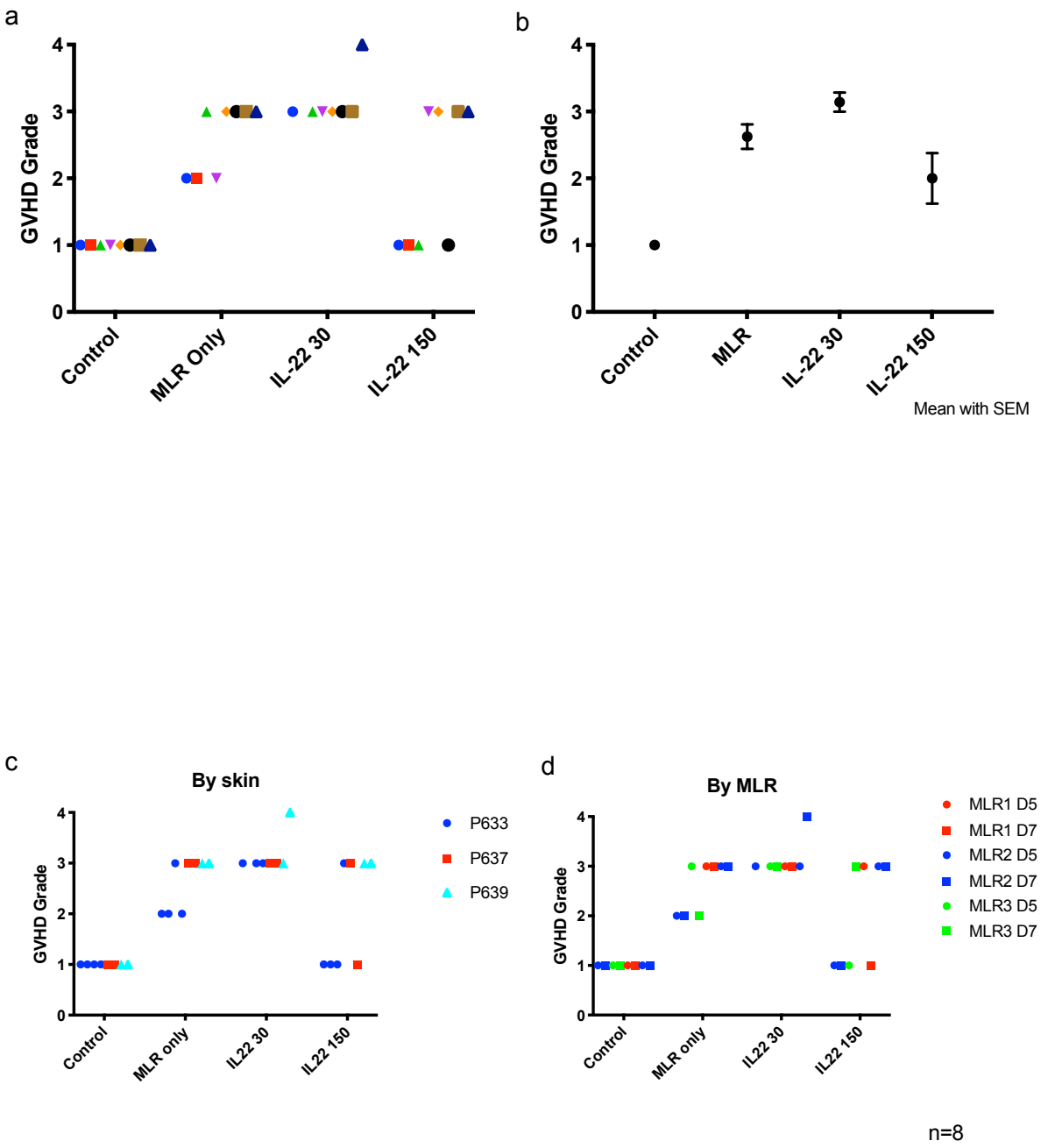

Figure 4.5 MLR supernatant reliably produced damage consistent with GVHD. The impact of differing concentrations of rIL-22 on this skin damage could then be assessed. (a) results of individual experiments with increasing concentrations of rIL-22, (b) mean values with SEM, (c) data colour coded by skin donor, (d) data colour coded by MLR supernatant. IL-22 30 refers to the addition of 30pg/ml rIL-22, and IL-22 150 to 150pg/ml rIL-22. A reduction in GVHD grade was seen only with the addition of 150pg/ml rIL-22, in 50% of experiments performed.

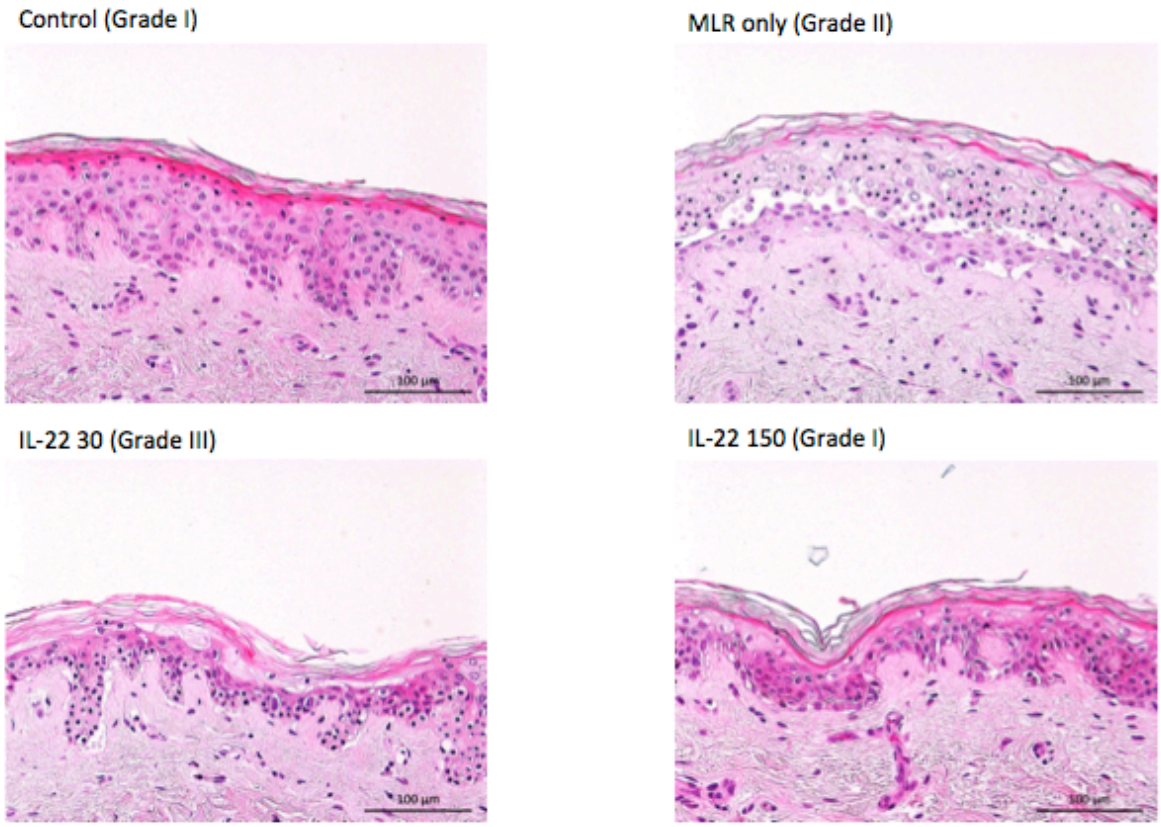

Figure 4.6 Examples of H&E stained sections, showing the differing GVHD Grades. In addition to the criteria required to define the Grades, the MLR alone produces evidence of severe epidermal damage. 30pg/ml rIL-22 illustrates the subepidermal cleft formation of Grade III GVHD, while 150pg/ml rIL-22 shows vacuolisation only.

Although the MLR supernatant used did not appear to influence the outcome, I next considered the effect of differences in baseline supernatant IL-22, and whether this might explain the separation of 'responders' from 'non-responders'. Three different MLRs, with supernatant stored at two time points had been used in the model, giving the potential for different baseline IL-22 concentrations in the explant. The IL-22 concentration of each supernatant was therefore measured by ELISA, as described in 3.2.4 (data not shown). The baseline IL-22 concentration in the supernatants ranged from 8.5pg/ml to 123pg/ml. This difference alone, however, did not explain whether or not a response was observed. MLR2 D5 and MLR2 D7, for example each resulted in one response and one non-response, when different skin donors were used. Interestingly, no response was observed with MLR1 D5, however, the supernatant with the lowest IL-22 concentration.

The cytokine content of each MLR supernatant was investigated using Luminex, and R was used to create heatmaps (work performed by A Resteu). Clustering was performed both by cytokine and by MLR. No pattern could be found to explain GVHD responders versus non-responders. Examples of the heatmaps generated are shown in Figure 4.7. Perhaps the most interesting observation is the heterogeneity seen between MLRs. I was unable to find any pattern to explain why some samples responded to the addition of rIL-22 and others did not.

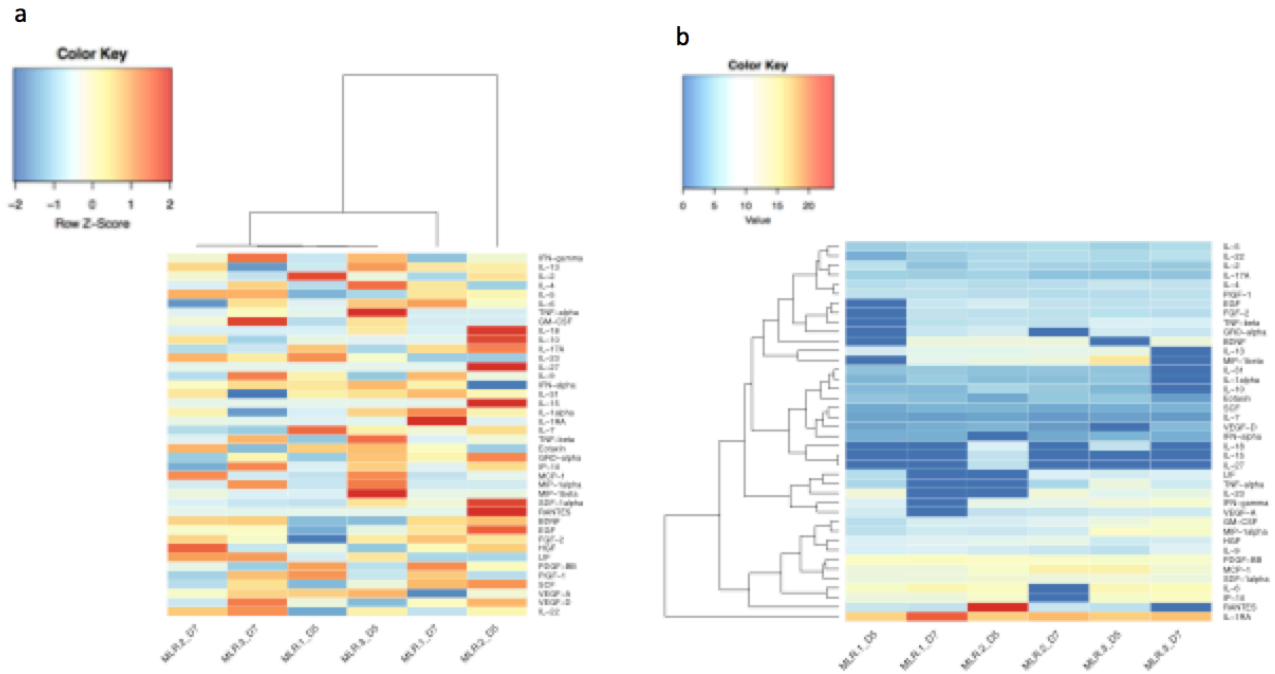

Figure 4.7 Heatmaps demonstrating the heterogeneity of cytokine expression in the MLR supernatants. The cytokines measured are shown on the Y axes. a) MLR supernatants clustered by MLR, b) MLR supernatants clustered by log2 cytokines. No pattern could be found based on MLR cytokine content to explain GVHD responders versus non-responders when rIL-22 was added to the skin explant model.

Baseline IL-22 and rIL-22 were then combined, to allow calculation of total IL-22 concentration. Total IL-22 concentration in the MLR/MLR plus 30pg/ml IL-22 ranged from 8.5-153pg/ml. Total IL-22 in the MLR plus 150pg/ml ranged from 158.5-273pg/ml (Figure 4.8). Improvement in GVHD grade was only observed when the total IL-22 concentration was greater than 190pg/ml.

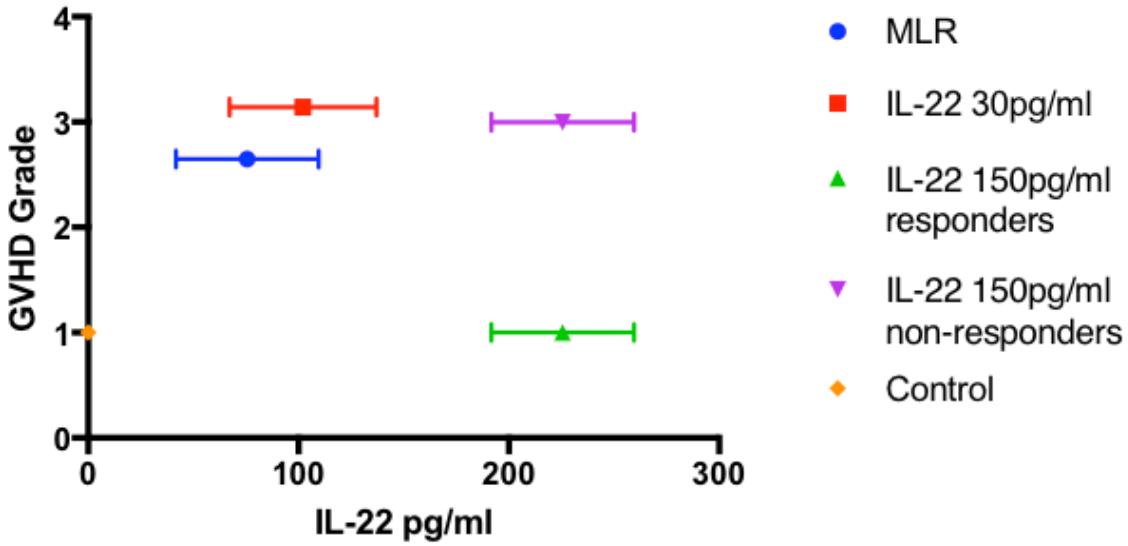

Figure 4.8 Impact on GVHD grade of combined baseline and rIL-22 concentration. Improvement in GVHD grade was only observed when the total IL-22 concentration was greater than 190pg/ml.

The total IL-22 concentration in the MLR supernatant with or without 30pg/ml rIL-22 was in the same range as that measured in the serum of patients with GVHD, while the addition of 150pg/ml rIL-22 resulted in a supra-physiological concentration. This raises the possibility of a biphasic response to IL-22, with a therapeutic effect only seen at concentrations above the physiological range.

4.3.4 IL-22R in the skin explant model

Finally, having seen an increase in IL-22R staining in pathological biopsies when compared to a healthy control (Figure 3.13), I investigated whether IL-22R was similarly increased in the skin explant model in order to explore the possibility that IL-22R induction might impact on which 'patients' responded to the addition of rIL-22. An additional slide was made, and stained for the IL-22R from the Control, MLR only and IL-22 150pg/ml blocks from experiment 4.3.3. The range of results was narrower than that seen with the patient biopsies (Figure 4.9). No significant difference in percentage of IL-22R positive cells was seen between responders and non-responders (two way ANOVA), although this may be due to the small sample size. There is a trend towards increased IL-22R staining in the responder group at a rIL-22 concentration of 150pg/ml.

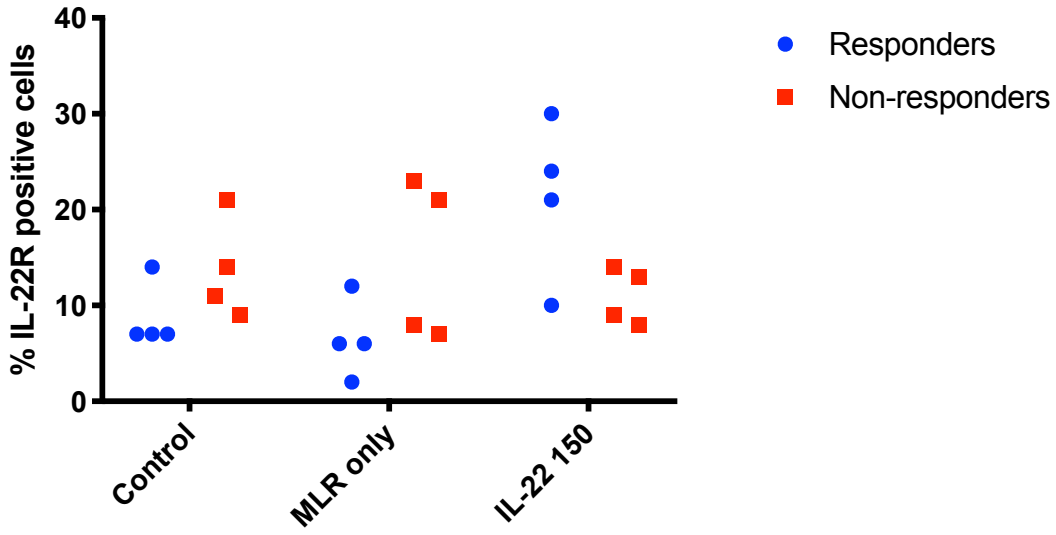

Figure 4.9 IL-22R staining in the skin explant model. No significant difference is seen between responders and non-responders for any of the conditions (two way ANOVA).

4.4 Discussion for Chapter Four

The aim of this chapter was to explore the potential for therapeutic use of IL-22 in an *in vitro* model. This work has demonstrated that the addition of rIL-22 alone to healthy skin cultured in vitro does not cause GVHD-type skin damage, even when added at supra-physiological concentrations. In a non-inflammatory model, no effect was seen with the addition of rIL-22. In contrast to the worsening symptoms seen in IBD (Li et al., 2014) in the preliminary experiments performed with an IL-22 blocking antibody, no change in GVHD grade has been observed.

In the inflammatory model that was created using the MLR supernatant, however, the addition of rIL-22 did have an effect. A trend towards a pro-inflammatory effect was seen with the addition of a low concentration of rIL-22, and a reduction in GVHD grade at higher concentrations, although neither reached statistical significance. It was interesting to observe that no benefit was achieved until IL-22 concentrations were higher than those measured in the serum of patients with aGVHD. This raises the possibility of a biphasic response to IL-22, with a therapeutic effect only seen above the physiological range.

Interrogation of the cytokine content of the MLR supernatants did not reveal a 'responder' phenotype to explain why some experiments did, and some did not, respond to the addition of rIL-22. Although numbers are very small, the trend towards higher IL-22R staining seen in the 'responder' experiments suggests up-regulation of the IL-22R, and thus up-regulation of the IL-22/IL-22R system may contribute to the response. This observation warrants further investigation, due to the potential clinical impact of being able to predict which patients would respond to treatment with IL-22.

Taken together these results suggest that, in this model, IL-22 is not required for GVHD, and that IL-22 alone does not cause GVHD. When added to an inflammatory system with the potential for causing GVHD, however, a biphasic response is seen, where IL-22 is pro-inflammatory at low concentrations and anti-inflammatory at high concentrations. Increased IL-22R staining in responders would be consistent with a theory of increased activation of the IL-22/IL-22R system being required to produce an anti-inflammatory effect.

The major benefit of the skin explant model is the ability to perform multiple reproductions of the same experiment. The MLR supernatant, in particular, reliably produced a minimum of

Grade II GVHD, in contrast to the use of true patient/donor pairs, where not all experiments result in GVHD (Wang et al., 2006). In addition, the model combines multiple factors, giving a 'read-out' that is comparable to a genuine clinical finding. While the experiments are performed *in vitro*, it also has the benefit of being a human model.

The limitations include the fact that the Lerner criteria are relatively blunt, with only five potential Grades of GVHD. This means that some of the finer differences between samples were lost. In addition, due to the avascular nature of the experiment, there is no ability to recruit additional effector cells etc., so it is possible that some potential effects that would be observed *in vivo* are not seen.

Given the synergistic effect that has been demonstrated in a murine model between donor derived IL-22, and type I interferon, resulting in increased STAT1 phosphorylation and GVHD severity (Lamarthee et al., 2016a), it would be interesting to investigate whether at higher concentrations of IL-22, this pathway becomes 'saturated', with a return towards proportionally increased STAT3 phosphorylation. I would propose that future work should include investigation of the relative phosphorylation of the STAT1 versus STAT3 pathway, with differing concentrations of IL-22. I would also propose further exploration of changes in gene expression, to assess whether there is a phenotype that predicts for either response or non-response to rIL-22. This could be further developed for other treatments (e.g. corticosteroids) to explore whether it is a generic 'response' phenotype.

Finally, it would be interesting to repeat these studies in a gut explant model. In two murine models (Hanash et al., 2012, Mertelsmann et al., 2013) the effects of IL-22 inhibition or supplementation were seen in the liver and intestine, but not the skin. It may be that it is possible to demonstrate a more significant response to IL-22 in a human gut than a human skin model. To my knowledge, there is currently no validated human intestinal explant model for the investigation of GVHD. The Phase I study currently underway at Memorial Sloane Kettering will test the efficacy of rIL-22 in gut GVHD in a clinical setting.

Chapter 5 General Discussion, Conclusions and Future Work

GVHD remains a limiting factor in the use of HSCT in a clinical setting, both as a result of direct morbidity and mortality and because the difficulty in separating GVHD from GVL means we are not able to harness the full effect of the donor immune system. This thesis began with the hypothesis that recipient ILC3-derived IL-22 protects target organs from GVHD by protecting the recipient's tissues from conditioning induced damage, resulting in reduced T cell activation. The main aims were to further clarify the role of recipient and donor ILCs and IL-22 in a human transplant setting, especially given the known contrasting effects of IL-22, in order to develop a clearer understanding of their potential therapeutic use.

For ILC-derived IL-22 to play a role in human GVHD requires the presence of IL-22-secreting ILC in the blood and ideally the target organs of GVHD. I have demonstrated that ILCs are reduced, and in many cases eradicated from the peripheral blood by transplant conditioning, and that by Day 28 post HSCT they are predominantly of donor origin. Given the importance of Alemtuzumab as a method of T cell depletion in the United Kingdom, having demonstrated CD52 expression on ILCs, consistent with the finding of Gross et al (Gross et al., 2016), it was interesting to observe the difference in circulating ILC recovery between those patients who did and those who did not receive Alemtuzumab.

As discussed, I was unable to accurately subset ILCs in the early post-transplant period, due to the very small number of cells circulating at this time. When investigating the role of ILC recovery on IL-22 production, I have therefore had to use total ILC numbers as a proxy for ILC3 (IL-22 producing) recovery. Taking this into account, however, I have found no correlation between ILC recovery and serum IL-22 concentration. The size of the population does, however, suggest that ILC3s are unlikely to play a major role in GVHD. I have not found evidence of a difference in (donor) ILC recovery between patients who did and did not develop Grade II-IV aGVHD. There are many potential reasons for this. ILC recovery (at least total ILC recovery) may not impact on GVHD development. It may be that circulating ILCs do not impact on GVHD development, but in target organs their recovery is important, although it is worth noting that I have not found evidence for an increase in the number of ILCs in the skin of patients with GVHD. Nor have I been able to show that ILCs are responsible for the production of IL-22 in this setting. It may be that any difference is masked by confounding factors, such as the use of Alemtuzumab, or the presence of viral reactivation. Given the effect of Alemtuzumab, the proposed impact of lymphopenia on IL-7, and ILC

dependence on IL-7, the impact of IL-7 concentration on ILC recovery and aGVHD was investigated. I found no correlation between IL-7 concentration and ILC recovery. Nor did I find that early IL-7 concentration post HSCT correlated with subsequent aGVHD development, in contrast to previously published data (Dean et al 2008). I propose differences that might have been seen in my cohort have been masked by the effect of Alemtuzumab on IL-7.

In relation to the initial hypothesis regarding the role of IL-22 in HSCT, I have found no evidence that IL-22 is induced, in either the skin or the blood, by transplant conditioning, regardless of intensity. It remains possible, however, that transplant conditioning induces local IL-22 production in either the gut or liver. One of the most surprising findings of this project, was the absence of change in gene expression seen in the skin with reduced intensity conditioning. Although the Ferrara three-phase model of GVHD development is based on full intensity conditioning (Ferrara et al., 2009), it has been presumed that similar mechanisms are responsible for GVHD in reduced intensity conditioning. I believe my data challenge both the concept of IL-22 being induced by conditioning and subsequently protecting target organs from aGVHD, and that the three-phase model is the mechanism behind GVHD development in reduced intensity conditioning.

I have, however, shown that serum IL-22 is increased in patients with GVHD and demonstrated increased IL-22R expression in the skin of patients with GVHD, suggesting involvement of the IL-22-IL-22R system. I have not been able to conclusively demonstrate whether IL-22 plays a pathological or protective role in aGVHD. A number of observations, however, lend weight to it playing a pathological role at physiological levels. The first of these is that peak serum IL-22 appeared to precede the development of clinically apparent aGVHD in a number of cases, although there may be a time lag between the onset of development of GVHD in the target organs and the appearance of clinical features. There may also be a delay in the patient presenting to a physician and the development of GVHD being recorded. Secondly, the up-regulation of CXCL9, CXCL10 and STAT1 in the gene expression profiling of skin biopsies from patients with aGVHD when compared to healthy controls is consistent with the findings of Lamarthée et al, (Lamarthee et al., 2016a) who demonstrated the importance of IL-22 in this system in a mouse model. Finally, in a skin explant model, IL-22 concentrations in the range that I had measured in the serum of patients with GVHD were associated with the presence of histological changes consistent with aGVHD, and the addition of low dose IL-22 was associated with a trend towards increasing

GVHD Grade. IL-22 does not, however, appear to be necessary for the development of aGVHD. Not all of the patients with aGVHD had elevated serum IL-22 concentrations, and preliminary results using an IL-22 blocking antibody in the skin explant model would support this finding.

Further data to support the importance of IL-22 in the development of GVHD came from the SNP study. Published data on high and low IL-22 producing phenotypes (De Luca et al., 2013), with the results of a number of murine IL-22 knockout models (Hanash et al., 2012, Mertelsmann et al., 2013, Couturier et al., 2013, Lamarthee et al., 2016a), would predict that a low IL-22 producer recipient with a high IL-22 producer donor (a CCTT genotype at rs2227485) would result in excess GVHD deaths. This was the finding of a competing risks analysis of 524 patient-donor pairs, comparing a CCTT genotype with all other patients combined.

It was not until IL-22 concentrations reached supraphysiological levels in the skin explant model that a reduction in the grade of GVHD was seen. This would suggest, taken with the additional findings above, that if IL-22 has potential as therapy for aGVHD, this will be at supraphysiological concentrations. Given the lack of alternative treatments available for steroid refractory GVHD, and the potential for treating the target organs without targeting the lymphocytes, further investigation is warranted. Careful consideration would, however, need to be given to the pharmacodynamics and pharmacokinetic properties of any drug, given the potentially detrimental properties at lower concentrations. It will be interesting to observe the outcomes of the current trial of F-562 in patients with lower gastrointestinal GVHD (NCT02406651).

In addition to the difficulties posed by the small cell numbers, I think the biggest limitation of this project is the heterogeneity of transplant patients. Patients begin conditioning therapy having been treated for different diseases, with varying protocols and for different time periods. Transplant protocols themselves vary, depending on disease, donor, graft source and age of patient. Post-transplant complications and treatment required for these vary widely. In contrast to this heterogeneity, however, GVHD represents a stereotypic response to immune effector cell injury. One potential method for overcoming the heterogeneity, to investigate ILC reconstitution only, would be to look in an autologous setting. As graft versus host disease was a central theme of this project, all the work was performed on samples from patients receiving allogeneic transplants. The autologous setting would, however, offer a

potentially much more homogenous patient and treatment cohort. For example, patients with multiple myeloma receiving a first autologous SCT have usually been treated with a limited number of induction regimens, and standardly receive single agent melphalan as conditioning (Auner et al., 2018). In addition, the fact that these patients do not usually develop GVHD or viral reactivation means that the first 100 days post transplant is also much more homogenous than that of a patient post allograft. My data would suggest that the absence of T cell depletion from this regimen is likely to result in more rapid ILC reconstitution, and potentially combining this patient cohort with an alternative method of detection such as CyTOF, would enable further investigation of the kinetics of different ILC subset recovery.

This work has, perhaps, raised as many questions as it has answered, and there are many aspects that warrant further investigation. One is further analysis of the gene expression data in both conditioning and GVHD, using gene pathway analysis to further explore up- versus down-regulation in both settings. Following on from this, a potentially fruitful avenue of exploration is further investigation of the downstream pathway of IL-22, in particular the relative phosphorylation of STAT1 versus STAT3 in GVHD, and whether this is altered by the IL-22 concentration. This could be undertaken using a combination of clinical samples, and the skin explant model. A better understanding of the impact of IL-22 on this pathway in GVHD would be beneficial to our ability to use this pathway as a therapeutic target.

A further variable in the IL-22 pathway not addressed in this project is IL-22BP. The binding protein is an inhibitor of IL-22 as it prevents binding to the trans-membrane receptor. While I have shown a rise in IL-22 in the serum in GVHD, it would be interesting to investigate whether this is mirrored by a fall in IL-22BP (as seen in the DSS model of colitis) or a rise (as seen in humans with IBD). IL-22BP represents another potential target for modulation of the IL-22/IL-22R system, but the normal response in GVHD needs first to be established. To my knowledge, only one group has published data on IL-22BP in the context of GVHD (Lounder et al., 2018). The study investigated IL-22 levels in children with gastrointestinal GVHD. An inverse correlation between IL-22 and IL-22BP levels at Day 30 was shown, but not an association with gastrointestinal GVHD. No further information regarding IL-22BP is given.

Finally, this project was performed using peripheral blood and skin samples, in part because of all the organs affected by GVHD, skin is the least invasive to sample. It is possible, however, that the IL-22-IL-22R axis plays a bigger role in gut or liver GVHD, as has been suggested in two murine models (Hanash et al., 2012, Mertelsmann et al., 2013). It would be

interesting to investigate whether this finding translates into human GVHD, by investigating ILC composition, IL-22R staining and IL-22 concentration in other target organs. The biggest difficulty that I would envisage in undertaking this work would be access to pathological samples. In our institution, a limited number of sigmoid colon biopsies and very few liver biopsies are performed each year. A validated gut or liver GVHD explant model would potentially enable work on the impact of the therapeutic use of IL-22 in this setting.

Despite many recent advances in the field of haemato-oncology, allogeneic stem cell transplant remains the only curative option for a number of haematological conditions, and GVHD continues to pose a significant barrier to successful outcomes. Targeting of the IL-22/IL-22R system provides an exciting option for the future, but further knowledge of the system in the context of GVHD is needed to maximise any future benefit.

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