The Genetic and Functional Significance of Non-HLA Polymorphisms in Haematopoietic Stem Cell Transplantation

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Abstract

Haematopoietic stem cell transplantation (HSCT) is currently the only curative treatment for many patients with malignant and non-malignant haematological diseases. However, its success is greatly reduced by the development of complications, including graft-versus-host disease (GVHD), infection and relapse. Human leukocyte antigen (HLA)-matching of patients and donors is essential in HSCT, but does not completely prevent such complications. Thus, it is extremely likely that non-HLA genes also have an impact.

Naturally occurring polymorphisms within non-HLA genes have been suggested to contribute to some of the genetic disparity that exists between transplant patients and donors, consequently, they may have the potential to influence HSCT outcome. As a result, this study investigated the significance of non-HLA polymorphisms in HSCT.

Polymorphisms in the genes of interleukin (IL) 2 (*IL2*, -330 T/G), 4 (*IL4*, -590 C/T), 6 (*IL6*, -174 G/C) and 10 (*IL10*, -592 A/C, -1082 G/A), IL-1 receptor antagonist (IL-1Ra (*IL1RN*), intron 2 VNTR), tumour necrosis factor α (TNFα (*TNFA*), intron 3 (GA)n), TNF receptor II (TNFRII (*TNFRSF1B*), -196 M/R), interferon γ (IFNγ (*IFNG*), intron 1 (CA)n), vitamin D receptor (*VDR*, intron 8 A/C, exon 9 T/C) and oestrogen receptor (*ESR1*, intron 1 A/G, intron 1 C/T) have been studied extensively in HLA-matched sibling transplantation, however, relatively little is known about their role in unrelated donor HSCT. This study therefore examined these polymorphisms in the unrelated donor setting. *VDR* intron 8 A/C, *IFNG* intron 1 (CA)n and *IL6* -174 G/C were found to associate with GVHD in our cohort. The *VDR* intron 8 A,A and *IFNG* intron 1 3,3 genotypes correlated with severe acute GVHD (grades III-IV), whilst the *IL6* -174 G,G genotype correlated with chronic GVHD. No genetic associations were demonstrated with any other clinical outcome.

This study also examined the +2044 G/A polymorphism in the gene encoding IL-13 (*IL13),* an immunoregulatory cytokine that has been implicated in GVHD. This polymorphism has been widely studied for disease associations; however, its role in HLA-matched sibling and unrelated donor HSCT is currently unknown. In our transplant cohort the *IL13* +2044 A allele was found to correlate significantly with both

acute and chronic GVHD. Higher IL-13 expression was also observed in patients with these conditions, although this altered expression could not be directly attributed to *IL13* +2044 G/A. The prognostic significance of pre-transplant IL-13 serum levels was also investigated. No associations were observed between IL-13 expression and GVHD. Elevated pre-transplant IL-13 serum levels did, however, correlate with post-transplant relapse and Hodgkin disease in two independent cohorts. Again, this altered expression could not be directly attributed to the *IL13* +2044 G/A polymorphism.

This study clearly demonstrates that non-HLA polymorphisms influence the outcome of both HLA-matched sibling and unrelated donor HSCT. Thus, in the future, genotyping with respect to a panel of non-HLA polymorphisms may be used to complement HLA typing, increase the ability to predict the risk of transplant complications and allow post-transplant treatment strategies to be tailored to an individual.

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Author's Declaration

The material contained in this thesis is entirely the work of the author, unless otherwise stated. The contents have not been previously submitted for any other degree with Newcastle University or any other university.

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List of Abbreviations

AA	Aplastic Anaemia
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
APC	Antigen presenting cell
BMT	Bone marrow transplantation
CBA	Cytometric bead array
CD	Cluster of differentiation
CGP	Cytokine gene polymorphism
CLL	Chronic Lymphoblastic Leukaemia
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
CsA	Cyclosporine A
CSF2	Granulocyte macrophage colony-stimulating factor gene
CTL	Cytotoxic T cell
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EBSS	Earle's balanced salt solution
ELISA	Enzyme-linked immunosorbent assay
ESR	Oestrogen receptor gene
GVH	Graft-versus-host
GVHD	Graft-versus-host disease
GVHR	Graft-versus-host reaction
GVL	Graft-versus-leukaemia
HD	Hodgkin disease
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
IFN	Interferon
Ig	Immunoglobulin
IHG	Induced heteroduplex generator
IL	Interleukin

IL-1Ra	Interleukin-1 receptor antagonist
KDa	Kilodalton
LPS	Lipopolysaccaride
MAC	Myeloablative conditioning
MHC	Major histocompatibility complex
mHAG	Minor histocompatibility antigen
MLC	Mixed lymphocyte culture
MM	Mycophenolate Mofetil
MTX	Methotrexate
NHL	Non-Hodgkin Lymphoma
NK	Natural killer
NO	Nitric oxide
OD	Optical density
OR	Odds ratio
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
RFLP	Restriction fragment length polymorphism
RIC	Reduced intensity conditioning
RPMI	Roswell Park Memorial Institute 1640 culture medium
RT	Room temperature
SEM	Standard error of the mean
SIB	Sibling
SNP	Single nucleotide polymorphism
SSCP	Single stranded conformational polymorphism
TBI	Total body irradiation
Th	Helper T lymphocyte
TNF	Tumour necrosis factor
TNFRSF	Tumour necrosis factor superfamily receptor gene
TRM	Transplant-related mortality
VDR	Vitamin D receptor
VNTR	Variable number of tandem repeats

Chapter 1. Introduction

Haematopoietic stem cells (HSCs) are immature blood cells found in the bone marrow, from which all other types of blood cells evolve and differentiate. Due to their ability to self-renew, a small number of HSCs can expand and generate a very large number of progeny HSCs. This phenomenon is employed in haematopoietic stem cell transplantation (HSCT) to reconstitute the haematopoietic system of a transplant patient in order to overcome a functional deficit (Wodnar-Filipowicz 2008).

HSCT is currently the only curative treatment for many malignant and non-malignant haematological diseases. Despite this, a high transplant-related mortality (TRM) is associated with the procedure and its success is greatly limited by the development of complications, such as graft-versus-host disease (GVHD), infection and relapse (Appelbaum 2007). Although significant advances have been made in the field of transplantation it is still uncertain which patients will develop such complications. As a result, the identification of methods that permit the prediction of HSCT outcome prior to transplantation is becoming important. As transplant complications are immunologically mediated, naturally occurring variations (polymorphisms) within genes associated with an individual's capability to mount an immune response have emerged as possible predictive markers and are currently being studied for associations with complication development and thus transplant outcome. Due to the complexity of HSCT it is unlikely that a single predictive genetic marker will exist. The ultimate goal of this research is therefore the identification of a profile of genetic markers that can be used in combination with identified clinical risk factors (prognostic index) prior to transplantation to inform on HSCT outcome, so that patients at high risk of complications can be identified early and treatment strategies can be tailored to the individual or adjusted accordingly. Such research may also lead to the identification of potential novel targets for future treatment modalities.

1.1 Haematopoietic Stem Cell Transplantation

The atomic bomb essentially marked the beginning of interest in HSCT, with the detonation and subsequent effects of nuclear devices provoking great interest in the effects of irradiation on humans; particularly on bone marrow function (Perry and Linch 1996; Baron and Storb 2004). However, the era of HSCT actually began with the landmark observation by Jacobson *et al,* (1949) that the lethal effects of ionizing radiation on bone marrow could be prevented by shielding the spleens of mice with lead (Jacobson *et al.* 1949). In 1951 it was demonstrated that this protection could also be conferred by intravenous infusion of bone marrow (Lorenz *et al.* 1951). It was not however, until the mid-1950s that the protective effect of bone marrow was attributed to HSCs within the infusion (Barnes and Loutit 1954; Main and Prehn 1955). This discovery and subsequent studies in mouse models led to the initial attempts at human HSCT (Thomas *et al.* 1957). Although these transplants were largely unsuccessful they did provide firm evidence that the infusion of stem cells from one individual to another was possible. Research continued, and eventual progress made in studies of primates and canines renewed hope that patients with haematological diseases could be treated by HSCT (van Bekkum 1977; Storb and Thomas 1985). Finally, in the late-1960s/early-1970s the first successful stem cell transplants were reported (Bach *et al.* 1968; Thomas *et al.* 1975). Nowadays, HSCT is routinely used to treat diseases, such as acute and chronic leukaemias, lymphomas, anaemias and immunological deficiencies.

Since these initial studies there has been a substantial growth in HSCT (Figure 1.1), with a dramatic increase in both the annual number of HSCT procedures and the number of diseases for which HSCT is considered appropriate. Current estimates suggest that 55-60,000 transplants are undertaken worldwide each year and HSCT now offers a potential for cure or long-term control of a number of diseases where other treatment options fail (Wingard *et al.* 2002; Appelbaum 2007; Eapen and Rocha 2008). A better understanding of the underlying disease, transplant strategy and supportive care has permitted this escalation and consequently changed the role of HSCT from a desperate therapeutic manoeuvre to a curative treatment modality (Baron and Storb 2004).

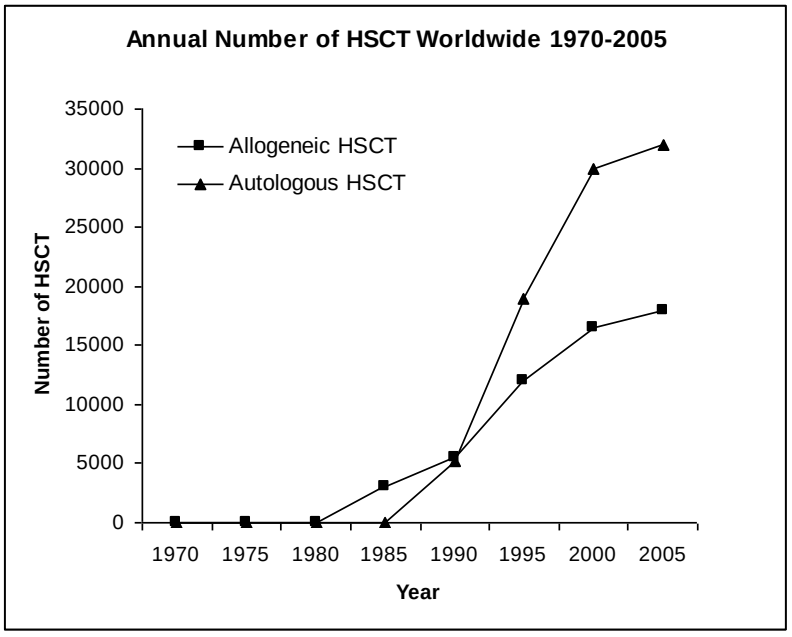

Figure 1.1 Annual numbers of allogeneic and autologous HSCT performed worldwide 1970-2005. Adapted from (Appelbaum 2007).

HSCT may be autologous (stem cells from the patient), syngeneic (stem cells from a genetically identical donor) or allogeneic (stem cells from a genetically distinct donor). An autologous transplant involves the reinfusion of the patient's own cells to provide a therapeutic benefit. This process has inherently fewer complications than allogeneic transplantation, although there is a risk that the stem cell graft may contain residual tumour cells that can cause disease relapse (Bjorkstrand *et al.* 1996; Lowenberg and Voogt 1996). Sibling donors that are genetically identical (syngeneic) are the ideal in HSCT, however, since few people have identical twins, this transplant type is relatively rare. Patients without a genetically identical relative have the option of an allogeneic HSCT from a suitably matched family member, usually a sibling or from an unrelated donor. This thesis concentrates on allogeneic HSCT from HLA-matched sibling or unrelated donors. Autologous and syngeneic transplantation are not covered in this current study

Allogeneic HSCT is initiated with a period of conditioning that prepares the patient for transplantation. Traditionally, this has involved the administration of total body irradiation (TBI) alone or in combination with chemotherapeutic agents such as

cyclophosphamide, melphalan and busulphan (target proliferating cells) at a level that destroys all lymphoid and myeloid cells of the body (myeloablative conditioning, MAC). The conditioning regimen has three main objectives: creation of space for the incoming graft, immunosuppression to prevent rejection of the donor cells by any residual host immune system and disease eradication. Whilst conditioning is necessary for HSCT, myeloablative regimens are associated with considerable toxic side effects involving the gastrointestinal tract, liver and kidneys, that are responsible for the majority of TRM (Bacigalupo *et al.* 2000; Gratwohl 2008). Thus, more recently there has been an emergence of HSCT using low intensity conditioning. Such regimens aim to reduce the complications associated with myeloablation by employing smaller doses of radiation and chemotherapy. Low intensity conditioning is based mainly on the chemotherapeutic agent fludarabine (induces lymphocyte apoptosis) and is considered as either non-myeloablative (fludarabine + low dose irradiation (2 Gray (Gy)) or reduced intensity (RIC, fludarabine + cyclophosphamide, melphalan or busulphan)(Gratwohl 2008). Unlike conventional myeloablative preparations, low intensity regimens are primarily immunosuppressive, run lower risks of serious infections and TRM, but depend purely on the graft for disease eradication. These newer transplant strategies are therefore employed mainly for the elderly and other patients who would otherwise be considered too weak to withstand myeloablation (Copelan 2006; Gratwohl 2008).

The transplant procedure itself involves the infusion of HSCs into the peripheral blood, where, after briefly circulating in the bloodstream, they migrate to the bone marrow cavity and engraft ($> 0.5x10^9$ neutrophils/L and $> 50x10^9$ platelets/L for 3 consecutive days) (de Lima and Champlin 2001). After several weeks of growth in the marrow, the expansion of stem cells is sufficient to normalize the blood count and reconstitute the immune system. Stem cells from the haematopoietic system are the progenitors of subsequent blood cell lineages, that are able to self-renew and differentiate into many different cell types and can be obtained from a variety of sources; bone marrow, peripheral blood and cord blood (Wodnar-Filipowicz 2008). Traditionally, stem cells for HSCT were obtained from the bone marrow, harvested from the rear of the pelvis (iliac crests) via needle aspiration under general anaesthetic. Nowadays however, stem cells from the peripheral blood are most commonly employed in HSCT. Peripheral blood stem cells (PBSC) are isolated and collected at the bed side through a process known as apheresis. Growth factors such as granulocyte colony-stimulating factor (G-

CSF) mobilise stem cells from the marrow cavity into the peripheral circulation and thus are used to boost the yield of PBSCs collected (Larghero *et al.* 2008). Compared with marrow, PBSC collections are less invasive and associated with a more rapid haematologic recovery (engraftment), but they have been linked with late complications of HSCT (Jansen *et al.* 2005). The third source of stem cells in transplantation is the blood from the umbilical cord and placenta. Cord blood is enriched with stem cells, but the small quantity of blood normally collected makes this source more suitable for paediatric transplants. Techniques such as ex-vivo expansion and the use of two or more cord blood units from different donors are being explored in order to permit the routine use of cord blood transplants in adults (Piacibello *et al.* 1998; Majhail *et al.* 2006). This thesis concentrates on allogeneic HSCT undertaken using stem cells from bone marrow or peripheral blood. Cord blood transplants are not covered in this current study.

1.2 The Major Histocompatibility Complex

A major advancement in the success of HSCT came with the discovery of the major histocompatibility complex (MHC). The first HSCT performed based on knowledge of the MHC was carried out in 1968 to treat an infant with severe combine immunodeficiency (Gatti *et al.* 1968). The earliest animal models of solid organ transplant used skin grafts to demonstrate that rejection occurred due to lymphocytic infiltration of the foreign tissue if organs were transplanted across strain barriers (Medawar 1944). The immunological problems associated with allo-grafting were shown to result from genetically encoded differences in tissue type initiating an immune (T cell) response. This led to the characterisation of the MHC.

The MHC is a tightly linked cluster of genes encoding cell surface glycoproteins, which can present foreign antigens to circulating T-cells resulting in a highly specific mechanism for T cell activation (Goulmy 1988). The human MHC is located on the short arm of chromosome 6 and spans 3.6 million base pairs of DNA (Consortium 1999). With over 200 genes identified, the MHC is the most gene-dense region in the genome and encodes some of the most polymorphic proteins. Polymorphisms within the MHC are thought to be driven by the continuous response of the immune system to infectious pathogens (Parham 1999).

The MHC is divided into three regions, class I, class II and class III. Class I and class II molecules are closely related in both structure and function and are encoded by the human leukocyte antigen (HLA) genes. This large cluster of genes were originally defined in transplantation experiments by their ability to promote vigorous rejection of grafts between different members of the same species (Snell 1948). Both HLA class I and class II molecules have two domains that resemble immunoglobulin domains and two domains that fold together to create a peptide-binding cleft. Both play a role in the binding of foreign peptides for recognition by the immune system. However, differences in their structure allow them to serve distinct functions in antigen presentation to different T cell subsets (Janeway *et al.* 1999). HLA Class I molecules are present on all nucleated cells and accommodate intracellular derived peptides of 8-11 residues, which when bound are recognised by CD8+ (cytotoxic) T cells and natural killer (NK) cells (York and Rock 1996). HLA class II molecules accommodate larger extracellular derived peptides of 10-30 residues, which when bound are recognised by CD4+ (T-helper) T cells. The expression of class II molecules is restricted primarily to antigen presenting cells (APCs), such as B cells, dendritic cells (DC) and macrophages (Boss 1997).

HLA class I and class II genes are the most polymorphic genes present in the genome. For some HLA genes over 500 different alleles have been identified. The high degree of allelic variants between individuals has significant implications in transplantation, since the chance of a complete match between a patient and an unrelated donor is extremely low and the degree of HLA disparity correlates closely with the rate of mortality after HSCT (Anasetti *et al.* 1995; Szydlo *et al.* 1997). To date ten HLA class I genes have been described: HLA-A, -B, -C, -E, -F, -G, -H, -J, -K, and –L. Of these, only HLA-A, -B. -C, -E, -F and -G are translated into proteins, the remaining four are pseudogenes. Characterisation of HLA-A, -B, and -C has demonstrated extensive polymorphisms for these genes and their encoded proteins. The expression of HLA-E, -F, and -G however, is more discriminate and these genes are significantly less polymorphic (Table 1.1) (Klein and Sato 2000). Five groups of HLA class II genes have also been described: HLA-DR, -DQ, -DP, -DM and -DO. Of these, HLA-DR, -DQ and -DP are the most polymorphic and associate with peptides for presentation (Klein and Sato 2000). In terms of matching individuals for HSCT, the polymorphic nature of HLA class I and II genes has great implications. Traditionally, only HLA-A, -B, and HLA-DR were considered as these were easier to identify and thought to be the

most polymorphic. More recently however, the application of DNA-based typing methods has highlighted the need to consider other genes which are also extensively polymorphic, such as HLA-C, -DQ, and -DP (Little and Madrigal 1999; Flomberg *et al.* 2004; Shaw *et al.* 2007).

HLA Class I	Number of Alleles
HLA-A	767
HLA-B	1178
HLA-C	439
HLA-E	9
HLA-F	21
HLA-G	43

Table 1.1 Number of identified HLA class I alleles as of January 2009. (Data obtained from IMGT-HLA database http://www.ebi.ac.uk/imgt/hla/). HLA, Human Leukocyte Antigen

The class III MHC region, which is flanked by the class I and II regions contains no HLA genes, but 57-60 structural genes that span 654-759kb of genomic DNA. This region encodes molecules that are critical to immune function, but they have very little in common structurally and functionally with class I and II molecules (Goldsby *et al.* 2003). Class III region products include the complement components C4, C2 and BF and the inflammatory cytokines tumour necrosis factor (TNF), lymphotoxin and heat-shock proteins. Other genes within the class III MHC region include essential genes involved in growth, development and differentiation and enzymes involved in metabolism (Klein and Sato 2000). The physiological roles of the majority of the genes within the class III region are yet to be determined; however, many autoimmune, neurological, reproductive and malignant disorders have been associated with the MHC.

1.3 Graft versus Host Disease

GVHD is an immunologically mediated disease that is the major limitation to the success of allogeneic HSCT. Despite progress in the understanding of GVHD and advances in HLA typing techniques, conditioning regimens and post-transplant prophylaxis strategies, the disease still occurs in 30-60% of transplants and most patients who develop severe manifestations succumb to it or to treatment related complications (Barret 1987; Goker *et al.* 2001; Vogelsang *et al.* 2003).

GVHD affects multiple target organ systems and occurs when immunologically competent T cells are introduced into an immunologically incompetent host. T cells within bone marrow or PBSC transplants recognise mismatched major (HLA) and/or minor (mHag) histocompatibility antigens of the transplant patient as foreign, become activated and initiate an immune response (allo-response) (Gale 1985; Goker *et al.* 2001).

The first description of GVHD came from animal experiments documenting haematopoietic reconstitution following lethal marrow irradiation. Mice that received syngeneic stem cell transplants recovered from radiation toxicity, 'primary disease'; whereas mice that received stem cells from different murine strains recovered from their primary disease but went on to develop 'secondary disease'. This condition was characterised by weight loss, diarrhoea, liver abnormalities and eventual death (Barnes *et al.* 1956). The occurrence of human GVHD was first demonstrated in 1963, when Mathe *et al* (1963) reported the development of severe dermatitis, weight loss and diarrhoea in a patient transplanted for leukaemia (Mathe *et al.* 1963). Following this, in 1966 Billingham outlined three prerequisites for the development of GVHD (Billingham 1966):

1) A graft containing a sufficient number of immunologically competent cells.
2) Histoincompatibility between the recipient and donor.
3) A host immune system that is incapable of mounting an effective immune response against the graft.

Although there have been slight modifications to these factors since their proposal, the basic principles still apply.

GVHD occurs in two forms; acute and chronic. The distinction between the two conditions was traditionally based on the time of onset; less or more than 100 days post transplant. However, since acute GVHD can present beyond 100 days when low intensity conditioning is employed or when immunosuppression is withdrawn, characteristics of chronic GVHD can occur before day 100 and symptoms common to both forms of the disease exist; this distinction is no longer plausible (Ringden and Deeg 1997; Devergie 2008). Nowadays, acute and chronic GVHD are determined by specific clinical and histological characteristics rather than by time of onset alone (Table 1.2).

Category	Time of manifestation	Acute GVHD features	Chronic GVHD features
Acute GVHD			
Classic	≤ 100 days	Yes	No
Pesistent, recurrent, late onset	> 100 days	Yes	No
Chronic GVHD			
Classic	No time limit	No	Yes
Overlap syndrome	No time limit	Yes	Yes

Table 1.2 Definition of acute and chronic GVHD (Devergie 2008). GVHD, graft-versus-host disease

1.3.1 *Acute GVHD*

Acute GVHD usually occurs within weeks of transplantation (< 100 days) and ranges from mild and self-limiting to an extremely severe and potentially fatal disorder. It is currently responsible for 15-40% of mortality and is the major cause of morbidity following allogeneic HSCT. Acute GVHD is characterised by damage primarily to the skin, liver and gastrointestinal tract, but it can also affect the reconstitution and functioning of the patient's now donor-derived immune system due to immune mediated destruction of the bone marrow stroma. The onset of acute GVHD often coincides with engraftment (Vogelsang *et al.* 1988; Hart and Fearnley 1997; Sun *et al.* 2007).

Clinical Manifestations and Grading

An itchy erythematous maculopapular rash is usually the earliest clinical manifestation of acute GVHD (Figure 1.2). The rash initially involves the palms, soles, face and trunk, but if the GVHD advances it spreads and can cover the entire body surface. Lesions are tight and pruritic, and in severe disease the skin may blister and become intensely painful. Surface area desquamation can occur, that leads to denudation and protein loss (Glucksberg *et al.* 1974; Vogelsang *et al.* 2003; Devergie 2008). Liver involvement is characterised by cholestatic hepatopathy with or without jaundice. Onset of liver GVHD usually coincides with a rise in serum levels of bilirubin, alkaline phosphatase and transaminases, and symptoms including fatigue, easy bruising and confusion are often indications of hepatic dysfunction. In extreme cases of GVHD, hepatic failure can occur (Crawford 1997; Vogelsang *et al.* 2003; Devergie 2008). Acute GVHD of the gastrointestinal tract usually presents at the same time as or shortly after the onset of skin GVHD and primarily manifests as nausea, vomiting and watery diarrhoea. Advanced GVHD is often associated with severe abdominal pain, bloody diarrhoea and massive enteral fluid loss. Diarrhoea results from water and protein exudation across a damaged mucosa and in severe disease may have a stringy appearance due to sloughing of intestinal mucosa (Mowat 1997; Vogelsang *et al.* 2003; Devergie 2008).

Concomitant signs of acute GVHD include fever, weight loss and a decrease in performance status. Lymphoid organs, mucous membranes, endocrine glands and the bronchi may also be involved in the disease, but these are not included in the clinical staging and grading (Devergie 2008).

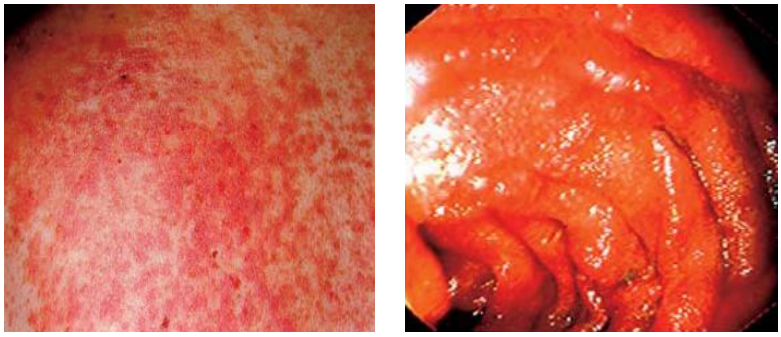

Figure 1.2 Clinical appearance of acute GVHD involving the skin and upper intestinal mucosa. Left: The diffuse erythematous maculopapular rash typical of acute GVHD of the skin. Right: An oedematous and reddened mucosa seen in GVHD of the gastrointestinal tract. (Riddell and Appelbaum 2007).

The first clinical grading system for acute GVHD was developed in 1974 (Glucksberg *et al.* 1974), however, in recognition of it's limitations this system was later modified in a consensus conference on acute GVHD grading (Table 1.3) (Przepiorka *et al.* 1995). Overall acute GVHD grade is based on the extent of skin, liver and gastrointestinal tract damage and ranges from I to IV, with the severity of acute GVHD increasing with grade. In brief, grade I denotes mild disease and a good clinical prognosis; grade II indicates moderately severe disease and grade III and IV are the most severe life-threatening stages, with multi-organ involvement and increased mortality.

	Extent of organ involvement		
	Skin	**Liver**	**Gut**
Stage			
1	Rash on <25% of skin	Bilirubin 2-3mg/dl	Diarrhea >500ml/day or persistent nausea
2	Rash on 25-50% of skin	Bilirubin 3-6mg/dl	Diarrhea >1000ml/day
3	Rash on >50% of skin	Bilirubin 6-15mg/dl	Diarrhea>1500 ml/day
4	Generalised erythroderma with bullous formation	Bilirubin >15mg/dl	Severe abdominal pain with or without ileus
Grade			
I	Stage 1-2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	-	Stage 2-3 or	Stage 2-4
IV	Stage 4 or	Stage 4	-

Table 1.3 Consensus conference grading of acute GVHD. Clinical acute GVHD overall is assessed by the minimum organ involvement required to confer that grade (Przepiorka *et al.* 1995).

Histopathological Features

The characteristic histological appearance of acute GVHD is apoptosis of epithelial cells in the skin, liver and gastrointestinal tract. GVHD lesions in these target organs are the same, consisting of apoptotic cells associated with immune cell infiltrates (satellite cell necrosis) (Devergie 2008). In the skin, the dermo-epidermal junction is the most severely affected, with epidermal and basal cell vacuolisation and necrosis. In patients with severe skin GVHD sub-epidermal cleft formation and/or complete dermal-epidermal separation may occur (Vogelsang *et al.* 2003). Pathological changes in the liver primarily affect the epithelium of the biliary canal and include lymphocytic infiltration and degenerative necrosis that leads to bile duct damage or loss, whilst in the gastrointestinal tract, necrosis around the basal crypts results in abscess formation and epithelial desquamation (McCarthy 1998; Devergie 2008).

In the first few weeks following HSCT the microscopic lesions induced by chemotherapy or conditioning regimens may be very similar to the lesions associated with GVHD, consequently acute GVHD is often difficult to diagnose early in the post-transplant period (Devergie 2008).

Pathophysiology

Murine transplant models have permitted the elucidation of the mechanisms involved in acute GVHD and the roles of particular cell types and soluble mediators implicated in the process. The pathophysiology of acute GVHD is considered to occur in three phases (Figure 1.3); the first of which is instigated by pre-transplant conditioning regimens, which damage host tissues and activate inflammatory cytokines. The second phase is initiated by the infusion of allogeneic stem cells and consists of donor T cell activation, proliferation and differentiation. Finally, the third phase is the effector phase, where the histopathological changes of acute GVHD become apparent (Chao 1997; Ferrara *et al.* 1999).

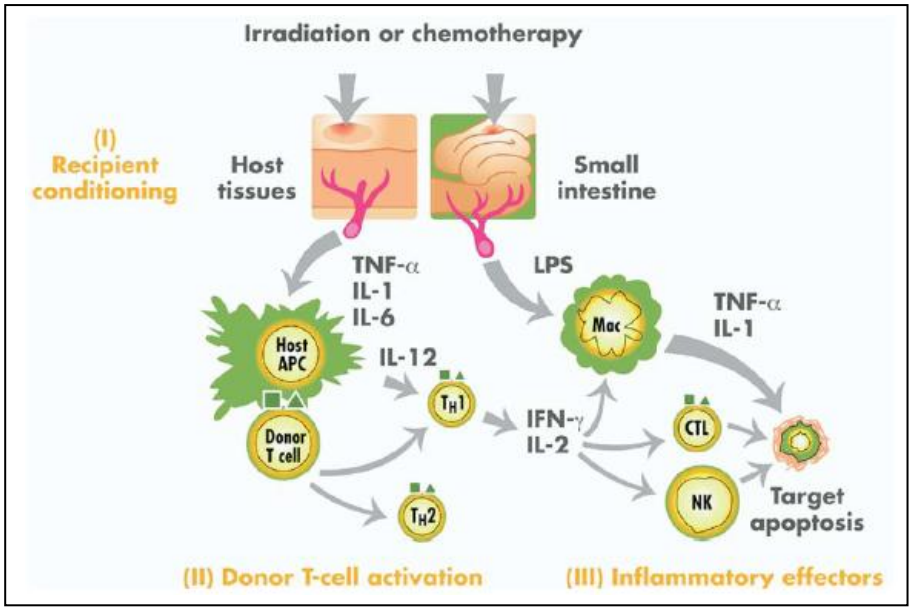

Figure 1.3 The pathophysiology of acute GVHD. Acute GVHD development is considered as a three phase process: (1) injury to host tissues by the conditioning regimen, resulting in the release of inflammatory cytokines; (2) infusion of the stem cell graft followed by donor T cell activation, proliferation and differentiation and (3) cellular and inflammatory attack, producing skin, liver and gastrointestinal tract damage. APC, antigen presenting cells; CTL, cytotoxic T lymphocyte; T_H, T helper; Mac, macrophage; NK, natural killer cell; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumour necrosis factor α; LPS, lipopolysaccharide (Devetten and Vose 2004)

Phase I: Conditioning Regimen

The earliest phase in the development of acute GVHD begins when conditioning regimens employed to prepare the patient for transplantation, cause profound tissue damage and activation of the immune response in host tissues, including the intestinal mucosa and the epithelial surfaces of the skin and liver. Underlying disease and infections may also contribute to this damage. Activated host cells respond to the tissue damage by secreting considerable amounts of inflammatory cytokines, such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6) (Xun *et al.* 1994). This cytokine release increases the expression of adhesion molecules (ICAM-1), costimulatory molecules and HLA antigens in the host (Pober *et al.* 1986; Leeuwenberg *et al.* 1988; Norton and Sloane 1991; Thornhill *et al.* 1991). These are essential for the localisation and activation of immune cells. The secretion of inflammatory cytokines also activates host APCs; which present foreign antigens to T cells and thus are vital for the development of GVHD. In murine models, cytokine release results in the activation

of host APCs, and the complete abolition of GVHD is possible by inactivation of these cells (Shlomchik *et al.* 1999). Host cells also secrete chemokines, which aid in the migration of other immune cells, including neutrophils and monocytes to the sites of tissue damage.

Damage to the gastrointestinal tract by conditioning regimens is also important in the initiation of GVHD, as irradiation and chemotherapeutic agents increase the permeability of the intestinal mucosa and allow the translocation of immuno-stimulatory microbial products, such as lipopolysaccharide (LPS) into the circulation (Johansson *et al.* 2001). LPS further enhances the activation of host APCs and stimulates host macrophages and other tissues to produce excessive amounts of inflammatory cytokines, particularly TNFα. Administration of a synthetic analogue that inhibits LPS-mediated NF-κB activation decreases the amount of TNFα produced and reduces intestinal injury in murine models of GVHD (Cooke *et al.* 2001).

An increased risk of GVHD has been correlated with intensive (myeloablative) conditioning regimens that cause extensive tissue damage and the excessive release of inflammatory cytokines. This relationship has been demonstrated in both human HSCT and animal transplant models (Gale *et al.* 1987; Clift *et al.* 1990; Hill *et al.* 1997). Moreover, delaying the infusion of donor stem cells until well after the administration of intensive conditioning regimens appears to decrease the risk of acute GVHD (Johnson *et al.* 1993; Johnson and Truitt 1995; Barrett *et al.* 1998).

Phase II: Donor T cell activation, proliferation and differentiation

Activation of an allo-response begins when T cells within the infused stem cell graft interact with APCs and become activated through the recognition of allo-antigens bound to HLA molecules (Figure 1.4) (Sun *et al.* 2007). After allogeneic HSCT, both host and donor derived APCs are present in the patient. Donor T cells can recognise allo-antigens on either host (direct presentation) or donor (indirect presentation) APCs, however, direct presentation is vital for the initiation of GVHD (Shlomchik *et al.* 1999). When a transplant patient and donor are not HLA-identical, donor T cells recognise host HLA molecules as foreign and a profound graft-versus-host reaction ensues. In HLA-identical transplants, GVHD occurs through donor T cell recognition of mHag differences (immunogenic peptides derived from polymorphic intracellular proteins and

presented on MHC class I and class II molecules) (Vogelsang *et al.* 2003). T cell recognition and activation involves the interaction between alloantigen-HLA complexes on the surface of APCs and antigen specific T cell receptors (TCRs) (Sakihama *et al.* 1995). Upon activation, donor T cells produce interleukin-2 (IL-2), which stimulates allo-reactive T cells to proliferate and differentiate into effector cells.

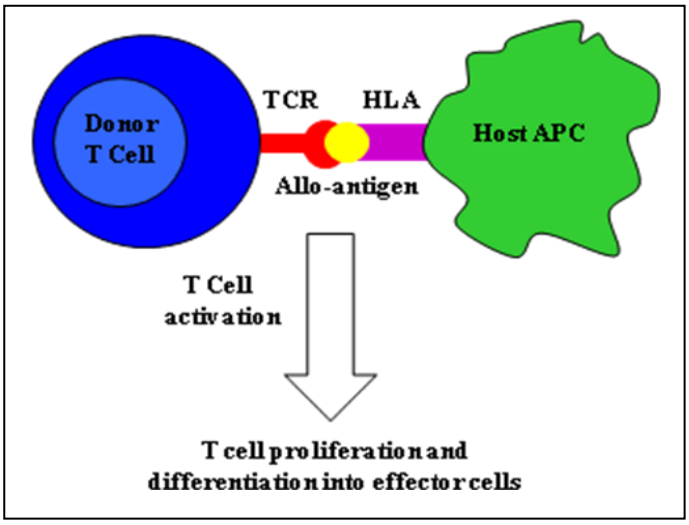

Figure 1.4 Donor T cell interaction with host APC. Molecular diagram of a CD4+ donor T cell recognising a foreign protein antigen (allo-antigen) via the T cell receptor in the context of a HLA class II molecule. TCR, T cell receptor; HLA, human leukocyte antigen; APC, antigen presenting cell

Donor T cell activation requires two signals, TCR-alloantigen-HLA binding and a costimulatory signal from APCs that requires cell to cell contact. This costimulatory signal determines the outcome of the activation sequence; if the signal is delivered T cells become activated, however, if it is absent tolerance (anergy) to the allo-antigen develops (Goker *et al.* 2001). Several molecules can provide costimulation to T cells, but the B7 antigens are currently the best characterised. Theses antigens (B7-1 (cluster of differentiation (CD) 80) and B7-2 (CD86)) bind to two T cell surface receptors, CD28 and CTLA-4. CD28 supplies a positive signal and if it is absent anergy develops. Conversely, CTLA-4 provides a negative signal; if it is not delivered, T cell activation occurs with uncontrolled cytokine production and T cell proliferation (Freeman *et al.* 1993; Green *et al.* 1994). Experimental data from murine HSCT models suggest that

the blockade of positive costimulatory molecules reduces GVHD, whereas antagonism of negative signals exacerbates the severity of the disease (Blazar *et al.* 1997; Blazar *et al.* 2003). Blockade of the B7-CD28 interaction with CTLA4-Ig has been demonstrated to prevent T cell activation and inhibit both acute and chronic GVHD (Blazar *et al.* 1994; Ogawa *et al.* 2001).

Activated donor T cells that secrete IL-2 and interferon-gamma (IFNγ) steer the subsequent immune response in the T-helper1 (T_H1) pro-inflammatory direction; activating cytotoxic (CD8+) T cells and NK cells, and enhancing IL-1 and TNFα production by host macrophages (Nestel *et al.* 1992). Both of these cytokines have been demonstrated clinically and experimentally to be important mediators of acute GVHD (Theobald *et al.* 1992; Allen *et al.* 1993; Via and Finkelman 1993; Szebeni *et al.* 1994). On the other hand, interleukin-10 (IL-10) and interleukin-4 (IL-4) secreting donor T cells enhance the proliferation of T-helper2 (T_H2) cells and drive an anti-inflammatory immune response. High spontaneous production of IL-10 by PBMCs prior to HSCT has be shown to associate with a decreased incidence of acute GVHD and transplantation of polarised T_H2 cell populations fail to induce acute GVHD to HLA class I or II antigens (Krenger *et al.* 1995; Holler *et al.* 2000). Consequently, the balance between T_H1 and T_H2 cytokines is critical for the development or prevention of acute GVHD.

Regulatory T cell subsets (Treg, Tr1 and NK1.1$^+$) may also help determine the response of donor T cells to allo-antigens. These cells can control immune responses by inducing anergy or by suppressing the expansion of allo-reactive T cells, and as a result of their actions have emerged as possible regulators of the GVHD pathway (Ferrara *et al.* 1999; Sun *et al.* 2007).

Phase III: Effector phase – target organ damage

The third phase of acute GVHD is perhaps the most complex and has only recently becoming appreciated. Initially it was thought that the cytolytic function of cytotoxic T cells directly caused the majority of damage in acute GVHD. However, it has now become apparent that several effector mechanisms are more likely to cause the damage observed. Target tissue damage can be caused directly through inflammatory mediators, in particular TNF-α and IL-1 secreted from monocytes and macrophages

(Piquet *et al.* 1987; Abhyanker *et al.* 1993). TNF-α can cause direct tissue damage by inducing necrosis of target cells or by initiating tissue destruction via apoptosis commonly mediated through the TNF-α-Fas antigen pathway (Laster *et al.* 1988). The critical role of cytokines in the pathogenesis of acute GVHD has also been highlighted in a study that demonstrated the occurrence of severe acute GVHD as a result of inflammatory cytokines in the absence of allo-antigen expression on host target tissue (Teshima *et al.* 2002). Syngeneic HSCT was utilised to generate mice in which all cells except marrow-derived APCs lacked either HLA class I or class II molecules, a further allo-graft from a mismatched donor resulted in 100% mortality due to acute GVHD. Further studies have demonstrated an increase in cytokine expression in the skin and PBMCs with subsequent increase in the severity of acute GVHD (Abhyanker *et al.* 1993; Tanaka *et al.* 1993).

Although cytokines clearly play a key role in the morbidity and mortality of acute GVHD, the tissue damage observed in GVHD target organs is not adequately explained by the systemic release of cytokines alone; intravenous infusion of TNFα and IL-1 fail to produce the immune cell infiltration of the liver and skin seen in acute GVHD. As cytotoxic T cells have the ability to kill virtually all nucleated cells, cell-mediated cytotoxicity is thought to contribute to the destruction of these target organs. T cells exert their cytotoxic effects by either the release of soluble mediators such as TNF-α or by direct contact with the target cell via receptor-ligand interactions (Ferrara *et al.* 1999). Contact-dependent cytotoxicity can occur through perforin/granzyme B-mediated cytolysis or Fas/Fas Ligand (FasL)-mediated apoptosis. Both of these pathways result in cell death through the activation of the caspase cascade (Figure 1.5) (Lowin *et al.* 1994; Via *et al.* 1996).

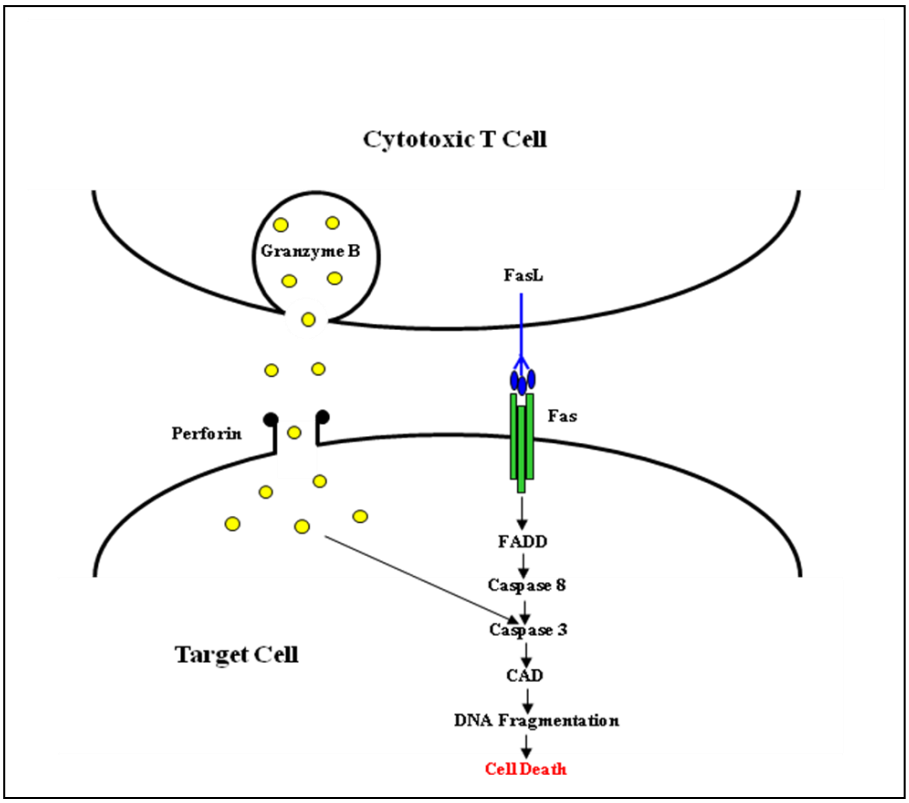

Figure 1.5 Perforin/granzyme B and Fas/FasL Pathways. Contact-dependent cytotoxicity through the perforin/granzyme B and Fas/FasL pathways results in cell death via the activation of the caspase cascade (Modified from (Ferrara *et al.* 1999). FADD, Fas-associated protein with death domain; CAD, caspase-activated DNase

Although perforin/granzyme B- and Fas/FasL-mediated pathways are important in effective cell-mediated cytotoxicity, they are not the sole effector mechanisms involved in GVHD. Significantly higher morbidity and mortality is observable in transplanted FasL-deficient B6.*lpr* mice compared with control B6 mice (van den Brink *et al.* 2000). Transplantation of perforin-deficient T cells results in a marked delay in the onset of GVHD, however all signs of the disease are still induced (Levy *et al.* 1995; Baker *et al.* 1996). Also, T cells from perforin/granzyme and FasL cytotoxic double deficient (*cdd*) mice are capable of inducing significant GVHD after transplantation into MHC-mismatched recipients conditioned with lethal TBI (Jiang *et al.* 1998; Martin *et al.* 1998) and GVHD target damage is observable in mice that lack allo-antigen expression on the epithelium (Maeda *et al.* 2005). Consequently, the actions of other effector molecules, such as the death receptor (DR) ligands TRAIL (TNF-related apoptosis-

inducing ligand) and TWEAK (TNF-like weak inducers of apoptosis) are currently being explored.

While cell-mediated tissue damage results mainly from the cytolytic actions of cytotoxic T cell, other effector cells, such as NK and TH17 cells may also contribute to GVHD damage (Klingemann 2000; Carlson *et al.* 2009).

1.3.2 *Chronic GVHD*

Chronic GVHD is the most common late complication of HSCT; occurring in approximately 60-80% of patients and apart from disease relapse is the major cause of late death post-transplant (Socie *et al.* 1999). This form of GVHD is still primarily a T cell-mediated disease, but is a much more pleiotropic syndrome (Shulman *et al.* 1980; Ratanatharathorn *et al.* 2001; Horwitz and Sullivan 2006).

Clinical Manifestations and Grading

Chronic GVHD can present itself in three ways; progressing as a continuation of pre-existing acute GVHD, developing as a separate entity after complete resolution of acute GVHD or *de novo* without any previous indication of acute GVHD (Sullivan *et al.* 1981; Flowers *et al.* 1999). It is a multi-system disease, but mainly affects the skin and mucous membranes. Clinically, chronic GVHD can often resemble non-specific autoimmune diseases such as Sjogrens syndrome or systemic sclerosis. Early stages of the condition present as a lichen planus-like rash, with later stages closely resembling scleroderma with skin atrophy and ulceration (Aractingi and Chosidow 1998). Dryness of the eyes and oral ulcers are also common. Chronic GVHD of the liver typically presents with cholestatis, whilst the involvement of the gastrointestinal tract results in dyspepsia and weight loss (Horwitz and Sullivan 2006). In addition to these specific organ system effects, chronic GVHD has profound effects on the immune system. Involution of thymic epithelium has been observed along with loss of secondary germinal centres in lymph nodes (Ghayur *et al.* 1990). Functionally, cellular immunity is affected by the disease with reduced B cell proliferation, reduced antibody responses to antigenic stimuli and defects in CD4+ cell numbers and functions (Ferrara and Deeg 1991b; Ratanatharathorn *et al.* 2001). Consequently, patients with chronic GVHD

experience long periods of immunosuppression and are therefore highly susceptible to opportunistic infection.

The staging of chronic GVHD has traditionally been based on observations made in a Seattle study involving 20 patients (Table 1.4) (Shulman *et al.* 1980). The presence of chronic GVHD is recorded as limited or extensive depending on clinical observations. Although this system is crude, it is helpful in delineating those patients that require treatment (extensive chronic GVHD) from those who can be managed expectantly (limited chronic GVHD) (Vogelsang 2001). To aid grading the Karnovsky score can be added into the assessment (Atkinson *et al.* 1989). This grading system does however pose problems in terms of capturing the depth and degree of affected organs. An updated grading system has been introduced that arranges patients into categories based on their clinical characteristics at the time of diagnosis. The additional factors for chronic GVHD grading in this system include: extensive skin involvement (>50% of body surface), thrombocytopenia (<100,000 platelets/mcl), progressive onset of chronic GVHD and Karnovsky performance status of less than 50% at primary treatment failure (Akpek *et al.* 2001; Akpek *et al.* 2003). Another potential advancement in chronic GVHD grading has been proposed by Filipovich *et al.* (2005). This grading system is based on the number of organs involved and the severity within each organ system (mild/moderate/severe). Despite the recommendations, current clinical practice however continues to support the use of the limited versus extensive grading system.

Limited Chronic GVHD	Extensive Chronic GVHD
Either or both Localised skin involvement Hepatic dysfunction	Generalised skin involvement *and/or* hepatic dysfunction *and one more of* i) chronic aggressive hepatitis ii) eye involvement iii) oral mucosa involvement iv) another target organ involvement

Table 1.4 Chronic GVHD grading system (Shulman *et al.* 1980). GVHD, graft-versus-host disease

Histopathological Features

Many of the histological changes of cutaneous chronic GVHD resemble those of acute GVHD. These include lymphocytic infiltration in the superficial dermis, vacuolisation, epidermal cell necrosis and apoptosis of basal keratinocytes. The diagnosis of chronic GVHD is often on the basis of hypergranulosis and hyperkeratosis (Horn *et al.* 1997). In the later stages of the disease sclerodermatous type changes become apparent with epidermal atrophy and progressive destruction of skin appendages (Aractingi and Chosidow 1998).

Pathophysiology

The mechanisms involved in chronic GVHD are still largely unclear. Despite advances in the understanding of acute GVHD, the same advances have not been made with the chronic form of the disease. The possible explanation for this is the lack of *in vitro* and animal models that resemble the condition. Although, allo-reactivity forms the basis for the pathogenesis of chronic GVHD, the exact phenotype and origination of the allo-reactive cells is still unclear. It is also unclear whether these cells are patient specific or simply react as a result of common epitopes shared by the graft and the patient (Claman *et al.* 1985; Horwitz and Sullivan 2006). The latter fits with the clinical symptoms of systemic sclerosis and would support the theory of chronic GVHD being an autoimmune phenomenon. The presence of allo-reactive T cells in lesions of chronic GVHD, the clinical similarities with autoimmune disorders and the increased number of auto-antibodies observed in patients have led to the investigation of possible altered thymic selection in the disease process (Quaranta *et al.* 1999). In both experimental and clinical studies of chronic GVHD, thymic atrophy, lymphocyte depletion and loss of thymic epithelial secretory function has been demonstrated. Thus, abnormal thymopoiesis resulting in the retention of auto-reactive clones may occur (Atkinson *et al.* 1982; Beschorner *et al.* 1982; Tutschka *et al.* 1982; Weinberg *et al.* 2001).

1.3.3 *Clinical Risk Factors of GVHD*

The first steps in the prevention of GVHD, where possible are to minimise risk factors associated with the development of the disease. There are several clinical parameters that affect the rate of GVHD.

Acute GVHD

It is well documented that the degree of HLA-mismatch between a donor and patient increases the rate of acute GVHD. The incidence of acute GVHD with only one haplotype mismatch increases as much as three-fold compared to fully HLA-matched transplants (Beatty *et al.* 1985). In unrelated donor HSCT, HLA disparity is of particular importance, as complete matching between patients and their unrelated donors is extremely low. Since the use of DNA-based typing, it is now known that only a single mismatch at HLA-A, -B, -C, -DRB1 or –DPB1 is required to substantially increase the risk of both GVHD and post-transplant mortality in this setting (Sasazuki T 1998; Flomberg *et al.* 2004; Chalandon *et al.* 2006; Lee *et al.* 2007; Shaw *et al.* 2007). Despite HLA-matching, acute GVHD can still occur as a result of mHag differences between patients and donors (Simpson *et al.* 2002). The incidence of acute GVHD has been shown to be significantly increased with greater than one mHag mismatch (Goulmy *et al* 1996). mHags located on the Y chromosome are male specific and have been shown to be targets for T cell-mediated killing of male cells and involved in GVHD (Vogt *et al* 2002). It therefore follows that sex mismatch between the patient and the donor is a risk factor for acute GVHD, with female donor-derived T cells recognising Y chromosome associated mHags of a male patient (Weisdorf *et al.* 1991; Gratwohl *et al.* 2001). Other mHags that are important in HSCT are HA-1, HA-2, HA-3, HA-4 and HA-5. Mismatches at these antigens have been shown to be responsible for acute GVHD following HLA-matched transplantation (Goulmy *et al.* 1996; Nesci *et al.* 2003).

Older age of an allogeneic HSCT patient has frequently been associated with an increased risk of GVHD (Weisdorf *et al.* 1991; Gratwohl *et al.* 1998; Cavet *et al.* 2001). Several possible reasons have been suggested including an increased susceptibility to infection, slower repair of damaged tissue and the reduced ability of the thymus to educate incoming donor T cells (Gluckman *et al* 1997). Older age of a donor,

particularly in the unrelated donor HSCT setting may also increase the risk of acute GVHD, however, currently data is conflicting (Goker *et al.* 2001; Hahn *et al.* 2008).

Other well documented clinical parameters associated with an increased rate of acute GVHD are myeloablative (high intensity) conditioning regimens, cytomegalovirus (CMV) positivity and low intensity GVHD prophylaxis strategies (Gratwohl *et al.* 1998; Hahn *et al.* 2008; Ljungman 2008).

Chronic GVHD

The most important risk factor for the development of chronic GVHD is the presence of prior acute GVHD (Perez-Simon *et al.* 2006). However, other factors, such as HLA disparity and unrelated donor HSCTs are also associated with a higher incidence of this condition (Ratanatharathorn *et al.* 2001). The incidence of chronic GVHD has been demonstrated to be as high as 80% in one antigen HLA-mismatched unrelated donor transplants (Horwitz and Sullivan 2006). As with acute GVHD, older patient age is associated, in fact recent studies have highlighted patient age as an independent risk factor for chronic GVHD (Carlens *et al.* 1998; Zecca *et al.* 2002). Among patients of HLA-identical HSCT the probability of developing chronic GVHD was significantly higher in adults over the age of 20 (Sullivan *et al.* 1991). The use of PBSCs in transplantation is now on the whole regarded to increase the incidence of chronic GVHD (Storek *et al.* 1997; Scott *et al.* 1998; Solano *et al.* 1998; Vigorito *et al.* 1998; Champlin *et al.* 2000; Snowden *et al.* 2000; Morton *et al.* 2001). This is be due to PBSC preparations having a significantly higher T and NK cell content than stem cells grafts taken directly from the bone marrow (Bensinger and Storb 2001). Conflict does however still exist, as a few studies have shown PBSC transplants to restore blood counts faster than bone marrow, without increasing the risk of GVHD (Powles *et al.* 2000; Bensinger *et al.* 2001).

1.3.4 *Prevention and Treatment of GVHD*

The primary approach in the treatment of acute GVHD is in prevention via the administration of prophylactic drugs. GVHD prophylaxis primarily involves immunosupression using cyclosporine A (CsA) alone or in combination with methotrexate (MTX) or mycophenolate mofetil (MM).

CsA has been used for acute GVHD prophylaxis since 1976 (Borel *et al.* 1976) and works by inhibiting T cell activation through the blockage of cytokine gene transcription (Herold *et al.* 1986). CsA binds with high affinity to cyclophilin, a ubiquitous cytosolic protein in T cells that is responsible for protein folding (Schmid 1995). The CsA-cyclophilin complex can associate with another cytosolic protein, calcineurin. In normal circumstances the engagement of the T cell receptor causes calcineurin to dephosphorylate transcription factors which are involved in the transcriptional activation of genes encoding cytokines including IL-2 (Timmerman *et al.* 1996). The direct association of the CsA-cyclophilin complex with calcineurin prevents the dephosphorylation of transcription factors and the subsequent expression and secretion of IL-2 in T cells. Inhibition of IL-2 production inhibits the clonal expansion of allo-reactive T cells.

MTX is a folate antagonist first developed for the treatment of malignancies in 1956 (Farber *et al.* 1956) and was subsequently the first GVHD prophylactic drug to be used as immunosuppression, after its efficacy was demonstrated in a canine model (Thomas *et al.* 1975). MTX exhibits both anti-inflammatory and immunosuppressive properties. MTX is converted to polyglutamate forms intracellularly by directly binding two to five polyglutamate groups (Fry *et al.* 1982). The polyglutamate derivatives of MTX inhibit dihydrofolate reductase (DHFR), which results in a depletion of thymidylate synthase; an enzyme that is essential in the production of pyrimidine nucleotides for *de novo* DNA synthesis (Chu *et al* 1990). MTX therefore interferes with DNA replication in dividing cells, including activated donor T cells. This leads to premature apoptosis and a reduced allo-response.

Several studies have been carried out comparing CsA and MTX as monotherapy for GVHD prophylaxis (Backman *et al.* 1988; Storb *et al.* 1988), with both drugs found to be equivalent in the likelihood of acute GVHD, chronic GVHD and overall survival.

Combination therapy with CsA and MTX has been compared to CsA monotherapy and was found to associate with reduced rates of acute GVHD and improved overall survival (Mrsic *et al.* 1990).

MM is the pro-drug of the potent immune-suppressant mycophenolic acid, a non-competitive reversible inhibitor of inosine monophosphate dehydrogenase; an enzyme that blocks *de novo* synthesis of guanosine nucleotides (Lipsky 1996). Lymphocytes depend on this pathway because they do not possess the salvage pathway of other cells. The toxicity profile of MM, particularly its lack of renal toxicity and cross-reactivity with CsA makes it an attractive prophylaxis candidate, however, a recent study has demonstrated that the addition of MM to CsA does not offer any benefit over CsA and MTX (Nash *et al.* 2005). Thus, CsA and MTX are typically the drugs of choice for combination therapy.

Immunosuppressive prophylaxis for GVHD effectively reduces the severity of the disorder in most individuals, but in doing so increases infection and decreases the beneficial graft-versus-leukaemia (GVL) effect, which helps to prevents relapse. Consequently, in many of the cases of GVHD associated death, the cause of death is infection or relapse rather than the GVHD itself (Goker *et al.* 2001).

Alternatively, acute GVHD can be prevented by T cell depletion. This can be achieved *in vitro* by physiological and immunological methods or *in vivo* with the use of antibodies such as Campath-1H (anti-CD52) and anti thymocyte globulin (ATG) (Hale *et al.* 1998; Bacigalupo 2007). These T cell antibodies not only allow the host immune response to be reduced; which favours engraftment, but as they are still in the circulation at the time of transplantation they also down-regulate donor T cells and thus reduce GVHD (Bacigalupo 2007). The removal of donor T cells is the most effective strategy for the prevention of GVHD; however, it is associated with significant complications including: an increased risk of relapse, an increase risk of engraftment failure and an increase risk of post-transplant infections (Hale *et al.* 1988). T cell depletion is therefore often only used for patients considered to be at a high risk of severe acute GVHD, such as older patients and those receiving transplants from an unrelated donor.

Despite the use of immunosuppressive prophylaxis and T cell depletion as preventative measures against acute GVHD, it is still highly likely that the condition will develop. Addition of corticosteroid therapy to the GVHD prophylaxis regimen is standard first-line treatment in such cases. Typically, methylprednisone is administered at a dose of 2mg/kg/day; however, a higher does (10mg/kg/day) can be used for patients who develop severe acute GVHD (grades III-IV) (Bacigalupo 2007; Morris and Hill 2007). Steroid treatment is usually given over a five day course, as this is normally sufficient to identify responsive and steroid-refractory (partial/non-responsive) patients. Methylprednisone therapy can be tapered in responders, whereas additional (second-line) therapeutic strategies are required for steroid-refractory patients (Bacigalupo 2007). ATG alone or in combination with methylprednisone is one second-line treatment option. Some encouraging results have come from phase II trials (Roy *et al.* 1992; Aschan, 1994); especially when ATG was administered early after GVHD diagnosis (MacMillan *et al*. 2002), however, evidence that this treatment improves survival is still lacking. Other second-line strategies include additional immunosuppression with MM and antibody therapy against IL-2 (denileukin difitox, inolinomab, basiliximab and daclizuman), TNF (infliximab) or the TNF receptor (etanercept) (Bacigalupo 2007). A more novel second-line procedure is extracorporeal photopheresis (ECP). This is an immunotherapeutic modality that involves the treatment of blood mononuclear cells with 8-methoxypsoralen activated by UV light. This chemical binds irreversibly to the DNA of T cells, damaging them and thus down regulating the allo-response in GVHD. Greinix *et al.* (2002) have reported a 82% response rate for patients with severe skin acute GVHD treated with ECP and steroids, 61% for gastrointestinal involvement and 61% for liver involvement. However, responses were not observed until 12 weeks after treatment.

Although there is no specific prophylaxis for chronic GVHD the incidence of previous acute GVHD is a risk factor for the subsequent onset of the chronic form of the disease. However, acute GVHD prophylaxis in several early studies has shown no effect on the rate of chronic GVHD (Storb *et al.* 1986; Storb *et al.* 1989). T cell depletion is associated with reduced rates of the condition, but as previously discussed the problems associated with this method prevents widespread use of this application (Hale *et al.* 1988). The main focus therefore is on the treatment of chronic GVHD rather than the prevention of the disease. The nature of chronic GVHD as a multi-system disorder and its association with immunosuppression means that the disease is difficult to manage.

The standard first-line therapy for chronic GVHD is a combination of CsA (10mg/kg/day) and prednisolone (1mg/kg/day). This approach has achieved a degree of success and is considered to have a survival advantage (Sullivan *et al.* 1988). More recently tacrolimus has been used interchangeably with CsA, although no direct comparison has been performed (Fraser and Baker 2007). The duration of therapy is determined by response, but typically is prolonged, continuing for close to 12 months even in those achieving complete resolution. As with acute GVHD, steroid and immunosuppressive therapies can be tapered in responders. So far, the addition of other immunosuppressive agents, such as thalidomide, to this standard first-line therapy has not led to any significant difference in chronic GVHD response rates or survival (Koc *et al.* 2000; Arora *et al.* 2001).

Approximately 15% of transplant patients receiving standard first-line therapy for chronic GVHD fail to achieve complete resolution and require further treatment (Koc *et al.* 2002). Currently however, there is no standard second-line therapy for steroid-refractory chronic GVHD, thus management of these patients is extremely problematic. MM is probably the most commonly used second-line treatment, although other therapeutic agents do exist. Several phase II trials of thalidomide treatment have been performed. The largest study (80 patients) reported overall response rates of 26%, but 36% of patients had to discontinue the drug because of side-effects (Parker *et* al. 1995). Other studies have however demonstrated more encouraging data, with both complete and partial responses being observed (Vogelsang *et al.* 1992; Kulkarni *et al.* 2003). Switching from CsA to tacrolimus in the setting of steroid-refractory disease has also been reported, but with varying success (Carnevale-Schiana *et al.* 2000; Kanamaru *et al.* 1995). Sirolimus has been used in combination with calcineurin inhibitors and corticosteroids. Relatively high overall response rates (complete and partial) have been documented following this treatment strategy, although only a very small number of patients were able to discontinue all immunosuppressive therapies and adverse effects were quite common (Couriel *et al.* 2005; Johnston *et al.* 2005). Other studied possible second-line treatments for chronic GVHD include hydroxychloroquine, pravastatin, ECP and monoclonal antibodies, such as rituximab (anti-CD20), daclizumab (IL-2 receptor inhibitor) and infliximab (TNFα inhibitor). Again, good overall response rates have been associated with these therapies, although the majority of responders achieved partial rather than complete resolution (Fraser and Baker 2007).

1.4 Infection

Infection is another major complication of HSCT and occurs mainly as a result of intensive pre-transplant conditioning. Myeloablative regimens cause prolonged neutropenia, immunodeficiency and damage to mucosal tissue such as the mouth, gastrointestinal tract and the skin (Bacigalupo *et al.* 2000; Gratwohl 2008). This creates the ideal environment for opportunistic bacterial, fungal and viral pathogens. Measures that can be taken against these infections include isolation of the patient in a clean room, gut decontamination or prophylaxis with antibiotic, antifungal or antiviral drugs. The most common source of viral infection following HSCT is infection with CMV, which can lead to potentially life threatening CMV disease and is caused primarily by viral reactivation in seropositive patients. Clinical trials of the drug glanciclovir and its oral derivative valganciclovir have proved effective in the pre-emptive treatment of CMV disease, however, CMV remains an issue for seropositive HSCT patients, especially after mismatched or unrelated transplants (Ljungman 2008).

1.5 Disease Relapse

Disease relapse can occur when small numbers of residual leukaemic cells evade destruction. These cells are able to grow unchecked in most patients because the immune system does not recognise them as foreign (Kolb 2008). The observation that disease relapse occurs more frequently after T cell depletion of the donor, lead to the realisation that some of the therapeutic effects of HSCT was due to an anti-tumour or 'GVL' effect. Barnes and Loutit (1956) first suggested GVL after experiments in mice showed that allogeneic HSCT recipients had a lower incidence of leukaemic death than syngeneic recipients. GVL occurs when T cells from an allogeneic donor recognise foreign mHags or tumour-specific antigens expressed by leukaemic cells within the patient and generate an immune response to eliminate them (Horowitz *et al.* 1990). Disease relapse following allogeneic HSCT can be reversed by infusing more of the donor's lymphocytes (donor-lymphocyte infusion, DLI), as the T cells within the infusion restore the GVL effect. Due to the presence of mature T cells within the infusion DLI is however associated with an increased incidence of GVHD (Kolb 2008).

1.6 Non-HLA Polymorphisms

In HSCT, complications including GVHD, infection, disease relapse and TRM can occur even when transplant patients and donors are HLA-matched. As a result, it is extremely likely that non-HLA genes also have an impact. Naturally occurring polymorphisms within such genes have been suggested to contribute to some of the genetic disparity that exists in HSCT and may therefore have the potential to influence transplant outcome.

Polymorphisms within the 5′ or 3′ regulatory sequences of genes, such as single-nucleotide polymorphisms (SNPs), microsatellites or variable number of tandem repeats (VNTRs), can alter transcription factor binding, and hence the level of protein produced. Such polymorphisms in the genes of cytokines can result in individuals producing higher or lower cytokine levels (Bidwell *et al.* 1998; Bidwell *et al.* 1999). Their impact therefore affects both the quality and quantity of, for example, the cytokine or cytokine receptor, and in the transplant setting, may influence the 'cytokine storm' associated with GVHD. Cytokine gene polymorphisms have been investigated in a number of diseases where there is an immune pathology, such as autoimmune diseases, cancer and transplantation. Immunosuppressed solid-organ transplant patients with a 'high-producer' TNFα (*TNFA*) genotype and 'low-producer' IL-10 (*IL10*) genotype have been shown to be more likely to reject their graft (Turner *et al.* 1997). These findings led to studies in the HSCT field, with *TNFA* and *IL10* gene polymorphisms being the first to be investigated for associations with transplant outcome. In recent years, the number of different polymorphisms studied has increased and expanded to encompass other immune molecules as well as cytokines. The vast amount of research performed to date has demonstrated a role for non-HLA genes in transplantation and highlighted their potential use as predictive markers of HSCT outcome.

The following sections will summarise non-HLA polymorphism results concerning cytokines and other immune molecules. However, the remainder of this thesis will focus mainly on the roles of cytokines and cytokine genes in HSCT.

1.6.1 *Cytokine and Cytokine Receptor Genes*

Tumour Necrosis Factor α

TNFα is a multifunctional pro-inflammatory cytokine produced mainly by monocytes and macrophages, which has the ability to up-regulate adhesion and MHC molecule expression and can directly induce cell death. It also possesses anti-tumour activity and plays a major role in the GVHD immune response (Lichtman *et al.* 1996). The gene encoding TNFα (*TNFA*) is highly polymorphic and located within the class III region of the MHC complex on chromosome 6, close to the HLA region and lymphotoxin and heat shock protein (*HSP)* genes.

TNFA polymorphisms have been widely studied with respect to HSCT outcome, particularly in the HLA-matched sibling setting. One of these polymorphisms is a SNP within the gene promoter region at position –308 (*TNFA* –308 G/A). This SNP has been linked with a diverse number of diseases and inconsistently associated with GVHD following HLA-matched sibling transplantation. Several small studies have reported a positive association with acute GVHD (Takahashi *et al.* 2000; Wang *et al.* 2002)); however, the majority of the larger studies found no such association (Middleton *et al.* 1998; Socié *et al.* 2001; Nordlander *et al.* 2002; Rocha *et al.* 2002; Lin *et al.* 2003; Mullighan *et al.* 2004)). *TNFA* –308 G/A, along with the *TNFB* +1069 C/T polymorphism, has also been linked with toxic complications, with the highest incidence of such complications in *TNFA* and/or *TNFB* heterozygous patients (Bogunia-Kubik *et al.* 2003).

Another SNP within *TNFA* is located at position +488 (*TNFA* +488 G/A). The A allele of this SNP has been associated with GVHD in two independent HLA-matched sibling cohorts. Patients with this allele developed significantly higher grades of acute GVHD than *TNFA* +488 A-negative patients. The same allele has also been associated with chronic GVHD and early death (Mullighan *et al.* 2004).

A number of microsatellite polymorphisms occur within *TNFA*: *TNFa, TNFb, TNFc*, *TNFd* and *TNFe* (Nedospasov *et al.* 1991; Udalova *et al.* 1993; Holzinger *et al.* 1995). The *TNFd3* and *TNFa2* alleles have been associated with higher TNFα production, whereas the *TNFa6* allele correlates with decreased production (Pociot *et al.* 1991;

Turner *et al.* 1995; Turner *et al.* 1997). In terms of HSCT, the *TNFd3* homozygous genotype is associated with an increased incidence of acute GVHD and early death following HLA-matched sibling transplantation (Middleton *et al.* 1998; Cavet *et al.* 1999), whereas the *TNFd4* allele correlates with the development of moderate to severe acute GVHD in this setting (Nordlander *et al.* 2002). Spink *et al* (2003) however, has recently reported the existence of sub-alleles of the *TNFd* polymorphism (contain slight sequence differences) and demonstrated that a high proportion of *TNFd* alleles have been incorrectly assigned as *d3* rather than one of these sub-alleles. This finding has major implications for existing disease associations with *TNFd3*, but as of yet these have not been investigated further.

An important factor potentially influencing any association between *TNFA* polymorphisms and GVHD is the location of the gene within the MHC class III region. *TNFA* polymorphisms are in linkage disequilibrium with HLA class I and class II genotypes. As certain HLA genotypes are known to influence HSCT outcome, association with *TNFA* genotype may be secondary to or interact with the HLA associations (Mullighan and Bardy 2004; Shaw *et al.* 2004). *TNFA* –863 C/A and –857 C/T polymorphisms in donors and/or patients have been correlated with a higher incidence of GVHD and a lower rate of relapse. By restricting the analysis to HLA A-, HLA B- and DRB1-matched pairs, the effect of HLA linkage disequilibrium with *TNFA* was revealed. GVHD outcome was still linked with patient genotype; however, the association with relapse was no longer observed (Ishikawa *et al.* 2002a).

TNFA polymorphisms have also been examined in the unrelated donor HSCT setting. The *TNFd* microsatellite polymorphism has been associated with several transplant outcomes, including GVHD, TRM and survival (Keen *et al.* 2004; Bettens *et al.* 2006). TNFα serum levels and *TNFd* genotypes have been correlated with acute GVHD, with patients homozygous for the *TNFd*3 or *TNFd*4 alleles having significantly higher TNFα levels during conditioning and more frequently developing acute GVHD (Remberger *et al.* 2003). More recently, *TNFd* has been associated with TRM rather than GVHD (Keen *et al.* 2004). The presence of the *d*4 allele in conjunction with the *TNFA* 1031C allele in either patients or donors significantly increased the incidence of TRM. A further allele associated with the *TNFd*4/1031C haplotype, *TNFa*5, is also linked to increased TRM. *TNFd* has also been associated with HSCT survival. A proportional

decrease in survival rates has been reported for transplant patients possessing *TNF d*3/*d*3, *d*4 and *d*5 respectively compared to TNFd1/d2d3 genotypes (Bettens *et al.* 2006).

The actions of TNFα are mediated by two receptors; a type I (TNFRI) receptor and a type II receptor (TNFRII). The second of these receptors is found on most cell types, but is more abundant on endothelial and haematopoietic cells and on TNFα binding induces apoptosis amongst CD8+ cells and stimulates haematopoietic cell (particularly T cell) proliferation (Zheng *et al.* 1995; Stark *et al.* 2003). The TNFRII (*TNFRSF1B)* gene is located on chromosome 1 at position 1p36. A SNP occurs within exon 6 of this gene at codon 196 that results in an amino acid substitution, methionine (M)→arginine (R). Consequently, two alleles for this polymorphism exist; M and R. Research performed to date has shown that the R allele is linked to the development of systemic lupus erythematosus (SLE) and in both the HLA-matched sibling and unrelated donor HSCT settings is associated with an increased incidence of acute and chronic GVHD (Morita *et al.* 2001; Ishikawa *et al.* 2002b; Stark *et al.* 2003).

Interleukin-10

IL-10 is a potent inhibitor of T-cell proliferative responses and suppresses pro-inflammatory cytokine production. The gene of this cytokine maps to chromosome 1 (1q31-32) and is highly polymorphic, containing numerous microsatellites and five SNPs, that have been resolved into conserved haplotypes representing high-level (GCC), intermediate-level (ATA) and low-level (ACC) producers of IL-10 (Edwards-Smith *et al.* 1999).

Several studies have examined the role of *IL10* polymorphisms in HSCT; however, at present, the data is conflicting. Initial studies demonstrated that the ACC haplotype and the longer *IL10* –1064 $(CA)_n$ microsatellite (>12) alleles associated with the development of severe acute GVHD (grade III–IV) in HLA-matched sibling transplant patients treated with CsA alone or CsA plus MTX (Middleton *et al.* 1998; Cavet *et al.* 1999). While in a larger HLA-matched sibling transplant cohort the ATA/ATA diplotype in the patient has been shown to decrease the risk of acute GVHD (Lin *et al.* 2003). A further study has demonstrated that the presence of *IL10* diplotypes (ACC/ACC vs ATA/ATC vs ATA/ATA) increase the incidence of chronic GVHD and individuals possessing the ATA haplotype required longer immunosuppression and

were more susceptible to invasive pulmonary aspergillosis (Kim *et al.* 2005; Seo *et al.* 2005). Several other studies have also reported differing associations between *IL10* polymorphisms and the risk of GVHD (Socié *et al.* 2001; Rocha *et al.* 2002; Mullighan and Bardy 2004).

In unrelated donor HSCT, an association between allele length at the *IL10* -1064 $(CA)_n$ microsatellite and survival has been reported: longer patient *IL10* alleles (>12) correlated with decreased survival (Bettens *et al.* 2006). In addition, in a mixed HLA-matched sibling and unrelated donor transplant cohort, the donors of chronic GVHD patients frequently possessed a greater number of CA repeats (allele 13 or above) (Takahashi *et al.* 2000). There also appears to be a significant link between TRM and the *IL10* GCC haplotype in this transplant setting. Presence of the donor R2-GCC haplotype has been shown to increase the incidence of TRM, while possession of the R3-GCC haplotype (donor-derived) decreases the risk (Keen *et al.* 2004). This finding conflicts with current HLA-matched sibling studies, as the majority of these have found associations with GVHD rather than TRM (Cavet *et al.* 1999; Ishikawa *et al.* 2002a; Lin *et al.* 2003).

Interferon γ

IFNγ is a pleiotropic cytokine with potent pro-inflammatory actions that is important in both the innate and adaptive immune response, and is essential for the induction and regulation of antimicrobial and anti-tumour immunity (Yang *et al.* 2005). Within the first intron of the *IFNG* gene on chromosome 12q14-15 there is a $(CA)_n$ microsatellite polymorphism. This polymorphism has two common alleles, 2 and 3, both of which exhibit differential IFNγ production *in vitro*. Allele 2 is associated with increased levels of IFNγ, whereas allele 3 has been linked with decreased production. *IFNG* microsatellite alleles have also been linked with a variety of immunological diseases and, in addition, in HLA-matched sibling transplantation an association between patient intron 1 $(CA)_n$ 3,3 homozygosity and an increased risk of acute GVHD has been reported (Cavet *et al.* 2001). Other studies have also shown an association between the possession of the intron 1 $(CA)_n$ 3,3 genotype or the lack of the intron 1 $(CA)_n$ 2,2 genotype and acute or chronic GVHD in this transplant setting (Mlynarczewska *et al.* 2004; Bogunia-Kubik *et al.* 2005). The mechanism behind these associations is

currently unknown, but may be explained if IFNγ has a negative feedback regulatory role, as seen in some murine models of GVHD (Brok *et al.* 1993). Possession of the IFNγ intron 1 $(CA)_n$ 3,3 genotype has also been associated with an increased risk of Epstein-Barr virus (EBV) reactivation following HLA-matched sibling or unrelated donor HSCT (Bogunia-Kubik *et al.* 2006).

Interleukin-1 Family

There are ten members in the IL-1 family, of these IL-1α and IL-1β (*IL1A* and *IL1B*) are agonists, and IL-1RA (*IL1RN*) is a specific receptor antagonist; which is relatively unique in cytokine biology. Binding of IL-1 to its receptor leads to induction of a wide range of genes including other cytokines, chemokines, nitric oxide synthase and type 2 cyclooxygenase, all of which are involved in the inflammatory response and stimulation of lymphocytes. The *IL1* family and its polymorphisms have therefore been associated with a wide range of inflammatory diseases (Cullup and Stark 2005).

The *IL1A* -889 C/T and *IL1RN* intron 2 VNTR polymorphisms have been extensively investigated in HLA-matched sibling transplantation. Possession of the *IL1RN* intron 2 VNTR allele 2 by transplant donors has been shown to down-regulate IL-1 production and consequently correlate with less-severe acute GVHD. In contrast, the same allele in the patient has been correlated with the development of acute GVHD (Cullup *et al.* 2001; Rocha *et al.* 2002). The presence of the *IL1RN* intron 2 VNTR allele 2 or the *IL1A* –889 C allele in the donor has also been correlated with chronic GVHD (Cullup *et al.* 2003). *IL1* polymorphism research in the unrelated donor setting is extremely limited; only one study performed on a small paediatric cohort has been reported to date. In this study, the presence of the *IL1A* –889 T allele in either the patient or donor was associated with significantly improved survival and decreased TRM. No association with acute GVHD was demonstrated; however, trends were observed between the incidence of chronic GVHD and the *IL1A* –889 C allele and allele 2 of the *IL1RN* intron 2 VNTR (MacMillan *et al.* 2003a).

Interleukin-2

IL-2 is a pro-inflammatory cytokine that plays a key role in immune response production; particularly in T cell activation, proliferation and differentiation, and has

been implicated in the initiation and maintenance of GVHD (Ferrara and Deeg 1991a). A T/G SNP exists within the *IL2* gene promoter region at position –330. The G allele of this polymorphism associates with early and sustained elevation of IL-2 production (high-producer genotype) and an increased risk for acute GVHD after unrelated donor HSCT (MacMillan *et al.* 2003b).

Interleukin-6

IL-6 is a pro-inflammatory cytokine that plays a central role in immune, inflammatory and acute phase responses. It is produced by numerous cell types, induces the development of cytotoxic T cells in the presence of IL-2 and synergises with IL-13 to promote haematopoietic stem cell differentiation and maturation. Consequently, IL-6 plays a major role in the biology of GVHD and HSCT (Lichtman *et al.* 1996).

The *IL6* gene is located on chromosome 7p21 and, like many other cytokine genes, has been found to be polymorphic, containing both microsatellites and SNPs (Terry *et al.* 2000; Ferrari *et al.* 2003). A G/C SNP exists in the promoter region at position –174. In both normal and HSCT populations, the G allele of this SNP correlates with higher serum IL-6 levels (Fishman *et al.* 1998). Several studies have also found the *IL6* -174 G,G genotype to be associated with both acute GVHD and chronic GVHD following HLA-matched sibling transplantation (Cavet *et al.* 2001; Socié *et al.* 2001; Mullighan and Bardy 2004; Karabon *et al.* 2005).

Interleukin-7 Receptor, Interleukin-18 and Interleukin-13

More recently studied cytokine gene polymorphisms include those within the *IL7* receptor and *IL18* genes. IL-7 is a 25kDa glycoprotein that plays an important role in the regulation of lymphopoiesis and signals through IL-7 receptors (IL-7R). These receptors consist of a γc and an α chain. Sequencing of the α chain has revealed the existence of four SNPs (+510 C/T, +1237 A/G, +2087 T/C and +3110 A/G), all of which result in amino-acid substitutions. In a recent study of 75 unrelated donor transplant pairs the *IL7R* +1237 A/G SNP within donors was shown to associate with survival and TRM, with the highest mortality being linked to the G,G genotype. No associations were observed in the HLA-matched sibling setting. Considering the

functions of IL-7, these findings seem to suggest a possible role for this SNP in T cell and immune reconstitution following unrelated donor HSCT (Shamim *et al.* 2006).

IL-18, a pro-inflammatory cytokine that induces Th1-type lymphocyte differentiation and cytotoxic T lymphocyte function has three polymorphisms at positions -137 G/C, -607 C/A and -656 G/T within its promoter region. Three main haplotypes exist, GCG, CAT and GAT. Cardoso *et al* (2004) have shown that the GCG haplotype in HSCT patients associated with a decreased risk of TRM at day 100, at 1 year post-transplant and with improved survival. *IL18* haplotypes had no effect on acute GVHD in this unrelated donor cohort (Cardoso *et al.* 2004). Associations between IL-18 and acute GVHD have, however, been demonstrated by well-established murine BMT models. Reddy *et al* (2003) have shown that neutralisation of IL-18 by antibodies increased acute GVHD-related mortality while administration of the cytokine significantly improved survival and reduced the pathological features of acute GVHD (Reddy *et al.* 2003)

Interleukin-13 (IL-13) is an immunoregulatory cytokine produced by B cells, mast cells, basophils, NK cells and DCs, but is predominantly secreted by activated Th2 CD4+ T cells (Brown *et al.* 1989; de Vries *et al.* 1996). It has many diverse functions, including the promotion of proliferation, immunoglobulin E (IgE) class switching and MHC class II expression in B cells (Punnonen *et al.* 1993; Chomarat and Banchereau 1998), the enhancement of monocyte and macrophage antigen presenting ability (Zurawski and de Vries 1994; de Vries 1998), the up-regulation of IgE receptor (CD23) expression on APCs (de Vries 1998) and the inhibition of pro-inflammatory cytokine production from monocytes and macrophages (de Waal Malefyt *et al.* 1993). In addition to these physiological roles, IL-13 has also been implicated in several pathological conditions. Most notably, in asthma; where it is capable of independently producing the entire asthmatic phenotype (Grunig *et al.* 1998; Wills-Karp *et al.* 1998; Shirakawa *et al.* 2000; Walter *et al.* 2001) and in allergy; where it up-regulates IgE production through the promotion of isotype class switching in B cells (Punnonen *et al.* 1993; Minton 2008). IL-13 has also been reported to play a role in several other pathological conditions, including inflammatory bowel disease (Kucharzik *et al.* 1996; Kadivar *et al.* 2004), parasitic worm infections (Urban *et al.* 1998; Chiaramonte *et al.* 1999), HIV infection (Montaner *et al.* 1993; Papasavvas *et al.* 2005) and chronic obstructive pulmonary disease (COPD) (Barnes 2000; van der Pouw Kraan *et al.* 2002; Hoshino *et al.* 2007).

In the transplant setting, IL-13 research is currently extremely limited. Elevated IL-13 levels have, however, been linked to chronic lung allograft rejection and the development of acute GVHD following HSCT (Jordan *et al.* 2004; Keane *et al.* 2007). In the latter study patients who developed severe acute GVHD (grades III-IV) had extremely high pre-transplant IL-13 production by their donor T cells; whereas those developing little or no acute GVHD had donors that produced no IL-13 prior to transplantation (Jordan *et al.* 2004). Several SNPs have been identified within *IL13,* although the +2044 G/A polymorphism in exon 4 is the most common and has been linked with inflammatory and allergy phenotypes (Heinzmann *et al.* 2000; Liu *et al.* 2000; Arima *et al.* 2002; Tsunemi *et al.* 2002; He *et al.* 2003; Heinzmann *et al.* 2003; Vladich *et al.* 2005; Zitnik *et al.* 2009). To date, however, no studies have been published associating this polymorphism with HSCT outcomes. The role of the *IL13* +2044 G/A polymorphism in both HLA-matched sibling and unrelated donor transplantation will therefore be examined in this current study.

1.6.2 *Innate Immunity Genes*

Other, non-cytokine genes associated with innate immunity have been assessed for their impact on HSCT outcome. There are a number of key molecules that allow mammals to sense pathogens, such as pattern recognition receptors (PRRs), which in turn activate the immune response via both the innate and adaptive pathways. These molecules obviously play a very important role in post-transplant complications, especially infectious episodes. Among these PRRs are a group of transmembrane receptors, Toll-like receptors (TLRs), which have a potent ability to activate APCs (Kaisho and Akira 2003; Kaisho and Akira 2004; Xu *et al.* 2004). APCs have ligands for different TLRs that are differentially expressed. TLR4 is a major receptor for LPS and has been implicated in the pathogenesis of GVHD. Polymorphisms in the *TLR4* gene have been associated with hyporesponsiveness to lipopolysaccharides. This leads to a reduced risk of acute GVHD, but consequently results in an increased risk of gram-negative bacteraemia. There is no hyporesponsiveness however in patients given prophylaxis for infection or GVHD (Lorenz *et al.* 2001). Polymorphisms in the *TLR1* and *TLR6* genes have been investigated in patients with invasive aspergillosis following HSCT, with patient genotype being associated with an increased risk of infection (Kesh *et al.* 2005). These results suggest that the impact of patient genotype is influenced by the period of

immunosuppression pre- and post-transplant before donor immune reconstitution occurs.

Intracellular PRRs, such as NOD-like receptors, which are associated with inflammatory bowel disease, are important molecules for study in HSCT because the gastrointestinal tract is damaged by conditioning regimens and is a target organ for GVHD. There are three SNPs in *NOD2/CARD15* (nucleotide-binding oligomerisation domains containing 2/caspase recruitment domain family number 15), SNP8, SNP12 and SNP13, which are involved in defective NF-κB responses. Possession of two or more of the *NOD2/CARD15* variants is associated with more severe GVHD and increased TRM in both HLA-matched siblings and unrelated donor transplants (Holler *et al.* 2004). In a second cohort of HLA-matched siblings (Holler *et al.* 2006), the genetic risk could be modulated through different types of gastrointestinal decontamination prior to transplantation. In T cell depleted transplants, possession of *NOD2/CARD15* SNPs is associated with lower disease free survival (Granell *et al.* 2007). These results illustrate that the altered immune response associated with *NOD* gene variants post-transplant is dependent on the type of immunomodulation (bacterial decontamination) and immunosuppression (T-cell depletion) administered.

The incidence of severe infections post transplant has been investigated by a number of groups. Severe bacterial infectious episodes are associated with the gene encoding myeloperoxidase (*MPO*), an enzyme released from neutrophils during inflammation. The A,G or A,A donor genotype of the *MPO* -463 G/A SNP rather than the wild-type G,G genotype have been associated the most severe infections. Mannose-binding lectin (MBL) binds a range of pathogens and opsonises them for phagocytosis. Human MBL is encoded by *MBL2* on chromosome 10. SNPs in the promoter region affect serum MBL levels, with low MBL levels giving an elevated risk of infection in immunocompromised individuals. In an Australian HLA-matched sibling HSCT cohort, *MBL2* mutations in the patient and/or donor genotype were associated with a risk of major infection following allogeneic transplant (Mullighan *et al.* 2002). However, this association was not observed in an independent study (Rocha *et al.* 2002).

FcγRIIa receptors provide a crucial link between cellular and humoral components of the immune response and are expressed in Langerhan cells, DCs, endothelial cells and

leukaemic cells. These receptors are genetically polymorphic. The *FcγRIIa*-R 131 genotype in the HSCT patients has been associated with an increased incidence of severe infection, and time to neutrophil recovery is delayed when the donor genotype is *FcRIIIb* HNA-1a/HNA-1b (Rocha *et al.* 2002). In a more recent study (van der Straaten *et al.* 2005), 73 acute monocytic leukaemia patients were analysed for *FcγRIIa* polymorphisms as candidates for acute GVHD. A mismatch between donor and patient *FcγRIIa*-R/R 131, *FcγRIIa*-H/H131 and *FcγRIIa*-R/H 131 genotypes were associated with an increased probability of GVHD. The presence of FcγRIIa allo-types on DCs, Langerhan cells and endothelial cells suggests that they may be candidate mHags due to recognition by donor T cells. This type of mismatching and potential relevance in GVHD of candidate mHags was also shown for the CD31 adhesion molecule (*PECAM1*) (Cavanagh *et al.* 2005), where the Val/Asn/Gly haplotype in the transplant donor significantly associated with the incidence of GVHD and donor heterozygosity at codon 125 or 563 associated with worse overall survival. Several other HLA-matched sibling studies have examined associations between *PECAM1* polymorphisms and HSCT outcome with inconsistent results (Behar *et al.* 1996; Nichols *et al.* 1996; Grumet *et al.* 2001; Cavanagh *et al.* 2005).

1.6.3 *Steroid Hormone Receptor Genes*

Steroid hormone receptors are implicated in the development of the immune system; consequently, these genes could have an impact on the reconstitution of the HSCT graft.

Vitamin D receptor (*VDR)* is the nuclear receptor of the steroid hormone vitamin D. Its gene has been mapped to chromosome 12q12-q14 and several polymorphisms have been reported to exist at this gene locus. The most common polymorphisms within the *VDR* gene are restriction fragment length polymorphisms (RFLPs). The *Apa1* A/C RFLP is found within intron 8 and has been associated with the incidence and severity of diseases of immune function, such as common variable immune deficiency. In terms of complications following HSCT, *Apa1* A has been found to correlate with both acute GVHD and lower overall survival following HLA-matched sibling transplantation (Middleton *et al.* 2002; Bogunia-Kubik *et al.* 2008).

Another steroid hormone receptor of interest in HSCT is oestrogen receptor. This receptor mediates the cellular response to oestrogens and related compounds and promotes a wide range of effects on haemopoiesis. The oestrogen receptor gene (*ESR1)* is located on chromosome 6q24 and within intron I two RFLPs, *PvuII* C/T and *XbaI* A/G exist. Both of these polymorphisms have been shown to associate with increased bone mineral density and the likelihood of developing Alzheimer's disease in Japanese individuals. In the context of HSCT, the C-T haplotype has been found to independently associate with both acute GVHD and lower overall survival following HLA-matched sibling transplantation (Middleton *et al.* 2003).

Chapter 2. Study Aims

The overall aim of this study was to investigate the genetic and functional impact of non-HLA polymorphisms on HSCT outcomes, including acute and chronic GVHD, overall survival, relapse incidence and TRM.

Polymorphisms in the genes of IL-2 (-330 T/G), IL-4 (-590 C/T), IL-6 (-174 G/C), IL-10 (-592 A/C, -1082 G/A), IL-1Ra (intron 2 VNTR), TNFα (intron 3 (GA)n), TNFRII (-196 M/R), IFNγ (intron 1 (CA)n), vitamin D receptor (intron 8 A/C, exon 9 T/C) and oestrogen receptor (intron 1 A/G, intron 1 C/T), that have been studied extensively in HLA-matched sibling transplantation were correlated with HSCT outcomes in an unrelated donor transplant population. The aim was to identify genetic markers that can be used pre-transplant to predict GVHD, overall survival, disease relapse and TRM in this setting.

The study also examined a polymorphism in the gene encoding IL-13, an immunoregulatory cytokine that has been implicated in the pathogenesis of GVHD. The *IL13* +2044 G/A polymorphism has been widely studied for disease associations; however its role in HSCT is currently unknown. To determine if *IL13* +2044 G/A is a predictive marker for HSCT outcomes, correlations between this polymorphism and GVHD, overall survival, disease relapse and TRM were investigated in both HLA-matched sibling and unrelated donor transplants. The *in vitro* human skin explant model of GVHD and peri-transplant serum samples were utilised to study IL-13 expression in acute and chronic GVHD and the functional significance of *IL13* +2044 G/A was examined using supernatant samples collected from phytohaemagglutinin (PHA) stimulated patient and donor peripheral blood mononuclear cells (PBMCs). In addition, correlations between pre-transplant IL-13 serum levels and GVHD, survival, disease relapse and TRM were investigated to determine the role they may have in the prediction of HSCT outcomes.

The specific aims for each part of this study are described in the relevant chapters.

Chapter 3. Materials and Methods

Only the company name of the supplier is included in the materials and methods section for reagents and materials utilised. For a detailed list of the supplying companies see Appendix A.

3.1 HSCT Patient and Control Populations

3.1.1 *HSCT Patients and Donors*

Investigations within this study, including non-HLA polymorphism analyses, acute GVHD model studies and the collection of clinical skin biopsies and serum samples from patients and donors (where applicable), were undertaken with informed consent as approved by the Joint Local Ethics Committee (JLEC) for the Newcastle upon Tyne Hospitals Trust (JLEC approval letters; Appendix B). The exact characteristics of each of the populations studied are documented in the relevant results chapters in which that particular cohort was employed.

3.1.2 *Volunteer Blood Donors*

Approval for the collection of peripheral blood samples from volunteer blood donors (control population for genetic studies) was granted under the remit of NHS Blood and Transplant (NHSBT), Holland Drive, Newcastle upon Tyne, UK and covered by ethics established by the NHSBT for an existing project. Individual blood donor consent however, was not necessary as the volume of blood collected from each individual was less than 10mls and the accrued data were anonymised.

3.1.3 *Laboratory Staff Volunteers*

Peripheral blood samples from laboratory staff volunteers were also utilised (control population for functional studies). All samples were taken with informed consent as approved by the JLEC.

3.1.4 *Definition of Outcome Measures*

The primary end points for the clinical aspects of this study were the development of acute or chronic GVHD. Acute GVHD was graded by consensus conference criteria (Przepiorka *et al.* 1995) and chronic GVHD was graded using the system proposed by Atkinson *et al.* (Atkinson *et al.* 1989). Although these grading systems, particularly that employed for chronic GVHD, were not the most up to date (section 1.3.2); they were the most relevant in terms of this study, as a large proportion of the clinical data utilised were obtained from transplants performed in the 1980s. Patients were considered assessable for acute GVHD if they survived ≥30 days post-transplant or died before day 30 with clinical acute GVHD (grades II-IV) and patients were deemed assessable for chronic GVHD if they survived ≥100 days. Overall survival was measured from the date of transplantation to the date of death or most recent clinic appointment. Relapse was classified as disease recurrence and TRM was defined as death from all causes other than relapse. Clinical data were available for all patients included in this study for at least 1 year following HSCT.

3.1.5 *HLA Typing*

The 776 patients/donors pairs recruited to our HSCT populations (HLA-matched sibling, n=347; unrelated donor, n=429) were transplanted at five different clinical centres across Europe (Austria, England, France, Germany and Spain). In order to ensure continuity of data, all transplant pairs underwent HLA typing prior to the performance of this study. As HLA-matching has significant implications in HSCT, particularly in unrelated donor transplantation, it was of great importance that as much HLA typing data as possible was obtained. Consequently, high resolution DNA-based typing (PCR with sequence specific oligonucleotide probes, PCR-SSOP) of the HLA-A, -B, -C, -DRB1 and –DQB1 genes was performed. HLA-DPB1 typing was not carried out, as at the time, the importance of this gene in HSCT had not yet been established (Shaw *et al.* 2007). HLA class I typing was performed by Dr G Fischer, Medical University of Vienna, Vienna, Austria and class II typing was performed by Dr M Uhrberg, University Clinic Düsseldorf, Düsseldorf, Germany. Of the original 776 patient/donor pairs, 55 unrelated donor transplant pairs were excluded from the study following typing, as incomplete HLA data was obtained. Of the remaining 721

patient/donor pairs, complete matching at all 5 loci (10/10 match) was observed in 78% (n=603).

3.2 Peripheral Blood Mononuclear Cell Preparation

Peripheral blood samples were collected from transplant patients and donors (40-60ml) prior to allogeneic HSCT and from volunteer blood donors and laboratory staff (10ml). The heparinized blood samples were diluted 1:1 with Earle's Balanced Salt Solution (EBSS, Gibco), layered onto lymphoprep (~5ml, Axis-Shield), and then separated by density gradient centrifugation (Jouan CR422, Fisher Scientific) at 800g (20^{o}C) for 15 minutes. The PBMCs at the plasma:lymphoprep interface were collected and washed twice in EBSS by centrifugation (MSE Centaur, VWR) at 380g for 5 minutes. The isolated cells were then re-suspended in cell culture medium for immediate use or in freezing solution for long term storage at -140^{o}C. Cell counting was performed using an Improved Neubauer haemocytometer (SLS) and viability was assessed using the trypan blue (Gibco) exclusion method.

3.3 Freezing Solution and Cryopreservation

Cells for cryopreservation were either pelleted and frozen at -80°C or stored as viable cells in freezing solution. The freezing solution employed consisted of 70% Rosewell Park Memorial Institute 1640 culture medium (RPMI 1640, Lonza), 20% heat inactivated foetal calf serum (FCS, Gibco) and 10% dimethyl sulfoxide (DMSO, NBS Biologicals). All components of the freezing solution were filter sterilised through a 0.2µm filter (Gelman Sciences). Viable cells were pelleted, re-suspended in cold freezing solution at a concentration of $1x10^{7}$cells/ml and transferred to 1.5ml cryovials (Nunc). Each vial was wrapped in bubble plastic and cryopreserved at -80^{o}C for 24 hours before being transferred to -140^{o}C for long term storage. When the cryopreserved cells were required for use they were thawed at 37^{o}C and rapidly diluted using warm cell culture medium.

3.4 Cell Culture Medium and Culture Conditions

Cell culture medium consisted of RPMI 1640 containing 100 IU/ml penicillin (Lonza), 100μg/ml streptomycin (Lonza), 2mM L-glutamine (Gibco) and either 20% heat-inactivated human autologous serum (RPMI-AS), 10% heat-inactivated human AB serum (RPMI-AB, PAA) or 10% FCS (RPMI-FCS). All cell cultures were incubated at 37^{o}C in a 95% air, 5% CO_2 humidified incubator (Galaxy B, SLS).

3.5 Genomic DNA Preparation

DNA was extracted from frozen PBMC pellets prepared using the methods described in sections 3.2 and 3.3. The cell pellets were thawed at room temperature (RT) and re-suspended thoroughly in 0.9ml Tris Sodium Ethylenediaminetetraactic acid (EDTA) buffer (TNE buffer). The re-suspended cells were lysed by the addition of 0.01ml 20% sodium dodecyl sulphate (SDS, Sigma) and nuclear proteins associated with DNA were digested by the addition of 0.01ml 10mg/ml proteinase K (Roche). After gentle inversion, complete lysis and digestion was ensured by incubation at 37˚C overnight.

After overnight incubation 0.9ml buffered phenol (Rathburn Chemicals) was added, the samples were mixed gently for 5 minutes to create an emulsion and then centrifuged at 500g for 1 minute (MSE Centaur 2, VWR). The phenol used in this extraction method acted as a solvent for nuclear proteins released by proteinase K and sank to the bottom of the tube. The supernatant was carefully removed to a clean 1.5 ml cryovial and 0.9ml chloroform:isoamyl alcohol was added. The samples were then mixed by vigorous inversions for 5 minutes. Following centrifugation at 500g for 1 minute, the supernatant was removed to a clean 15ml tube (Greiner). 0.5ml 7.5M ammonium acetate (BDH/Merck) was added to the supernatant. Samples were mixed by gentle inversion then incubated for 20 minutes at RT to allow the DNA to precipitate. 3ml ethanol (BDH/Merck) was added and the samples were mixed by inversion. Precipitated DNA at the acetate/ethanol interface was spooled onto a clean glass rod, aired dried for 5 minutes to allow alcohol evaporation and unwound into 0.5ml Tris EDTA (TE) buffer. The prepared DNA samples were left to re-suspend at RT for 48 hours.

Reagents

a) **1M Tris Stock Solution (pH 7.5):** 60.55g Tris (2-amino-2-(hydroxymethyl) 1,3-propanediol) (BDH/Merck) made up to 500ml with deionised water

b) **0.5M EDTA Stock Solution (pH 8.0):** 93.06g EDTA (BDH/Merck) made up to 500ml with deionised water. Titrate pH to 8.0 using sodium hydroxide pellets (BDH/Merck)

c) **TNE Buffer:** 4.39g sodium chloride (BDH/Merck), 5ml 1M Tris stock solution and 10ml 0.5M EDTA stock solution made up to 500ml with deionised water

d) **Chloroform:Isoamyl Alcohol:** 240ml chloroform (BDH/Merck) and 10ml isoamyl alcohol (Fisher Scientific)

e) **TE Buffer:** 10ml 1M Tris stock solution and 1ml 0.5M EDTA stock solution made up to 1L with deionised water

3.6 Genotyping Methods

Prior to the performance of any polymorphism genotyping, power calculations were carried out (performed by Dr H Bickeböller, University of Göttingen, Göttingen, Germany) to determine whether the transplant cohorts being employed in this study were large enough for all existing associations with HSCT outcome to be observed. Although there are no formal standards for power, a sample size with a calculated power >80% is regarded as adequate. Power calculations in this study were performed on all transplant cohorts undergoing genotyping analyses (1: unrelated donor cohort; 2: a mixed unrelated donor and HLA-matched sibling cohort) and used the minor allele frequency (MAF) of each of the non-HLA polymorphisms (*IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL13* +2044 G/A, *IL1RN* intron 2 VNTR, *TNFA* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T) being tested and the relative risk for each of the HSCT outcomes (GVHD, overall survival, disease relapse and TRM) being examined to determine the minimum sample size required. The calculated sample sizes ranged from 180-250 patients, with the largest populations being necessary for the *IL4* -590 C/T, *IL13* +2044 G/A and *TNFRSF1B* -196 M/R polymorphisms. The unrelated donor (n=374) and mixed HSCT (n=721) populations employed in this study were substantially larger than these minimum values,

consequently, both cohorts had calculated powers >80% and therefore reached adequacy.

Power calculations were also performed to determine whether any of the single-disease subgroups (acute lymphoblastic leukaemia, acute myeloid leukaemia, chronic lymphocytic leukaemia, chronic myeloid leukaemia, Hodgkin disease, non-Hodgkin lymphoma, myeloproliferative disorders, plasma cell neoplasm and aplastic anaemia) within the unrelated donor and mixed HSCT cohorts were large enough to permit non-HLA polymorphism analysis. Calculated minimum sample sizes ranged from 198-210 patients, with the smallest population being required for the *IL6* -174 G/C polymorphism. The largest single-disease subgroup (acute myeloid leukaemia) in the unrelated donor and mixed HSCT cohorts contained only 132 and 196 patients respectively; consequently, none of the subgroups reached the minimum values required for adequacy (calculated powers of <80%) and were therefore not analysed as separate populations.

3.6.1 *Polymerase Chain Reaction*

The polymerase chain reaction (PCR) is a widely employed cell-free method designed to generate large amounts of a desired DNA sequence and can be used on fragments of DNA that are initially present in extremely small quantities. The DNA region of interest is isolated by the use of primer sequences that are complementary to the sections of DNA flanking the target. A heat-stable form of the DNA polymerase enzyme, typically Taq polymerase, is then employed to synthesize complementary strands of the target DNA via enzymatic replication (Mullis *et al.* 1987).

There are three steps in the PCR reaction:

Denaturation: The DNA to be amplified is denatured by heating to 94˚C. This causes the double stranded DNA to dissociate into single strands. All enzymatic reactions are inhibited in this step in order to prevent the continuous extension of DNA strands already generated.

Annealing: During this step, primers hybridize to the complementary regions of DNA that flank the sequence being amplified. The temperature required for annealing is

dependent upon the primer length and sequence, but is typically 55-65˚C. Higher temperatures lead to increased PCR specificity.

Extension: Taq polymerase creates new DNA strands by extending the primers in the 5' to 3' direction using deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP) that are present in excess. This step of the reaction is carried out at 72˚C, as this is the ideal working temperature for the Taq polymerase enzyme.

Each set of three steps is referred to as a cycle. Repetition of this cycle (typically 25-35 times) results in an exponential increase in the number of copies of the desired DNA sequence (Strachan and Read 2000). The amplification of a particular DNA region in this way opens that area up for further investigations, such as DNA sequencing or polymorphism analysis as performed in this study.

DNA samples from our HSCT and control populations were analysed for polymorphisms in cytokine, cytokine receptor and steroid hormone receptor genes. PCR amplification was performed using the same basic method, however, primer sequences, reagent concentrations and thermal cycling conditions varied (Table 3.1). Briefly, PCR products were amplified from target DNA in a 10x potassium chloride (KCl) based buffer (Bioline), with dNTPs (Roche) at 200µM. Forward and reverse primers (MWG) at a final concentration of 1µM and 0.5 Units of Taq polymerase (Bioline) were used per 100µl of reaction mix. All reagents were combined in a master mix and 23µl were added to each genomic DNA sample (2µl (50ng)). Amplifications were performed using an ABI 2720 thermal cycler (AB). PCR products were then subjected to a polymorphism analysis method or separated directly using electrophoresis.

Table 3.1 Primer sequences, PCR conditions and analysis methods employed for polymorphism genotyping.

For each polymorphism, the location, nucleotide/amino acid change (where appropriate) and primer sequences are indicated. The PCR conditions and analysis methods employed are also recorded. PCR, polymerase chain reaction; VNTR, variable number tandem repeat; KCl, potassium chloride; dNTP, deoxynucleotide triphosphate; SSCP, single stranded conformational polymorphism; IHG, induced heteroduplex generator; RFLP, restriction fragment length polymorphism; $MgCl_2$, magnesium chloride

Gene and Polymorphism	Primers	PCR Reaction Mix	PCR Conditions (35-40 cycles)	Analysis Method	References
IL1RN Intron 2 VNTR	5'-CTCAGCAACACTCCTAT-3' 5'-TCCTGGTCTGCAGGTAA-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30seconds 60°C 1 minute 72°C 1 minute	2% agarose gel Ethidium Bromide	(Hurme and Sattila 1998)
IL2 -330 T/G rs2069762	5'-ACCACAATATGCTATTCACATGTTCA-3' 5'-TGATAGCTCTAATTCATGCAATTAACG-3'	10X KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 60°C 1 minute 72°C 1 minute	SSCP 15°C electrophoresis 12% acrylamide gel Siver Stain	(Hdud, 2005) Unpublished - Personal Communication
IL4 -590 C/T rs2243250	5'-ACTAGGCCTCACCTCACCTGATAGC-3' 5'-GTTGTAATGCAGTCCTCCTG-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 57°C 30 seconds 72°C 30 seconds	SSCP 10% acrylamide gel Silver Stain	(Walley and Cookson 1996)

IL6 -174 G/C rs1800795	5'-TGACGACCTAAGCTGCACTTTTC-3' 5'-GGGCTGATTGGAAACCTTATTAAGA-3'	1x TaqMan Universal Master Mix 1x Assay Mix Deionised Water	Initial Hold: 95°C 10 minutes Cycle: 92°C 15 seconds 60°C 1 minute	TaqMan	AB Assay by-Design P/N: 4331349
IL10 -592 A/C rs1800872	5'-GAAATCGGGGTAAAGGAGCC-3' 5'-AGTTCCCAAGCAGCCCTTCC-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 62°C 1 minute 72°C 1 minute	IHG 15% acrylamide gel Silver Stain	(Morse *et al.* 1999)
IL10 -1082 G/A rs1800896	5'-AATCCAAGACAACACTACTAAGGC-3' 5'-CTGGATAGGAGGTCCCTTAC-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 57°C 1 minute 72°C 1 minute	IHG 15% acrylamide gel Silver Stain	(Morse *et al.* 1999)
IL13 +2044 G/A rs20541	5'-CTTCCGTGAGGACTGAATGAGACGGTC-3' 5'-GCAAATAATGAGCTTTCAAGTTTCAGTGGA-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 56°C 1 minute 72°C 1 minute	PCR-RFLP *NlaIV* Digestion 11% acrylamide gel Silver Stain	(Graves *et al.* 2000) Modified
IFNG Intron 1 (CA)n Microsatellite	5'-GCTGTCATAATAATATTCAGAC-3' 5'-CGAGCTTTAAAAGATAGTTCC-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 60°C 1 minute 72°C 1 minute	8% acrylamide gel Silver Stain	(Pravica *et al.* 2000)

TNFA Intron 3 *LST1* (GA)n Microsatellite	5'-AGATCCTTCCCTGTGAGTTCTGCT-3' 5'-CATAGTGGGACTCTGTCTCCAAAG-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 60°C 1 minute 72°C 1 minute	8% acrylamide gel Silver Stain	(Udalova *et al.* 1993)
TNFRSF1B -196 M/R rs1061622	5'-ACTCTCCTATCCTGCCTGCT-3' 5'-TTCTGGAGTTGGCTGCGTGT-3'	10 x KCl Buffer dNTP (400µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 57°C 1 minute 72°C 1 minute	SSCP 12% acrylamide gel Silver Stain	(Komata *et al.* 1999; Stark *et al.* 2003)
VDR Intron 8 A/C Exon 9 T/C rs7975232 rs731236	5'-CAGAGCATGGACAGGGAGCAA-3' 5'-GGATGTACGTCTGCAGTGTG-3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 63°C 1 minute 72°C 1.5 minutes	PCR-RFLP *ApaI* Digestion *TaqI* Digestion 2% agarose gel Ethidium Bromide	(Hennig *et al.* 1999; Middleton *et al.* 2002)
ESRI Intron 1 A/G Intron 1 C/T rs9340799 rs2234693	5'CTGCCACCCTATCTGTATCTTTTCCTATTCTCC3' 5'TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA3'	10 x KCl Buffer dNTP (200µM) Forward Primer (1µM) Reverse Primer (1µM) $MgCl_2$ (1.5mM) Taq Polymerase (0.5U) Deionised Water	94°C 30 seconds 58°C 1 minute 72°C 1 minute	PCR-RFLP *XbaI* Digestion *PvuII* Digestion 1% agarose gel Ethidium Bromide	(Middleton *et al.* 2003)

3.6.2 *Restriction Fragment Length Polymorphism Analysis*

Polymorphisms that lie within the recognition sequence for a specific restriction enzyme can be investigated using restriction fragment length polymorphism (PCR-RFLP) analysis. Polymorphic allele identification by this method is dependent on the creation or destruction of a restriction enzyme cutting site. If such a site is present in one allele of a polymorphism and absent in the other, then digestion with the appropriate restriction enzyme will produce DNA fragments of differing lengths and electrophoretic mobilities; that can be distinguished by gel electrophoresis (Strachan and Read 2000). This type of analysis was employed to identify the allelic variants of polymorphisms within the *VDR* (Intron 8 A/C and Exon 9 T/C), *ESRI* (Intron 1 A/G and C/T) and *IL13* (+2044 G/A) genes. The *VDR* and *ESRI* polymorphisms were genotyped using established PCR-RFLP methods (Table 3.1), whilst the allelic variants of the *IL13* +2044 G/A polymorphism were identified using a method developed during the course of this study (section 3.6.3).

Digest mixtures were prepared by combining 5µl of PCR products with 2.5µl of deionised water, 1µl of 10x bovine serum albumin (BSA, Promega), 0.5µl of restriction enzyme and 1µl of the corresponding restriction enzyme buffer. After gentle mixing the digest samples were incubated at the appropriate temperature and then separated by gel electrophoresis (Figure 3.1). All restriction digests were performed with a 5-fold excess of enzyme to avoid partial digestion of the products and thus erroneous allele detection. The restriction enzyme employed and specific digestion conditions utilised for each PCR-RFLP are displayed in Table 3.2.

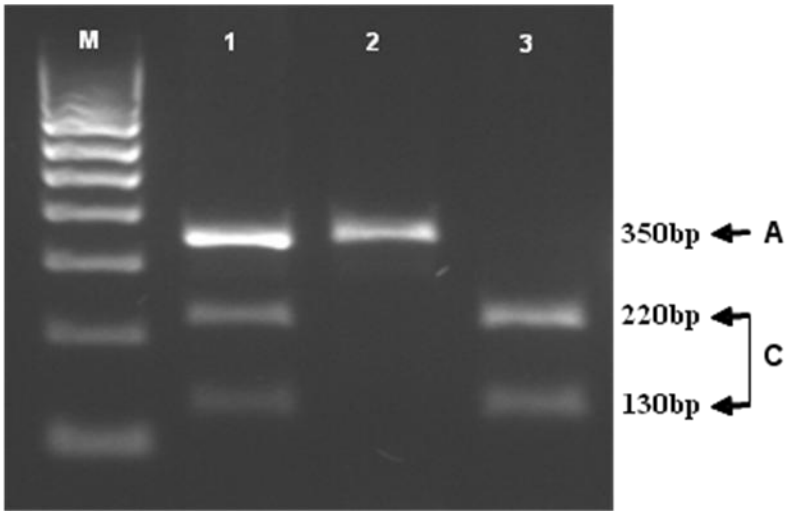

Figure 3.1 A representative 2% agarose gel showing polymorphism analysis of *VDR* Intron 8 A/C using the PCR-RFLP method. Alleles are identified according to the presence (C) or absence (A) of an *ApaI* restriction site. All three possible genotype combinations are shown: AA (lane 2), AC (lane 1) and CC (lane 3). M, DNA size markers (100bp Low Ladder, Sigma); bp, base pairs

Gene	Polymorphism	Restriction Enzyme	Conditions	Reference
VDR	Intron 8 A/C	*ApaI* (Promega)	2 hours at 25°C 2% agarose gel	(Hennig *et al.* 1999)
VDR	Exon 9 T/C	*TaqI* (Promega)	1 hour at 65°C 2% agarose gel	(Hennig *et al.* 1999)
ESRI	Intron 1 A/G	*XbaI* (Promega)	1.5 hours at 37°C 1% agarose gel	(Middleton *et al.* 2003)
ESRI	Intron 1 C/T	*PvuII* (Roche)	1.5 hours at 37°C 1% agarose gel	(Middleton *et al.* 2003)
IL13	Exon 4 G/A	*NlaIV* (NEB)	2 hours at 37°C 11% acrylamide gel	(Graves *et al.* 2000) Modified

Table 3.2 Restriction enzymes and digestion conditions employed for PCR-RFLP analysis. *VDR*, vitamin D receptor; *ESRI*, oestrogen receptor; *IL13*, interleukin-13

3.6.3 Method Development for *IL13* +2044 G/A Determination

Study Population: Newcastle Control Cohort

A total of 100 Newcastle volunteer blood donors were recruited to act as the control cohort for the genotyping aspects of this current study. The group comprised 53 males and 47 females, with a median age of 37 years (range 19-56). DNA samples were obtained using the methods described in sections 3.2, 3.3 and 3.5.

Controls were typed for the *IL13* +2044 G/A polymorphism using the method developed. In order to confirm that a reliable genotyping protocol had been established, allele and genotype frequencies were calculated and compared to those published in the literature.

PCR Amplification

PCR Conditions

The primer sequences used to amplify the 236bp fragment containing the *IL13* +2044 G/A polymorphism were as follows:

Forward Primer	5'-CTTCCGTGAGGACTGAATGAGACGGTC-3'
Reverse Primer	5'- GCAAATAATGAGCTTTCGAAGTTTCAGTGGA -3'

These primers were identical to those used in the previously published method by Graves *et al* (Graves *et al.* 2000). Each PCR employed a 25µl reaction mix containing 2µl of genomic DNA (50ng), 10x KCl buffer, dNTPs at 200µM, forward and reverse primers at 1µM and 0.5 Units of Taq polymerase. Samples were thermal cycled for 35 cycles of 94^{o}C for 30 seconds, 56^{o}C for 60 seconds and 72^{o}C for 60 seconds.

PCR Development

The specificity of the amplification was assessed by electrophoresis of the PCR products on a 1% agarose gel. The main problem encountered during method development was the presence of numerous high molecular weight products, suggesting

a lack of specificity in the PCR. A variety of reaction conditions were tested in an attempt to address this problem. The tested conditions were: variation in annealing temperature (55-60ºC), variation in primer concentration (0.5-1.5µM) and variation in dNTP concentration (100-500µM). The optimal reaction conditions found were those described overleaf.

Allele Discrimination

IL13 +2044 G/A allele discrimination was achieved using PCR-RFLP analysis (section 3.6.2). The restriction enzyme *NlaIV* digests the PCR product 26bp from the 5'end in all cases (internal control) and then a further 32bp from the 3' end for the more common G allele (Figure 3.2). The presence of this allele leads to the creation of the *NlaIV* recognition sequence; GGNNCC (where N indicates any base and the *IL13* +2044 G/A location is at G one). The restriction enzyme then digests the sequence in the middle, leaving a 178bp fragment (Graves *et al.* 2000; Chmiel *et al.* 2005).

Digested products were initially separated using 1% agarose gels. Successful digestion was confirmed, but adequate separation of the products was not achieved. In an attempt to improve the resolution, higher concentrations of agarose were utilised (2% and 3%). However, separation was still insufficient for accurate allele discrimination. Consequently, acrylamide gel electrophoresis was employed. As acrylamide has a smaller gel matrix in comparison to agarose, it was hoped that this method would permit greater separation of the digested products. In order to identify the optimal conditions for *IL13* +2044 G/A discrimination a variety of reaction conditions were examined. The tested conditions were: variation in the percentage of acrylamide (10-15%), variation in voltage (200-400V) and variation in electrophoresis temperature (15-30 ºC). The cleanest product separation was achieved using 11% acrylamide gels run overnight at a constant voltage of 200V and a constant temperature of 25 ºC.

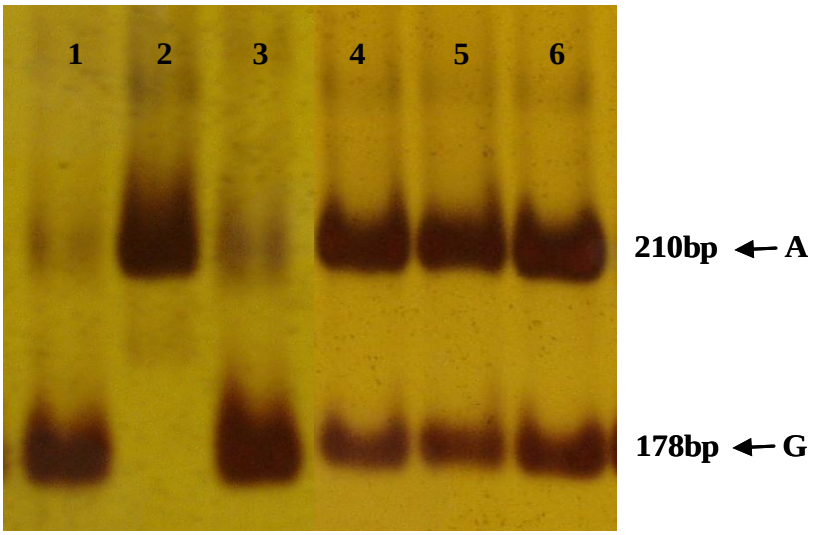

Figure 3.2 A representative 11% acrylamide gel showing polymorphism analysis of *IL13*+2044 G/A using the PCR-RFLP method. The two allelic variants (G or A) are identifiable and all three possible genotype combinations are shown: GG (lanes 1 and 3), GA (lanes 4, 5 and 6) and AA (lanes 2). bp, base pairs

Allele and Genotype Frequencies

The allele and genotype frequencies calculated for the Newcastle control cohort were very similar to those reported in the literature (P=0.860 and 0.910 respectively; Table 3.3) (Heinzmann *et al.* 2003; Maier *et al.* 2006). Thus, an accurate genotyping protocol for the *IL13* +2044 G/A polymorphism had been successfully established.

	Newcastle Control Cohort (n=100)	Published Control Cohort
Alleles		
G	0.81	0.79
A	0.19	0.21
Genotypes		
GG	0.66	0.68
GA	0.30	0.29
AA	0.04	0.03

Table 3.3 *IL13* +2044 G/A allele and genotype frequencies for the Newcastle control cohort.

In order to assess the reproducibility of the method, 25% of the control cohort was re-genotyped. No differences were observed between the two sets of data (P=1.000; Table 3.4).

	Newcastle Control Cohort (n=25)	
	Genotype 1	**Genotype 2**
***IL13* +2044 G/A**		
GG	15	15
GA	9	9
AA	1	1

Table 3.4 Reproducibility assessment of the *IL13* +2044 G/A genotyping method in 25% of the Newcastle control cohort. IL13, interleukin-13

The method developed for *IL13* +2044 G/A determination was therefore accurate and reproducible enough to be utilised in the remainder of this study for genotyping of our HSCT cohort.

3.6.4 *Single Stranded Conformational Polymorphism Analysis*

Single stranded conformational polymorphism (SSCP) analysis is a highly sensitive single tube assay employed for mutation detection (Orita *et al.* 1989; Nataraj *et al.* 1999). The method works on the principle that single-stranded DNA fragments adopt specific tertiary conformations that are dependent upon their nucleotide sequence. Thus, the presence of a polymorphism within DNA fragments results in the generation of products with different shapes and electrophoretic mobilities that can be distinguished by acrylamide electrophoresis. In this study SSCP analysis was employed to identify the allelic variants of polymorphisms within *IL2* (-330 T/G), *IL4* (-590 C/T) and *TNFRSF1B* (-196 M/R).

SSCP mixtures were prepared by combining PCR products and formamide load in a 1:1 ratio (5µl of each). Samples were denatured to single-stranded DNA at 98˚C for 5 minutes and then snap cooled on ice for 5 minutes to prevent re-annealing. After cooling, 4µl of products were loaded onto an acrylamide gel and separated by electrophoresis overnight at 400V (Figure 3.3).

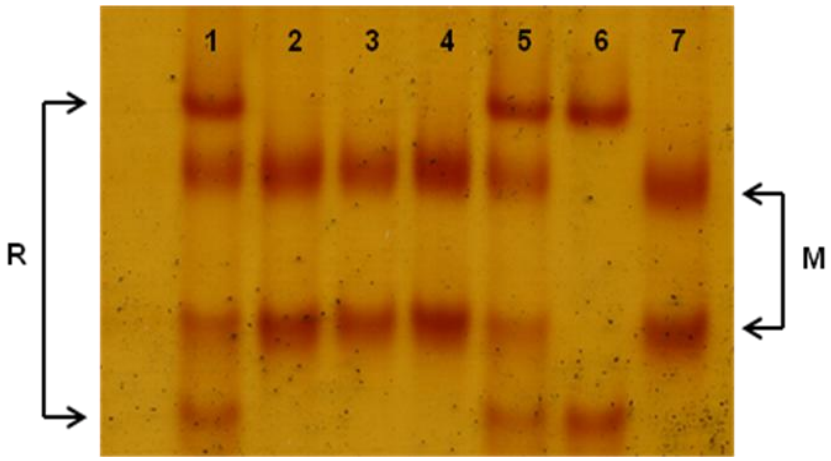

Figure 3.3 A representative 12% acrylamide gel showing polymorphism analysis of *TNFRSF1B* -196 M/R using the SSCP method. The two allelic variants (M or R) are identifiable and all three possible genotype combinations are shown: MM (lanes 2, 3, 4 and 7), MR (lanes 1 and 5) and RR (lanes 6)

3.6.5 *Induced Heteroduplex Generator Analysis*

Induced heteroduplex generator (IHG) analysis is a mutation detection method that uses synthetic DNA fragments (heteroduplex generators) to identify polymorphic alleles. Polymorphisms within PCR products are identified by the induction of conformational changes in the target DNA following inter-strand hybridization with heteroduplex generators. The conformational changes produced are specific for each of the allelic variants and due to differences in their electrophoretic mobility can be visualised following electrophoresis (Morse *et al.* 1999; Wood *et al.* 2001). IHG analysis was employed in this study to identify the allelic variants of two polymorphisms within the *IL10* promoter region (*IL10* -592 A/C and *IL10* -1082 G/A).

IHG mixtures were prepared by combining PCR products, heteroduplex generators (supplied by Dr L Keen, Department of Pathology and Microbiology, University of Bristol, Bristol, UK) and formamide load in a 1:1:1 ratio (5µl of each). Samples were then heated to 94°C for 4 minutes and ramp cooled to 37°C over 30 minutes (Morse *et al.* 1999). These conditions facilitated the production of allele specific heteroduplexes between the PCR products and generators; that were separated by acrylamide electrophoresis (Figure 3.4).

The heteroduplex generators employed contained poly-adenine (A) identifiers inserted immediately downstream of the *IL10* polymorphisms (Figure 3.5). These identifiers introduce a degree of mismatch between the PCR products and the generators, which varies depending on the nucleotide sequence of the products. The variation in the degree of mismatching between the strands alters the conformation and electrophoretic behaviour of the PCR products and consequently permits the identification of the *IL10* allelic variants.

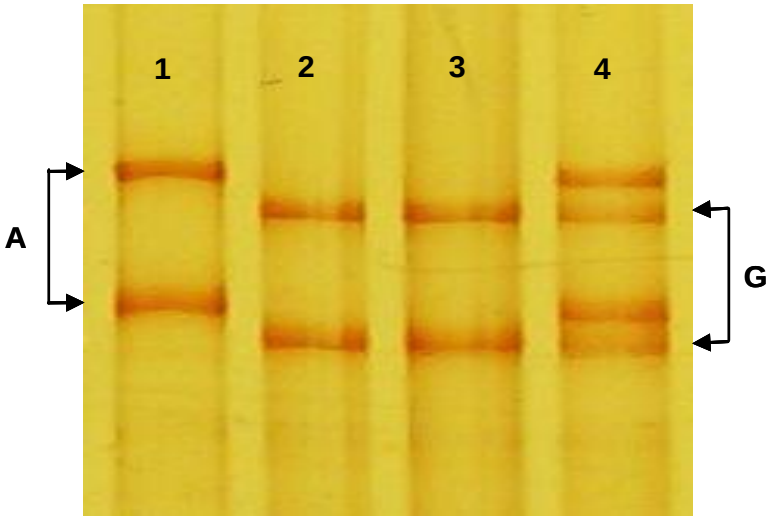

Figure 3.4 A representative 15% acrylamide gel showing polymorphism analysis of *IL10* -1082 G/A using the IHG method. The two allelic variants (G or A) are identifiable and all three possible genotype combinations are shown: GG (lanes 2 and 3), GA (lane 4) and AA (lane 1)

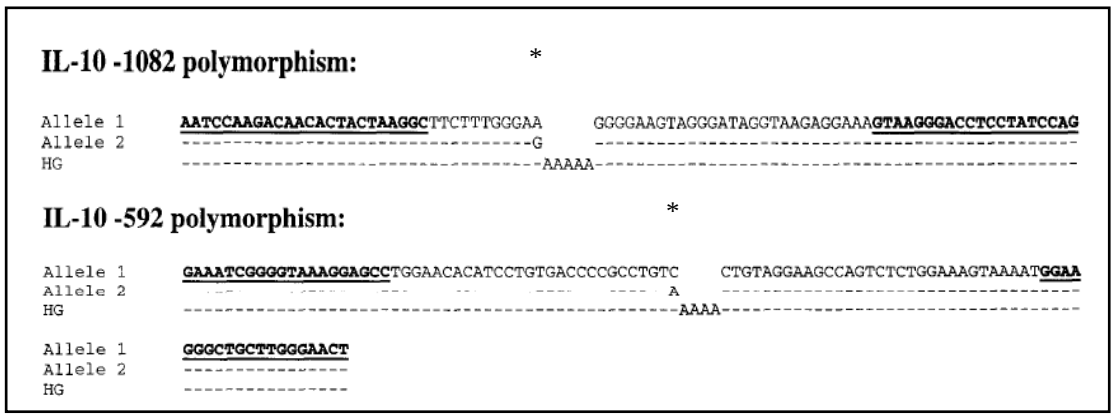

Figure 3.5 Nucleotide sequences of *IL10* promoter polymorphism alleles and heteroduplex generators. Polymorphism positions and heteroduplex generator identifiers (poly-adenine (A) inserts) are denoted using asterisks (Morse *et al*. 1999). IL-10, interleukin-10; HG, heteroduplex generators

3.6.6 *TaqMan Analysis*

TaqMan is a fluorescence-based PCR assay used for gene expression quantification and genetic polymorphism detection (Watson and Li 2005). Polymorphism identification using this method is achieved by including fluorescently labelled probes in the PCR. In order to differentiate between the allelic variants, each probe contains a different fluorescent dye (VIC or FAM).

Two probes are used in TaqMan analysis, each one specific for one of the polymorphic alleles. The probes consist of an oligonucleotide with a 5'-fluorescent reporter dye and a 3'-quencher dye. When a probe is intact, the proximity of the reporter dye to the quencher results in suppression of the fluorescent signal. During PCR, primers bind to specific sequences flanking the DNA to be amplified and the probe hybridises to a specific sequence within the amplified DNA. Taq polymerase extends the primers in the 5' to 3' direction and cleaves any bound probe with its 5'-3' nuclease activity. The reporter dye and quencher dye become separated during cleavage and a fluorescent signal, unique to each dye is produced (Figure 3.6). This process occurs in every PCR cycle, consequently, repetition of the cycle results in an exponential accumulation of PCR product and fluorescence (Watson and Li 2005).

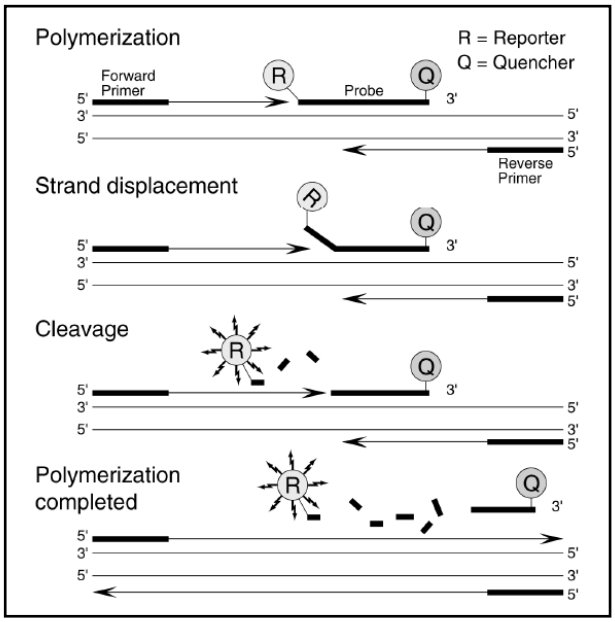

Figure 3.6 A schematic diagram of a single PCR cycle in TaqMan analysis. Taq polymerase extends the primers bound to the target DNA in the 5'→3' direction and cleaves any probe hybridised to the target. Cleavage separates the reporter dye from the quencher dye, which results in a fluorescent signal (Lyamichev *et al.* 1993).

TaqMan analysis of the *IL6* -174 G/C polymorphism was carried out using a custom TaqMan genotyping assay supplied by AB (P/N: 4331349). Primer and probe sequences employed are shown in Tables 3.1 and 3.5 respectively. PCR products were amplified from target DNA using 1x TaqMan universal PCR master mix (buffer, dNTPs, $MgCl_2$ and Taq polymerase) and 1x assay mix (TaqMan primers and VIC/FAM labelled probes). The reagents were combined in a master mix and 13µl were added to each genomic DNA sample (12µl). All DNA samples were diluted to 10ng in deionised water. PCR amplification was then performed using the ABI 7500 Real-time PCR (RT-PCR) system (AB). After an initial hold at 95^{o}C for 10 minutes, the samples were cycled for 40 cycles of 92ºC for 15 seconds and 60ºC for 1 minute. Following thermal cycling, fluorescence measurements were recorded and genotyping data was obtained after analysis with Sequence Detection System (SDS) software (AB, Figure 3.7).

	Probe Sequence	**Reporter Dye**
IL6 -174 G/C	5'-TCTTGCGATGCTAA-3'	VIC
	5'-TCTTGCCATGCTAAA-3'	FAM

Table 3.5 Probe sequences employed for *IL6* -174 G/C TaqMan analysis. FAM, 6-carboxyfluorescein

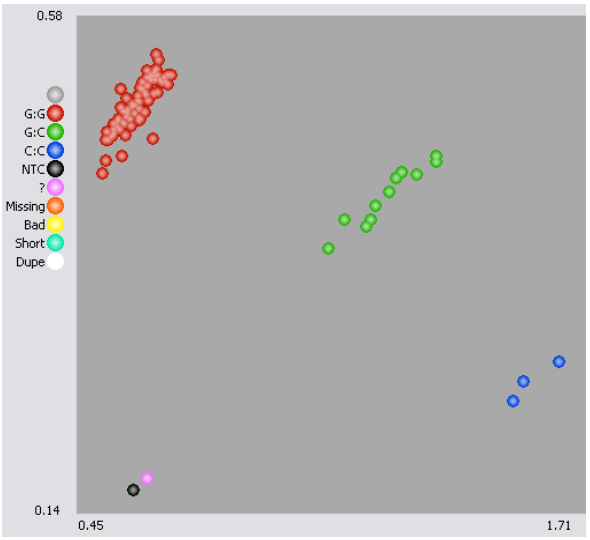

Figure 3.7 A representative plot showing allelic discrimination of *IL6* -174 G/C using a custom TaqMan genotyping assay (AB). Alleles are identified by the detection of VIC and/or FAM fluorescence. All three possible genotype combinations are show: GG, GC and CC. NTC, no-template control; ?, sample fail

3.6.7 *Sequencing of PCR Products*

To confirm that the PCR products generated by each genotyping method contained the appropriate polymorphism, control DNA samples were amplified and sequenced by the Molecular Biology Unit, Newcastle University, Newcastle upon Tyne, UK. The DNA sequences obtained were compared to the full sequence of the corresponding gene using MacVector software; direct alignment and polymorphism detection was achieved for all the samples analysed. The sequenced samples were then employed in all future PCR analyses as positive controls (homozygous and heterozygous).

Reagents

a) **dNTP:** 0.0125ml each dNTP stock (dATP, dCTP, dGTP, dTTP) in 0.95ml deionised water

b) **Formamide Loading Buffer:** 9ml formamide (Sigma), 0.5ml glycerol (BDH/Merck), 0.2ml 0.5M EDTA stock solution, 25mg bromophenol blue (Sigma) and 0.3ml distilled water

3.7 Gel Preparation

3.7.1 *Agarose Gels*

1%, 2% and 3% agarose gels were prepared by dissolving Microsieve Low Melt GTG agarose (0.64g, 1.28g and 1.92g respectively, Flowgen) and SeaKem LE agarose (0.16g, 0.32g and 0.48g respectively, Flowgen) in 80ml Tris Acetate EDTA (TAE) buffer using a heated magnetic stirrer (Fisher Scientific). Any volume lost during the heating process was replaced with deionised water. The agarose solution was then cooled under cold water, poured into a casting tray (Bio-Rad) around a plastic well forming comb (Bio-Rad) and left to set at RT for approximately 1 hour.

3.7.2 *Acrylamide Gels*

Glass casting plates (Bio-Rad) were cleaned with distilled water and chloroform; then coated in Sigmacote (Sigma) to allow gel removal after electrophoresis. The gel rig (Sequi-gel, Bio-Rad) was then assembled, ensuring the correct alignment of the plates, spacers and clamps to avoid leakage. Gel solutions of 8%, 10%, 11%, 12% and 15% were prepared using 30ml, 39ml, 43ml, 46ml and 49ml respectively of acrylamide stock solution (30% w/v acrylamide : 0.8% w/v bisacrylamide, Flowgen or 40% acrylamide/bis solution 19:1, Bio-Rad) and 15ml Tris Borate EDTA (TBE) buffer. All gel solutions were made up to 150ml with deionised water and degassed for 5 minutes. 0.1ml N-tetramethylethylenediamine solution (BDH/Merck) and 0.2ml 50% ammonium persulphate (BDH/Merck) solution were added to allow matrix formation and after gentle mixing the gels were cast between the glass plates. A plastic well forming comb was inserted and the gels were left to set at RT for approximately 1 hour.

Reagents

a) **TAE Buffer:** 4.84g Tris, 1.1ml glacial acetic acid (BDH/Merck) and 2ml 0.5M EDTA stock solution made up to 1L with deionised water

b) **TBE Buffer:** 10.8g Tris, 5.5g orthoboric acid (Sigma) and 0.58g EDTA made up to 1L with deionised water

c) **50% Ammonium Persulphate:** 0.5g ammonium persulphate in 1ml of deionised water

3.8 Staining Techniques

3.8.1 *Ethidium Bromide*

PCR products separated by agarose gel electrophoresis were stained with ethidium bromide (Sigma). 0.02ml 2mg/ml ethidium bromide was included in all molten agarose before casting and 0.01ml was also added to the electrophoresis buffer (TAE) prior to separation. Stained products were visualised using UV transillumination (AlphaImager, Alpha Innotech).

3.8.2 *Silver Staining*

Silver staining was employed to visualise PCR products separated by acrylamide gel electrophoresis. Gels were removed from the glass plates of the gel rig, washed twice in a fixing solution (10% ethanol; 0.5% acetic acid) for 3 minutes and then soaked in a silver nitrate solution (0.1% silver nitrate) for 15 minutes. During this 15 minute period the gel and staining solution were gently agitated to allow the positive charged silver ions to bind to the negatively charged DNA. Colour was developed using a solution of 1.5% sodium hydroxide and 0.15% formaldehyde. After agitation in developing solution for 20 minutes the gels were rinsed in tap water and sealed in plastic before visualisation on a light box.

Reagents

a) **Fixing Solution:** 100ml ethanol and 1ml glacial acetic acid made up to 1L with deionised water

b) **Staining Solution:** 0.5g silver nitrate (BDH/Merck) in 500ml deionised water

c) **Developing Solution:** 6g sodium hydroxide pellets and 0.6ml formaldehyde (Fisher Scientific) made up to 500ml with deionised water

3.9 Mixed Lymphocyte Cultures

The mixed lymphocyte culture (MLC) was the first widely used functional test of allo-reactivity in HSCT and provided the platform for the development of other cellular-based assays (Mickelson *et al.* 1993). Traditionally, MLCs were employed prior to HSCT to predict the incidence and severity of acute GVHD by determining the degree of allo-reactivity between the donor and patient in a transplant pair. Irradiated patient cells and non-irradiated donor cells are combined in culture and allo-reactivity is quantified by the incorporation of ^{3}H-thymidine into proliferating donor cells. Since the first application of the MLC, studies have demonstrated that the allo-reaction produced is not sensitive enough to accurately predict acute GVHD in either HLA-matched sibling (Lim *et al.* 1988; DeGast *et al.* 1992) or unrelated donor (Mickelson *et al.* 1993) HSCT; consequently, this technique is no longer widely used in the clinical setting. The MLC is still however an extremely useful tool in the research setting. A modified version of the assay was employed in this study to mimic the allo-response following HSCT.

MLCs were set up primarily between a HSCT patient and donor in the graft-versus-host (GVH) direction (allogeneic MLC); patient (host) PBMCs were irradiated and acted as stimulator cells for the unirradiated donor (graft) responder cells. As controls, autologous MLCs were set up using patient cells as both stimulator and responder.

As previously described (section 3.2), patient and donor PBMCs were separated from heparinized whole blood samples by density gradient centrifugation. $1x10^{7}$ stimulator cells (patient) were irradiated (20 Gy) and co-cultured with $1x10^{7}$ responder cells (donor) in 10ml 10% RPMI-AB in 25cm^{3} tissue culture flasks (Greiner). Each MLC was incubated at 37^{o}C with 5% CO_2 for 7 days.

After 7 days the culture was harvested for cells and supernatant by centrifugation at 380g for 5 minutes. The supernatant was removed and frozen in aliquots at –80^{o}C for IL-13 analysis by ELISA. The viable cells were counted by the trypan blue exclusion method and either used immediately in the *in vitro* human skin explant assay or cryopreserved for use at a later date.

3.10 *In Vitro* Human Skin Explant Assay

The human skin explants assay is an *in vitro* model that has been used over the years for the prediction of acute GVHD prior to transplantation (Vogelsang *et al.* 1985; Dickinson *et al* 1988; Sviland *et al* 1990; Dickinson *et al.* 1998; Dickinson *et al.* 1999). Prediction is based on the degree of damage observed when allo-reactive donor cells (previously stimulated in the MLC) and a pre-transplant patient-derived skin biopsy are combined in culture. The skin damage produced is a consequence of donor-derived allo-reactive cytotoxic T cells recognising specific allo-antigens present in the patient-derived skin as foreign (graft-versus-host reaction, GVHR). The resultant skin damaged is graded from I-IV using the classification of GVHD histopathology described by Lerner *et al.* (1974).

In addition to its use in the prediction of acute GVHD, the human skin explants assay has also developed into a useful research tool to investigate potential mechanisms in the pathogenesis of the disease. In this current study, the assay was performed prior to transplantation and was employed to examine the possible roles of IL-13 in acute GVHD.

Two 4mm punch biopsies of skin were taken from a patient's upper arm and placed in EBSS (1-2 hours) prior to their use in the assay. The skin biopsies were dissected using sterile instruments, removing the sub-dermal adipose tissue and leaving a portion of the dermis under the epithelium. The biopsies were then divided into a total of six pieces of approximately equal size.

Cells harvested from the previously described allogeneic and autologous MLCs (section 3.9) were diluted to $2x10^6$ cells and pelleted by centrifugation at 380g for 5 minutes. The pelleted cells were then re-suspended in 0.4ml 20% RPMI-AS.

The six skin sections prepared from the two punch biopsies were cultured in duplicate in a 96 well U-bottomed microtitre plate (Greiner) under three conditions; with allogeneic MLC cells, autologous MLC cells and 20% RPMI-AS. 0.2ml of each cell suspension or medium solution was added to the appropriate well. A skin section was then added to the bottom of each well. Full submersion of the sections ensured there was maximum contact between the skin and the MLC cells. To avoid any

contamination between the wells, forceps were sterilised by immersion in 70% ethanol and washed twice in EBSS before contact with any skin. Once prepared, each human skin explant assay was incubated at 37°C with 5% CO_2 for 3 days.

After 3 days the skin sections were removed with sterile forceps and submerged in 10% buffered formalin (supplied by the Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne, UK). The biopsies were paraffin-embedded, sectioned and stained with haematoxylin and eosin (H&E) in the Department of Pathology. The H&E stained sections were then graded for GVHR by Professor A.M. Dickinson and Dr X.N. Wang, and verified by Dr L Sviland (Figure 3.8). All explants were graded independently and whilst blinded to culture conditions and clinical outcome.

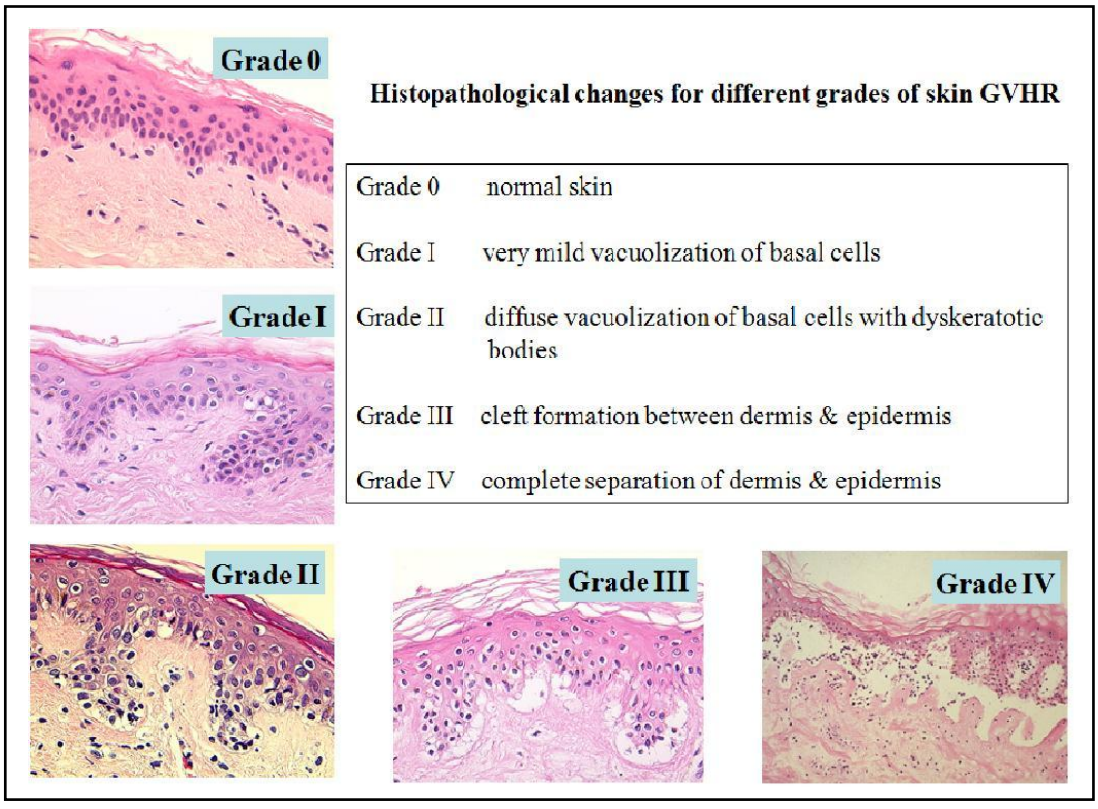

Figure 3.8 Grading system for the human skin explant assay. GVHR, graft-versus-host reaction

3.11 Mitogen Stimulation Assay

Mitogen stimulation allowed cytokine production responses by activated cells to be examined *in vitro*. Two mitogens were employed in these assays, lipopolysaccharide (LPS) and phytohaemagglutinin (PHA). LPS is a constituent of the cell wall of gram-negative bacteria that activates granulocytes and PHA is a plant-derived polysaccharide that activates lymphocytes, particularly T cells (Goldsby *et al.* 2003). PBMCs were cultured with LPS and PHA for 24 and 72 hours respectively. Unstimulated PMCS in 10% RPMI-FCS were also cultured for 24 and 72 hours (spontaneous cytokine release). These time points were chosen for optimal mitogenic responses and followed a method previously established by our research group (unpublished data).

Pre-transplant patient and donor PBMCs were obtained from heparinised blood using the previously described method (section 3.2) and re-suspended in 10% RPMI-FCS at a concentration of $1x10^6$ cells/ml. 0.1ml of the PBMC suspension was added to 60 wells of a 96 well U-bottomed microtitre plate. 0.1ml 10% RPMI-FCS was then added to 30 of the wells, 0.1ml 10% RPMI-FCS containing 200ng/ml LPS was added to 15 wells and 0.1ml 10% RPMI-FCS containing 10µg/ml PHA was added to the remaining 15 wells (Figure 3.9). The plate was then incubated at 37°C with 5% CO_2 for 24 hours.

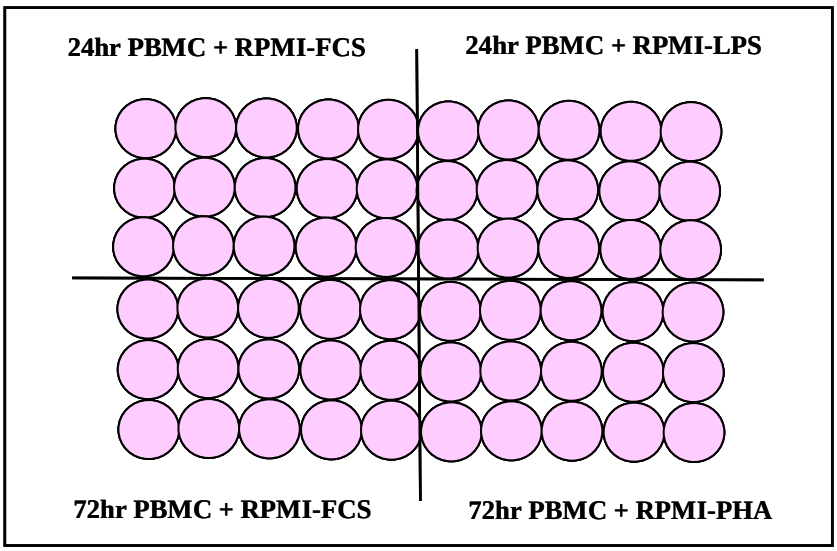

Figure 3.9 Plate layout for mitogen stimulation assay. PBMC, peripheral blood mononuclear cells; FCS, foetal calf serum; LPS, lipopolysaccharide; PHA, phytohaemagglutinin

After 24 hours the supernatants from 15 of the wells containing PBMCs and 10% RPMI-FCS were pooled, centrifuged at 380g for 5 minutes, aliquoted and frozen. This procedure was then repeated for the 15 wells containing PBMCs and 10% RPMI with LPS. The plate was incubated for a further 48 hours. After this time, the supernatant for the remaining two conditions (10% RPMI-FCS and 10% RPMI with PHA) were processed as above. All the samples were stored at -80°C for IL-13 analysis by ELISA.

Reagents

a) **200ng/ml LPS:** 17.5µl LPS stock solution (40µg/ml, Sigma) and 3.5ml 10% RPMI-FCS

b) **10µg/ml PHA:** 35µl PHA stock solution (1mg/ml, Inverness Medical) and 3.5ml 10% RPMI-FCS

3.12 Serial Serum Sample Collection and Preparation

Serum samples were obtained from blood collected from patients at eight time points across the transplant period (day -7 (pre-HSCT), day 0, day 7, day 14, day 28, 3 months, 6 months and 12 months) and from laboratory volunteers at eight time points (day -7, day 0, day 7, day 14, day 28, 3 months, 6 months and 12 months). The blood (6ml) was collected into an anticoagulant free vacutainer tube (BD) and allowed to clot at RT for 15-20 minutes and then at 4^{o}C for 2-4 hours. The serum was separated by centrifugation at 380g for 5 minutes, aliquoted and frozen at -80^{o}C for IL-13 analysis by ELISA.

3.13 Interleukin-13 Quantification by Enzyme-Linked ImmunoSorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay used to detect and quantify the amount of an analyte (antigen or antibody) in a variety of biological samples. Several different ELISA techniques exist however, they all rely on the same basic principle; an analyte is immobilised on a surface, either directly or via a capture antibody (sandwich ELISA) and an enzyme-labelled antibody is used to detect its presence. The detection antibody may bind directly (primary antibody) or indirectly via an unlabelled intermediary (secondary antibody) and on addition of an enzymatic substrate a visible signal is produced. The application of appropriate standards of

known concentrations permits the quantification of the analyte in the experimental system (Goldsby *et al.* 2003).

IL-13 expression was quantified in MLC supernatants, PHA stimulated supernatants and serum samples using two ELISA kits; Human IL-13 ELISA Development Kit (39-10,000pg/ml, PeproTech) and Human IL-13 Low Level ELISA Kit (0-40pg/ml, RayBiotech). All supernatants were initially tested neat using the PeproTech supplied ELISA kit. Samples with IL-13 measurements above the upper limits of this kit were diluted 1:10 with assay diluent and re-tested. Any sample producing IL-13 measurements below the kits lowest limits were re-assayed using the low level IL-13 ELISA kit supplied by RayBiotech. All serum samples were tested neat using the low level IL-13 kit. All samples were tested in duplicate and the mean absorbance for each set was used for quantification.

Assays were performed and all reagents prepared according to the manufacturer's instructions. Before commencing the ELISAs, reagents, microtitre plates, standards and samples were allowed to equilibrate to RT; temperature-dependent inter-well variation was therefore minimised.

Human IL-13 Development Kit (PeproTech)

A 96 well microtitre plate (Maxisorb, Nunc) was coated by incubating 0.1ml of anti-human IL-13 capture antibody (1µg/ml) in each well at RT overnight. After this incubation period, the plate was washed 4 times with a Tween-based wash buffer (Wellwash 4 Mk2, ThermoLabsystems). In order to minimise non-specific binding, 0.3ml blocking buffer (1% BSA) was introduced into each well and the plate was incubated at RT for 1 hour. After a further 4 washes, 0.1ml standards and samples in duplicate were added to the plate. Using assay diluent, a dilution series of the standard stock solution (1µg/ml)) was prepared. Solutions containing the following concentrations of IL-13 were used: 10,000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06 (pg/ml). Neat assay diluent was employed as a negative control (blank, 0pg/ml). The plate was incubated for 2 hours at RT and then washed 4 times. 0.1ml of biotinylated detection antibody (0.25µg/ml) was pipetted into each well and the plate was incubated for a further 2 hours at RT. The plate was washed 4 times and 0.1ml avidin-horseradish peroxidise (HRP) conjugate was added. After 30 minutes incubation

at RT, the plate was washed a further 4 times and 0.1ml 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) liquid substrate (Sigma) was added to each well. The plate was then incubated at RT for colour development.

After 20 minutes incubation, the absorbance of each well was measured spectrophotometrically at 405nm (A_{405}) with a correction for absorbance at 650nm (A_{650}). Absorbances were measured using a Multiskan Acsent plate reader in combination with Acsent software (ThermoLabsystems). Readings were considered acceptable when optical density (OD) measurements less than 0.2 units for the negative control and 1.2 units for the highest standard concentration were obtained. Mean values for A_{405} and A_{650} were calculated for each duplicate set, including all standards and controls and the appropriate negative control measurements were subtracted from these means (blank subtraction). Corrected A_{405} values for the standards and samples were then calculated (mean A_{405} – mean A_{650}). A standard calibration curve (Figure 3.10) was plotted and the IL-13 concentration of each sample was derived from the curve. Any dilution factor was taken into account at this point.

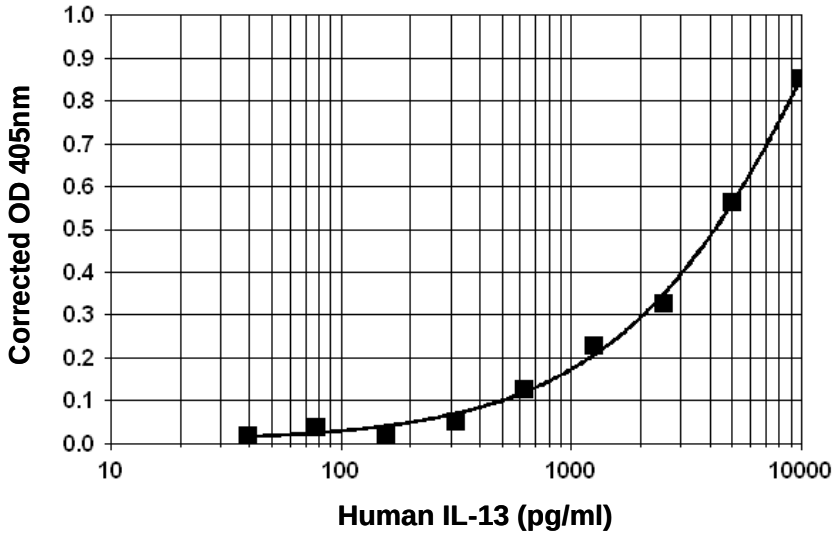

Figure 3.10 A typical standard curve generated using the Human IL-13 ELISA Development kit (PeproTech). Corrected OD at 405nm accounts for the subtraction of the OD at 650nm from the OD at 405nm. OD, optical density; IL-13, interleukin-13

Reagents

a) **10x Phosphate Buffered Saline (PBS):** 96g of PBS (Gibco) dissolved in 1L deionised water

b) **1x PBS:** 100ml 10x PBS made up to 1L with deionised water

c) **Wash Buffer:** 0.5ml Tween-20 (Sigma) and 1L 1x PBS

d) **Blocking Buffer:** 1g bovine serum albumin (BSA (fraction 5), Sigma) dissolved in 100ml 1x PBS

e) **Assay Diluent:** 10ml blocking buffer, 90ml 1x PBS and 0.05ml Tween-20

f) **Anti-Human IL-13 Capture Antibody (1µg/ml):** 0.1ml reconstituted capture antibody (lyophilised antibody in 1ml sterile deionised water, 100µg/ml) in 9.9ml 1x PBS

g) **Standard Dilution Series:** 0.1ml reconstituted recombinant human IL-13 (lyophilised standard in 1ml sterile deionised water, 1µg/ml) in 9.9ml assay diluent (10,000pg/ml, top standard). Remaining 8 standards were prepared using a doubling dilution series (0.5ml previous standard + 0.5ml assay diluent)

h) **Human IL-13 Detection Antibody (0.25µg/ml):** 0.025ml reconstituted detection antibody (lyophilised antibody in 0.25ml sterile deionised water, 100µg/ml) in 10ml assay diluent

i) **Avidin-HRP Conjugate:** 5.5µl avidin-HRP conjugate in 11ml assay diluent

Human IL-13 Low Level Kit (RayBiotech)

A dilution series of the standard stock solution (20ng/ml) was prepared using assay diluent. Solutions containing the following concentrations of IL-13 were employed: 40, 16, 6.4, 2.56, 1.03, 0.41, 0.16 (pg/ml). Assay diluent alone was used as a negative control (blank, 0pg/ml). 0.1ml of all standards and samples in duplicate were added to the pre-coated (anti-human IL-13 capture antibody) microtitre plate. The plate was incubated at RT for 2.5 hours then washed 4 times using the wash buffer provided. 0.1ml biotinylated anti-human detection antibody was added to each well and the plate was incubated for a further 1 hour at RT. Following 4 more washes, 0.1ml HRP-streptavidin was added and the plate was incubated for 45 minutes at RT. After a further 5 washes, 0.1ml 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to each well and the plate was incubated (RT) in the dark for 30 minutes to allow colour development.

The colour reaction was stopped by adding 0.05ml sulfuric acid (2M) to each well. The plate was gently agitated to ensure an even distribution of colour and the absorbances were measured at 450nm (A_{450}). Readings were considered acceptable when OD measurements of less than 0.03 for the negative control were obtained. The mean absorbance for each set of duplicate standards and samples was calculated and the mean A_{450} for the negative control was then subtracted from these values. A standard calibration curve (Figure 3.11) was plotted and IL-13 concentrations were derived. Any dilution factor was taken into account at this point.

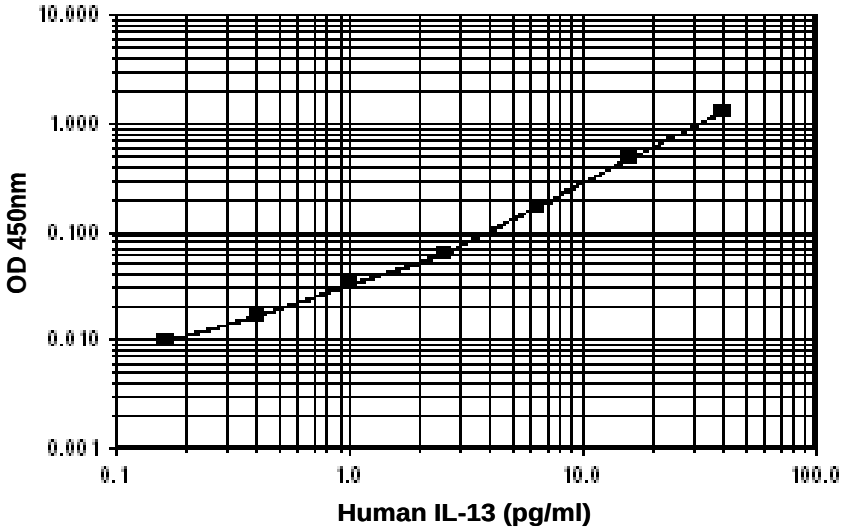

Figure 3.11 A typical standard curve generated using the Human IL-13 Low Level ELISA kit (RayBiotech Inc). OD, optical density; IL-13, interleukin-13

Reagents

a) **Wash Buffer:** 20ml wash buffer (20x) concentrate in 380ml deionised water

b) **Assay Diluent:** 15ml assay diluent (5x) concentrate in 60ml deionised water

c) **Standard Dilution Series:** 0.002ml reconstituted recombinant human IL-13 (lyophilised standard in 166µl assay diluent, 20ng/ml) in 0.998ml assay diluent (40pg/ml, top standard). Remaining 6 standards were prepared using a 2.5 dilution series (0.2ml of previous standard + 0.3ml assay diluent)

d) **Human IL-13 Detection Antibody:** 0.2ml reconstituted detection antibody (lyophilised antibody in 0.2ml assay diluent) in 15.8ml assay diluent

e) **HRP-Streptavidin:** 0.002ml HRP-streptavidin concentrate in 12ml assay diluent

3.14 Cytometric Bead Array

A cytometric bead array (CBA) is an immunoassay with the ability to detect and quantify multiple analytes in a single sample. The assay employs a series of beads with discrete fluorescent intensities to detect soluble analytes. Each bead population utilised provides a capture surface (coated with a capture antibody) for a specific analyte and is analogous to an individually coated well in an ELISA. Analyte detection is achieved by addition of a fluorescent dye and measurement of fluorescence-based emission using flow cytometry. The application of appropriate standards of known concentrations permits the quantification of the analytes in the experimental system.

The quantification of IL-2, IL-4, IL-5, IL-10, IL-13, IFNγ and TNFα in supernatants collected from harvested allogeneic MLCs (section 3.9) was carried out using a Human Soluble Protein CBA kit (20-10,000pg/ml, BD). The supernatants were tested neat and a standard dilution series for each cytokine was included in all assays. Due to the high reproducibility and expensive nature of the CBA kit, standards and samples were not tested in duplicate. 10% of the sample cohort was however re-assayed and no significant differences were observed between the values. IL-13 levels obtained using this method and standard ELISA (section 3.13.1) were also compared and again no significant differences were observed between the values; thus, the two methods employed for IL-13 quantification were comparable.

Assays were performed and reagents prepared according to the manufacturer's instructions. Before commencing, all reagents, standards, samples and tubes (BD) were allowed to equilibrate to RT.

A dilution series of the standard stock solution (5000pg/ml) was prepared using assay diluent. Solutions containing the following IL-2, IL-4, IL-5, IL-10, IL-13, IFNγ and TNFα concentrations were employed: 5000, 2500, 1250, 625, 312.5, 156, 80, 40, 20 (pg/ml). Assay diluent alone was used as a negative control (blank, 0pg/ml). 0.05ml of each standard and supernatant sample was combined with 0.05ml of capture beads and the tubes were incubated at RT for 1 hour. 0.05ml of phycoerythrin (PE) detection reagent was added and the tubes were incubated at RT for further 2 hours. 1ml of wash buffer was introduced and the tubes were centrifuged (MSE Centaur) at 200g for 5 minutes. The supernatant from each tube was then aspirated and discarded. 0.3ml of

wash buffer was added and the tubes were vortexed (Vortex-Genie 2, SLS) briefly to resuspend the beads. The fluorescent intensity of the standards and samples were measured using flow cytometry (FACSCalibur, BD) and IL-2, IL-4, IL-5, IL-10, IL-13, IFNγ and TNFα concentrations were calculated using standard curves (Figure 3.11) constructed following data analysis with FCAP Array software (BD).

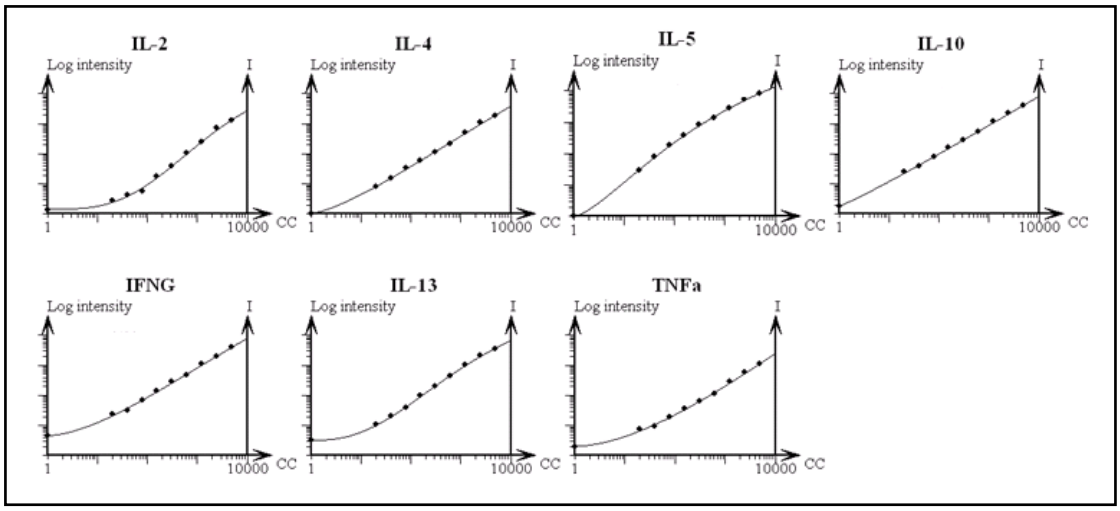

Figure 3.12 A typical set of standard curves generated using the Human Soluble Protein CBA kit (BD). IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-10, interleukin-10; IFNG, interferon gamma; IL-13, interleukin-13; TNFa, tumour necrosis factor alpha; I, intensity; CC, calculated concentration

Reagents

a) **Capture Beads:** 0.1ml each cytokine capture bead (IL-2, IL-4, IL-5, IL-10, IL13, IFNγ and TNFα) in 4.3ml capture bead diluent

b) **Standard Dilution Series:** Lyophilised standards (IL-2, IL-4, IL-5, IL-10, IL13, IFNγ and TNFα) in 2ml assay diluent (5000pg/ml, top standard). Remaining 8 standards were prepared using a doubling dilution series (0.5ml previous standard + 0.5ml assay diluent)

c) **PE Detection Reagent:** 0.1ml each cytokine PE detection (IL-2, IL-4, IL-5, IL-10, IL13, IFNγ and TNFα) in 4.3ml detection diluent

3.15 Statistical Analysis

Statistical analyses carried out throughout this study employed the computer software packages Graphpad Prism 5.0, SPSS 15.0 and NCSS 97 (supplied by Newcastle University).

HSCT allele and genotype frequencies were compared to control frequencies using the chi-square (χ^2) test and Fisher's exact test where appropriate. All genotypic data were analysed for associations with acute and chronic GVHD, overall survival, relapse incidence and TRM. GVHD analyses were carried out using the chi-square (χ^2) test and forward stepwise logistic regression, whilst Kaplan-Meier curves, the Log-Rank test and Cox regression were utilised for survival analyses. Relapse incidence and TRM were analysed using competing risks methods (cumulative incidence curves and the Log-Rank test). For all genotypic analyses that examined two or more genetic factors in a given population, multiple comparison corrections (Bonferroni adjustment) were performed (sections 4.3.3).

The Kolmogorov-Smirnov test was used to determine whether functional data was normally distributed – a Gaussian distribution. All normally distributed data was analysed using a parametric statistical test (paired/unpaired Student's t-test or One-way ANOVA), whilst any data that was not normally distributed was analysed by a non-parametric method (Wilcoxon Signed-Rank test, Mann-Whitney U test or Kruskal-Wallis test). Correlation calculations were performed using the Pearson's or Spearman's Rank Correlation tests.

Chapter 4. Non-HLA Polymorphisms in Unrelated Donor HSCT

While the influence of non-HLA polymorphisms on HSCT outcome has been studied extensively in HLA-matched sibling transplantation (see section 1.4), relatively little is still known about these polymorphisms in unrelated donor HSCT (Ishikawa *et al.* 2002; MacMillan *et al.* 2003a; MacMillan *et al.* 2003b; Remberger *et al.* 2003; Kean *et al.* 2004; Bettens *et al.* 2006). Since an HLA-matched sibling is available to fewer than 30% of patients (de Lima and Champlin 2001), unrelated donor HSCT constitutes a large proportion of the transplants performed each year, thus investigations into the impact of non-HLA polymorphisms in this setting are also important. Considering that the risk of GVHD and post-transplant mortality is substantially increased following unrelated donor HSCT (Anasetti *et al.* 1995), the identification of genetic markers that can predict these outcomes would be extremely beneficial.

Polymorphisms within the genes of IL-2 (-330 T/G), IL-4 *(*-590 C/T), IL-6 (-174 G/C), IL-10 (-592 A/C, -1082 G/A), IL-1Ra (intron 2 VNTR), TNFα (d intron 3 (GA)n), TNFRII (-196 M/R), IFNγ (intron 1 (CA)n), vitamin D receptor (intron 8 A/C, exon 9 T/C) and oestrogen receptor (intron 1 A/G, intron 1 C/T) have been demonstrated by our research group and numerous others worldwide to significantly associate with both GVHD and survival following HLA-matched sibling HSCT (Middleton *et al.* 1998; Cavet *et al.* 1999; Cavet *et al.* 2001; Cullup H 2001; Socie *et al.* 2001; Middleton *et al.* 2002; Rocha V 2002; Lin *et al.* 2003; Middleton *et al.* 2003; Stark *et al.* 2003; Mlynarczewska *et al.* 2004; Bogunia-Kubik *et al.* 2005b; Karabon *et al.* 2005; Rocha *et al.* 2009). Considering this, the aim of the current study was therefore to determine the role, if any that these polymorphisms play in the unrelated donor transplant setting.

4.1 Study Aims

i) To establish and compare the allele and genotype frequencies of the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms in a Newcastle control population with an unrelated donor HSCT cohort.

ii) To correlate transplant outcome, in terms of acute GVHD incidence and severity, chronic GVHD incidence, overall survival, relapse incidence and TRM with the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms by univariate and multivariate statistical analyses.

4.2 Statistical Analysis

Polymorphic allele and genotype frequencies calculated for the unrelated donor HSCT cohort were compared to those of Newcastle controls using the chi-square (χ^2) and Fisher's exact test where appropriate. Expected and observed genotype frequencies for the Newcastle controls, HSCT patients and unrelated donors were assessed for Hardy-Weinberg equilibrium using the chi-square (χ^2) test. Non-HLA polymorphisms were correlated with acute and chronic GVHD using the chi-square (χ^2) test and forward stepwise logistic regression. Overall survival was analysed using Kaplan-Meier curves and the Log-Rank test, followed by Cox regression. The incidence of relapse and TRM were analysed using cumulative incidence curves and the Log-Rank test (competing risk analysis).

4.3 Experimental Design

4.3.1 *Inclusion and Exclusion Criteria*

All patients and their donors recruited as part of a European Commission FP5 biotechnology project (Eurobank QLRI-CT-2000-00010, PI Professor A M Dickinson), that received an unrelated donor HSCT between November 1983 and December 2006 and had DNA and 1 year's clinical follow-up available were included in the analyses. The study was given JLEC approval (Appendix B) and informed consent was obtained from all patients and donors. Exclusion criteria were as follows:

i) HLA-matched sibling HSCT
ii) Transplantation not performed
iii) Incomplete HSCT outcome data
iv) Incomplete HLA typing data
v) Below the age of 16 at the time of transplant
vi) Transplantation for an immunological disease
vii) Transplantation with a source other than PBSC or bone marrow
viii) Two or more transplants performed

Once the inclusion and exclusion criteria had been taken into account, the unrelated donor HSCT cohort employed had been reduced from 374 patient/donor pairs to 362.

4.3.2 *Control Measures*

For all samples, successful PCR amplification was confirmed by agarose gel electrophoresis prior to the performance of any PCR-RFLP, SSCP or IHG analyses (section 3.6.1, 3.6.2, 3.6.3 and 3.6.4). Accurate ascertainment of polymorphic alleles was ensured by the inclusion of both homozygous and heterozygous control samples of known genotype (positive controls) in all PCR reactions, polymorphism analysis methods (PCR-RFLP, SSCP and IHG) and electrophoresis gels (section 3.6.6). Genotypes were determined with reference to these known samples and verified by an independent observer. In equivocal cases, samples were re-analysed (less than 5% of the cohort). All genotypes were determined whilst blinded to clinical outcome. Correct

allele determination was also ensured by the inclusion of negative controls, appropriate DNA size markers and by re-typing 25% of each cohort tested.

4.3.3 *Method of Analysis*

Prior to any correlation with clinical outcome, the accuracy of *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T genotyping in our unrelated donor HSCT cohort was assessed by the comparison of calculated allele and genotype frequencies with those of a Newcastle control group and previously published data. Only the major allele and genotype frequencies were calculated for the microsatellite (*TNFAd* intron 3 (GA)n, *IFNG* intron 1 (CA)n) and VNTR (*IL1RN* intron 2) polymorphisms examined in this study. In order to rule out selection bias and genotype deviation, patient, donor and control genotype frequencies were also tested for Hardy-Weinberg equilibrium.

For each of the non-HLA polymorphisms examined, the alleles and/or genotypes previously identified as significant factors in the HLA-matched sibling HSCT setting were individually correlated with the incidence and severity of acute GVHD, incidence of chronic GVHD and overall survival using univariate analysis methods (chi-square test (χ^2), Kaplan-Meier curves and Log-Rank test). Alleles/genotypes with P values ≤ 0.1 were then tested for independence against identified confounding clinical factors using multivariate analysis methods (forward stepwise logistic and Cox regression). In order to retain adequate power only the strongest factors, up to a maximum of 5, were entered into all multivariate analyses. P values <0.05 were considered statistically significant and values between 0.05 and 0.1 were considered indicative of a trend.

As thirteen non-HLA polymorphisms were examined in our unrelated donor HSCT cohort and the replication of any associations observed in a second independent cohort was not possible, multiple comparison corrections were performed in order to reduce the chance of type I errors (false positive results). These corrections were achieved using Bonferroni adjustment. This method reduces the chance of spurious results by lowering the level of statistical significance (typically 0.05) according to the number of

tests i.e. corrected values are achieved by dividing the P value obtained by the number of factors analysed; 13 in the case of the current study. Although Bonferroni adjustment is highly conservative and prone to over-correction, it can be applied simply to any multiple testing situation, has a high power in large populations such as ours and other than replication, is the most appropriate methods for ruling out associations by chance (Aickin and Gensler 1996; Perneger 1998).

Non-HLA polymorphism alleles/genotypes were also correlated with relapse incidence and TRM. As both of these outcomes are affected by other clinical events, competing risk analysis methods were required. These methods allow all possible associations to be observed by excluding the relevant competing event (relapse or TRM). Due to the extreme complexity of multivariate analyses for competing risks and the requirement for specialised statistical software, univariate analysis (cumulative incidence curves and Log-Rank test) alone was performed in this study. Again, P values <0.05 were considered statistically significant and values between 0.05 and 0.1 were considered indicative of a trend.

HLA disparity is a substantial risk factor in HSCT, particularly in the unrelated donor transplant setting; where the risk of GVHD and post-transplant mortality increases significantly with single mismatches at HLA-A, -B, -C, -DRB1 or –DPB1 (Sasazuki T 1998; Flomberg *et al.* 2004; Chalandon *et al.* 2006; Lee *et al.* 2007; Shaw *et al.* 2007). As a result, it was extremely likely that this factor would have a considerable impact on HSCT outcome in this study. Thus, prior to any analyses with the non-HLA polymorphisms, HLA disparity was tested for associations with GVHD. In our unrelated donor HSCT cohort HLA-mismatched transplants were found to correlate with a higher incidence of both acute and chronic GVHD ($P=0.015$ and $P=0.042$ respectively). The most accurate and reliable means of accounting for such associations involves stratification of the cohort into HLA-matched and HLA-mismatched transplant groups. However, this was not possible in the current study, as the mismatched group would be too small to analyse (n=114). Instead, associations with HSCT outcome were tested for in the whole unrelated donor cohort with HLA-mismatch entered into all multivariate analyses as a confounding factor. Due to the heterogeneity of the unrelated donor HSCT cohort employed, stratification should also be considered for clinical factors such as underlying disease, disease stage (early/advanced), conditioning regimen (low intensity/myeloablative), prophylaxis intensity (low/high) and graft manipulation

(T cell replete/T cell deplete). However, due to number constraints and as these factors had no impact on HSCT outcome in this study, stratification was not performed (section 4.6).

In recent years, there has been concern around the proliferation of genetic association studies, especially those in which results have not been replicated (Colhoun *et al.* 2003). In an attempt to address this problem, any significant correlation observed in this study was verified in a smaller second cohort (n=248). This cohort was a subset of the original unrelated donor transplant population and comprised the 10/10 HLA-matched patient/donor pairs only. This cohort was not independent and therefore not ideal; however, it was the most appropriate means of verification available. Time constraints did not permit the formation of a completely independent population of adequate size (power). Sample collection for the creation of a large independent verification cohort is currently being undertaken.

4.4 Study Populations

4.4.1 *Newcastle Control Cohort*

A total of 100 Newcastle volunteer blood donors were recruited to act as a control cohort for this study. The group comprised 53 males and 47 females, with a median age of 37 years (range 19-56).

4.4.2 *Unrelated Donor HSCT Cohort*

Genomic DNA was obtained from allogeneic HSCT patients and their unrelated donors for *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphism genotyping. This was an adult cohort (≥16 at the time of transplant) comprising 362 unrelated donor transplants. HLA matching between patients and donors was performed by high resolution DNA-based typing for HLA-A, B, C, DRB1 and DQB1. Complete matching at all 5 loci was observed in 248 (69%) transplant pairs.

Cohort characteristics:
The 362 HSCT patients included in this cohort were transplanted between November 1983 and December 2006 at clinical centres in Austria, England, France, Germany and Spain. All patients and donors were recruited and consented as part of the previously described 'Eurobank Project' (section 4.3.1).

Acute GVHD was deemed assessable in those alive ≥30 days post-transplant and developed in 237 patients. 125 patients had no acute GVHD (grade 0), 67 grade I, 102 grade II, 41 grade III and 27 had grade IV. Acute GVHD grades III and IV were considered severe. Chronic GVHD was assessable from 100 days post-transplant in 286 patients. 145 of these patients developed the condition. All transplant patients were prepared for HSCT with low intensity (n=138) or myeloablative conditioning (n=234) regimens and 61% (n=221) were transplanted with T cell depleted stem cell grafts. 275 patients were given high intensity GVHD prophylaxis (CsA plus MTX or MM), whilst

87 received CsA monotherapy. The overall survival rate for this cohort was 49% (n=179) and causes of death included disease relapse, infection and GVHD. Table 4.1 shows the demographic characteristics of the unrelated donor HSCT cohort examined in this study.

Table 4.1 Characteristics of the unrelated donor HSCT cohort (n=362). CMV, cytomegalovirus; CsA, cyclosporine A; MTX, methotrexate; MM, mycophenolate mofetil; TCD, T cell depletion

TRANSPLANT PATIENTS		
Median Age		41 years (range 16-67)
		Number in each group
Gender		
	Male	202
	Female	160
	Female to Male HSCT	52
CMV Status		
	Positive	178
	Negative	184
Underlying Disease		
	Acute Lymphoblastic Leukaemia	46
	Acute Myeloid Leukaemia	132
	Chronic Lymphocytic Leukaemia	7
	Chronic Myeloid Leukaemia	83
	Hodgkin disease	7
	Non-Hodgkin Lymphoma	24
	Myeloproliferative Disorders	22
	Plasma Cell Neoplasm	33
	Aplastic Anaemia	8
Conditioning Therapy		
	Low intensity	138
	Myeloablative	234
GVHD Prophylaxis		
	CsA	87
	CsA and MTX	185
	CsA and MM	90
	TCD	221
TRANSPLANT DONORS		
Median Age		36 years (range 19-64)
		Number in each group
Gender		
	Male	235
	Female	127
CMV Status		
	Positive	153
	Negative	209

4.5 Allele and Genotype Frequencies

Allele and genotype frequencies for the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms were determined in the Newcastle control cohort, HSCT patients and unrelated donors. The allele frequencies calculated for the three groups are shown in Table 4.2. The frequencies determined were comparable and did not differ significantly to those published in the literature (Walley and Cookson 1996; Middleton *et al.* 1998; Cavet *et al.* 1999; Cavet *et al.* 2001; Cullup *et al.* 2001; Middleton *et al.* 2002; MacMillan *et al.* 2003b; Middleton *et al.* 2003; Stark *et al.* 2003). Thus, the non-HLA polymorphisms examined in this study are not a consequence of disease, but occur naturally within all populations

All possible genotype combinations for each of the non-HLA polymorphisms examined were observed in the patient and donor groups and the genotype frequencies determined were similar to those calculated for the Newcastle control cohort (Table 4.3). Potential deviation from Hardy-Weinberg equilibrium was examined for using the chi-square (χ^2) test. No significant differences were demonstrated between the observed and calculated expected values for the patients, donors or controls (Table 4.4, P values only). Thus, the genotype frequencies observed in all three groups were in Hardy-Weinberg equilibrium; there was no selection bias and genotype deviation was absent. Furthermore, the observed genotype frequencies were comparable to those published in the literature (Walley and Cookson 1996; Middleton *et al.* 1998; Cavet *et al.* 1999; Cavet *et al.* 2001; Cullup H 2001; Middleton *et al.* 2002; MacMillan *et al.* 2003b; Middleton *et al.* 2003; Stark *et al.* 2003).

	Newcastle Controls n=100	HSCT Patients n=362	HSCT Donors n=362
Polymorphic Alleles			
IL2 -330 T/G			
T	0.73	0.71	0.72
G	0.27	0.29	0.28
IL4 -590 C/T			
C	0.84	0.84	0.82
T	0.16	0.16	0.18
IL6 -174 G/C			
G	0.39	0.41	0.41
C	0.61	0.59	0.59
IL10 -592 A/C			
A	0.25	0.27	0.25
C	0.75	0.73	0.75
IL10 -1082 G/A			
G	0.47	0.47	0.47
A	0.53	0.53	0.53
IL1RN intron 2 VNTR			
1	0.75	0.75	0.73
2	0.20	0.22	0.21
TNFAd intron 3 (GA)n			
d3	0.57	0.58	0.58
d4	0.29	0.27	0.27
TNFSRF1B -196 M/R			
M	0.72	0.72	0.72
R	0.28	0.28	0.28
IFNG intron 1 (CA)n			
2	0.44	0.43	0.44
3	0.25	0.25	0.24
VDR intron 8 A/C			
A	0.50	0.52	0.50
C	0.50	0.48	0.50
VDR exon 9 T/C			
T	0.60	0.58	0.58
C	0.40	0.42	0.42
ESR1 intron 1 A/G			
A	0.65	0.65	0.66
G	0.35	0.35	0.34
ESR1 intron 1 C/T			
C	0.47	0.47	0.48
T	0.53	0.53	0.52

Table 4.2 Non-HLA polymorphism allele frequencies for the Newcastle control cohort, HSCT patients and unrelated donors. IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

	Newcastle Controls n=100	HSCT Patients n=362	HSCT Donors n=362
Polymorphic Genotypes			
IL2 -330 T/G			
TT	0.50	0.49	0.50
TG	0.45	0.45	0.43
GG	0.05	0.06	0.07
IL4 -590 C/T			
CC	0.71	0.71	0.69
CT	0.26	0.26	0.27
TT	0.03	0.03	0.04
IL6 -174 G/C			
GG	0.14	0.13	0.13
GC	0.49	0.48	0.48
CC	0.37	0.39	0.39
IL10 -592 A/C			
AA	0.07	0.07	0.06
AC	0.35	0.37	0.34
CC	0.58	0.56	0.60
IL10 -1082 G/A			
GG	0.22	0.22	0.24
GA	0.49	0.49	0.46
AA	0.29	0.29	0.30
IL1RN intron 2 VNTR			
11	0.24	0.23	0.22
12	0.18	0.16	0.19
22	0.07	0.08	0.07
TNFAd intron 3 (GA)n			
d3d3	0.32	0.32	0.32
d3d4	0.17	0.19	0.19
d4d4	0.07	0.06	0.06
TNFSRF1B -196 M/R			
MM	0.54	0.54	0.54
MR	0.36	0.37	0.37
RR	0.10	0.09	0.09
IFNG intron 1 (CA)n			
22	0.18	0.18	0.17
23	0.44	0.42	0.47
33	0.22	0.24	0.21
VDR intron 8 A/C			
AA	0.24	0.26	0.25
AC	0.52	0.51	0.50
CC	0.24	0.23	0.25
VDR exon 9 T/C			
TT	0.33	0.35	0.35
TC	0.53	0.52	0.52
CC	0.14	0.13	0.13
ESR1 intron 1 A/G			
AA	0.42	0.45	0.43
AG	0.45	0.45	0.45
GG	0.13	0.10	0.12
ESR1 intron 1 C/T			
CC	0.22	0.23	0.22
CT	0.49	0.49	0.51
TT	0.29	0.28	0.27

Table 4.3 Non-HLA polymorphism genotype frequencies for the Newcastle control cohort, HSCT patients and unrelated donors. IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

	Newcastle Controls P Value	HSCT Patients P Value	HSCT Donors P Value
IL2 -330 T/G	0.892	0.911	0.879
IL4 -590 C/T	0.901	0.735	0.824
IL6 -174 G/C	1.000	0.978	0.945
IL10 -592 A/C	0.945	1.000	1.000
IL10 -1082 G/A	1.000	0.967	1.000
IL1RN intron 2, VNTR	0.752	0.721	0.697
TNFAd intron 3 (GA)n	0.853	0.978	0.901
TNFRSF1B -196 M/R	0.741	0.847	0.867
IFNG intron 1 (CA)n	0.752	0.641	0.689
VDR intron 8 A/C	0.861	0.895	0.887
VDR exon 9 T/C	0.978	0.894	0.732
ESR1 intron 1 A/G	0.881	0.971	1.000
ESR1 intron 1 C/T	0.811	1.000	0.955

Table 4.4 P values for Hardy-Weinberg equilibrium analysis of the non-HLA polymorphism genotype frequencies in the Newcastle control population (n=100), HSCT patients (n=362) and unrelated donors (n=362). No significant difference was demonstrated between the observed and calculated expected frequencies (data not shown). IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

4.6 Correlation of Clinical Factors with Acute and Chronic GVHD

It is well established that certain clinical factors have a considerable impact on HSCT outcome, particularly GVHD (section 1.3.4) (Beatty *et al.* 1985; Atkinson *et al.* 1990; Gratwohl *et al.* 1998; Nichols *et al.* 2002; Hahn *et al.* 2008). These factors are therefore potential confounding variables that need to be accounted for when genetic association studies are performed in the HSCT setting. As a result of this, the effects of clinical factors on the development of acute and chronic GVHD in our unrelated donor HSCT cohort were assessed prior to polymorphism analysis.

Eight clinical factors were tested for associations with acute and chronic GVHD. These were patient and donor age at HSCT (over 40 years), gender mismatch transplantation (female to male), CMV status (patient and/or donor positivity), HLA matching,

conditioning regimen (low intensity/myeloablative), prophylaxis intensity (low/high) and graft manipulation (T cell replete/T cell deplete). The results of the univariate analyses are shown in Table 4.5. No significant associations were demonstrated with patient or donor age over 40 years, myeloablative conditioning, low intensity prophylaxis and T cell depletion. Thus, these factors had no impact on HSCT outcome in this study and as a result were not included in any further analyses. Significant correlations were however; demonstrated between GVHD and female to male transplantation, CMV positivity and HLA-mismatch. These three clinical factors were therefore identified as confounding variables and needed to be accounted for when associations between the non-HLA polymorphisms and HSCT outcome were examined using multivariate analysis.

	Acute GVHD		**Chronic GVHD**	
	P	**OR**	**P**	**OR**
Clinical Factors				
Patient age >40 years	0.146	0.657	0.233	0.542
Donor age >40 years	0.389	0.250	0.367	0.384
Female/male transplantation	**0.017**	5.698	**0.035**	4.072
CMV positivity	**0.047**	3.614	**0.050**	3.680
HLA-mismatch	**0.015**	5.786	**0.042**	3.937
Myeloablative conditioning	0.359	0.868	0.623	0.103
Low intensity prophylaxis	0.481	0.091	0.353	0.442
T cell depletion	0.172	0.535	0.100	0.710

Table 4.5 Univariate analysis demonstrating the associations between clinical factors and acute and chronic GVHD. P values showing a significant association (≤0.1) are shown in bold. CMV, cytomegalovirus; HLA, human leukocyte antigen; GVHD, graft-versus-host disease; P, P value; OR, odds ratio

4.7 Correlation of Non-HLA Polymorphisms with Acute and Chronic GVHD

Associations between the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms and the incidence and severity of acute GVHD were examined in the entire cohort of 362 unrelated donor HSCT patients. Associations

between these polymorphisms and the incidence of chronic GVHD were analysed in 286 patients alive greater than 100 days post-HSCT. The results of the univariate analyses are shown in Tables 4.6. No associations could be demonstrated between the non-HLA polymorphisms examined and the overall incidence of acute GVHD (grades I-IV) or the incidence of clinical acute GVHD (grades II-IV). A strong correlation was however; demonstrated between the *VDR* intron 8 A/C polymorphism and the development of severe acute GVHD (grades III-IV). HSCT patients possessing the *VDR* intron 8 A,A genotype were more susceptible to the higher grades of acute GVHD than patients with the A,C or C,C genotypes (41% versus 16% respectively, P=0.015 OR=5.786). A similar association was also demonstrated for the *IFNG* intron 1 (CA)n polymorphism. HSCT patients possessing the *IFNG* intron 1 (CA)n 3,3 genotype were more susceptible to the higher grades of acute GVHD than patients without the genotype (48% versus 27% respectively, P=0.037 OR=4.071). In terms of chronic GVHD, patients possessing the *IL6* -174 G,G genotype were more at risk of developing the condition than patients with the G,C or C,C genotypes (80% versus 56% respectively, P=0.010 OR=5.634).

The associations identified by univariate analyses were then tested for independence against confounding clinical factors using multivariate analysis. The clinical factors entered into the analyses where those shown in section 4.6 to have a significant impact on the development of GVHD in this cohort. The factors included were: female to male transplantation, CMV positivity and HLA-mismatch. Acute GVHD grades I-IV and PBSC transplants were also included in the multivariate analysis for chronic GVHD, as both are considerable risk factors for the development of the condition (Atkinson *et al.* 1990; Bensinger *et al.* 2001). The results of the multivariate analyses are shown in Table 4.7.

Following multivariate analyses, the previously demonstrated associations between patient *VDR* intron 8 A,A and *IFNG* intron 1 3,3 genotypes and severe acute GVHD (grades III-IV) and patient *IL6* -174 G,G genotype and chronic GVHD retained their significance (P=0.005 OR=7.991, P=0.007 OR=5.507 and P=0.007 OR=6.562 respectively). Thus, the observed associations are independent of established clinical risk factors for HSCT outcome. As thirteen genetic factors were examined in our unrelated donor HSCT cohort and an adequately sized independent second cohort does not currently exist to validate these findings, multiple comparisons corrections

(Bonferroni adjustment) were performed in an attempt to rule out spurious positive results. After correction, the associations demonstrated for the *VDR* intron 8 A,A, *IFNG* intron 1 (CA)n 3,3 and *IL6* -174 G,G genotypes remained, however, their significance had been substantially reduced (corrected P values; P=0.065, P=0.091 and P=0.091 respectively (trends)).

	Acute GVHD Grade							
	I-IV		II-IV		III-IV		Chronic GVHD	
	P	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**
Patient								
IL2 -330 G	0.455	0.199	0.440	0.125	0.253	0.860	0.243	0.801
IL4 -590 T	0.254	0.784	0.143	0.722	0.216	0.524	0.359	0.868
IL6 -174 G,G	0.525	0.034	0.379	0.319	0.413	0.255	**0.010**	5.634
IL10 -592 A	0.587	0.002	0.236	0.988	0.476	0.191	0.677	0.001
IL10 -1082 G	0.577	0.002	0.371	0.801	0.414	0.231	0.356	0.388
IL1RN VNTR 2	0.535	0.026	0.490	0.053	0.373	0.347	0.481	0.091
TNFAd (GA)n 3,3	0.124	0.667	0.354	0.450	0.197	0.941	0.600	0.003
TNFRSF1B -196 R,R	0.509	0.043	0.413	0.182	0.407	0.225	0.311	0.580
IFNG (CA)n 3,3	0.601	0.003	0.470	0.103	**0.037**	4.071	0.226	0.698
VDR intron 1 A,A	0.239	0.504	0.100	0.991	**0.015**	5.786	0.556	0.020
VDR exon 9 T/C	0.288	0.676	0.267	0.694	0.331	0.479	0.423	0.223
ESR1 intron 1 G	0.526	0.990	0.364	0.397	0.234	0.928	0.259	0.995
ESR1 intron 1 C	0.222	0.721	0.567	0.002	0.213	0.677	0.414	0.255
Donor								
IL2 -330 G	0.193	0.957	0.573	0.001	0.287	0.650	0.564	0.013
IL4 -590 T	0.353	0.398	0.490	0.055	0.210	0.579	0.467	0.122
IL6 -174 G,G	0.443	0.201	0.530	0.028	0.250	0.859	0.353	0.442
IL10 -592 A	0.606	0.001	0.188	0.813	0.203	0.601	0.564	0.013
IL10 -1082 G	0.563	0.004	0.290	0.713	0.250	0.859	0.242	0.511
IL1RN VNTR 2	0.249	0.813	0.522	0.022	0.531	0.023	0.467	0.139
TNFAd (GA)n 3,3	0.245	0.852	0.273	0.756	0.198	0.681	0.402	0.263
TNFRSF1B -196 R,R	0.419	0.190	0.568	0.002	0.303	0.867	0.623	0.103
IFNG (CA)n 3,3	0.292	0.594	0.113	0.920	0.391	0.260	0.264	0.960
VDR intron 8 A,A	0.148	0.603	0.300	0.058	0.499	0.069	0.469	0.126
VDR exon 9 C	0.447	0.161	0.616	0.006	0.575	0.003	0.452	0.261
ESR1 intron 1 G	0.491	0.069	0.371	0.801	0.544	0.019	0.527	0.057
ESR1 intron 1 C	0.500	0.060	0.530	0.028	0.213	0.511	0.580	0.016

Table 4.6 Univariate analysis for acute and chronic GVHD in the unrelated donor HSCT cohort (n=362). Significant associations (P≤0.01) are shown in bold. GVHD, graft-versus-host disease; P, P value; OR, odds ratio; IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

	P Value	Odds Ratio
Acute GVHD III-IV		
Female/male transplantation	**0.047**	3.614
CMV positivity	0.242	0.997
HLA-mismatch	**0.032**	3.832
Patient *VDR* intron 8 A,A	**0.005**	7.991
Patient *IFNG* intron 1 3,3	**0.007**	5.507
Chronic GVHD		
Female/male transplant	0.137	0.847
HLA-mismatch	**0.020**	5.393
PBSC Transplants	**0.005**	8.137
Acute GVHD I-IV	**0.001**	15.798
Patient *IL6* -174 G,G	**0.007**	6.562

Table 4.7 Multivariate analysis for severe acute (grades III-IV) and chronic GVHD in the unrelated donor HSCT cohort (n=362). Significant associations (P<0.05) are shown in bold. GVHD, graft-versus-host disease; CMV, cytomegalovirus; HLA, human leukocyte antigen; VDR, vitamin D receptor; IFNG, interferon γ; PBSC, peripheral blood stem cell; IL6, interleukin-6

Several clinical factors were also shown to be significantly associated with GVHD following multivariate analyses. Female to male transplantation and HLA-mismatch correlated with acute GVHD (P=0.047 OR=3.614 and P=0.032 OR=3.832 respectively), whilst HLA-mismatch, PBSC transplantation and acute GVHD grades I-IV were linked to chronic GVHD (P=0.020 OR=5.393, P=0.005 OR=8.137 and P=0.001 OR=15.798 respectively). These findings acted as a further control in this study, by validating the cohort analysed. As all five factors are well recognised clinical risks in HSCT (Atkinson *et al.* 1990; Gratwohl *et al.* 1998; Bensinger *et al.* 2001), demonstrated associations with GVHD would be expected in an unbiased cohort.

In an attempt to verify the genetic associations demonstrated, the *VDR* intron 8 A,A, *IFNG* intron 1 3,3 and *IL6* -174 G,G genotypes were correlated with GVHD in a second cohort. This population was a subset of the original unrelated donor transplant cohort that comprised the 10/10 HLA-matched HSCT patient/donor pairs only (n=248).

Again, patient *VDR* intron 8 A,A and *IFNG* intron 1 3,3 genotypes were significantly linked with the development of severe acute GVHD (grades III-IV, P=0.008 OR=6.979 and P=0.011 OR=5.801 respectively) and patient *IL6* -174 G,G genotype correlated with chronic GVHD (P=0.018 OR=5.799). Although significance was reduced considerably following multiple comparison corrections, confirmation of the results in this cohort seems to suggest that the associations demonstrated are real. Replication of the results in an independent cohort is however necessary to completely rule out associations by chance.

4.8 Correlation of Non-HLA Polymorphisms with Overall Survival

Overall survival was defined as the total amount of time (in months) each patient survived post-HSCT and was measured from the date of transplantation to the date of death or most recent clinic appointment. The relationship between the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms and this outcome was examined in the entire cohort of 362 unrelated donor HSCT patients. Following univariate analysis, a significant association was demonstrated with the *IL6* -174 G/C polymorphism. Patients with the *IL6* -174 G,G genotype were found to have a lower overall survival than those with the G,C or C,C genotype (P=0.096 OR=2.174). No correlations could be demonstrated for the remaining non-HLA polymorphisms examined (Table 4.8).

The association observed for the *IL6* -174 G,G genotype was then tested for independence against confounding clinical factors using multivariate analysis. The factors included were female to male transplantation, CMV positivity and HLA-mismatch (section 4.6). Severe Acute GVHD (grades III-IV) was also included in the multivariate analysis, as this is a major cause of death following HSCT (Vogelsang *et al.* 1988). The results of the multivariate analyses are shown in Table 4.9.

Following multivariate analysis, only acute GVHD demonstrated an association; HSCT patients with a severe form of the condition (grades III-IV) had a significantly lower overall survival than those with minimal or no acute GVHD (P<0.0001 OR=32.734;

Figure 4.1). Patient carriage of the *IL6* -174 G,G genotype was again found to associate with lower overall survival compared to patient carriage of the G,C or C,C genotype, however, significance was not retained (P=0.101 OR=0.979). It therefore seems unlikely that the *IL6* -174 G/C polymorphism is an independent risk factor for overall survival. Although, testing of this variable in a larger cohort may strengthen this association and allow it to be retained following multivariate analysis.

	Overall Survival (months)	
	P value	**Odds Ratio**
Patient		
IL2 -330 G	0.248	0.895
IL4 -590 T	0.201	0.589
IL6 -174 G,G	**0.096**	2.174
IL10 -592 A	0.479	0.066
IL10 -1082 G	0.278	0.651
IL1RN intron 2, VNTR 2	0.328	0.425
TNFAd intron 3 (GA)n 3,3	0.399	0.212
TNFRSF1B -196 R,R	0.272	0.833
IFNG intron 1 (CA)n 3,3	0.241	0.822
VDR intron 8 AA	0.434	0.168
VDR exon 9 C	0.520	0.033
ESR1 intron 1 G	0.455	0.105
ESR1 intron 1 C	0.256	0.829
Donor		
IL2 -330 G	0.312	0.495
IL4 -590 T	0.515	0.044
IL6 -174 G,G	0.398	0.413
IL10 -592 A	0.569	0.188
IL10 -1082 G	0.285	0.625
IL1RN intron 2, VNTR 2	0.284	0.598
TNFAd intron 3 (GA)n 3,3	0.324	0.427
TNFRSF1B -196 R,R	0.445	0.128
IFNG intron 1 (CA)n 3,3	0.293	0.558
VDR intron 8 AA	0.487	0.056
VDR exon 9 C	0.595	0.054
ESR1 intron 1 G	0.257	0.731
ESR1 intron 1 C	0.305	0.515

Table 4.8 Univariate analysis for overall survival in the unrelated donor HSCT cohort (n=362 patient/donor pairs). Significant associations ($P \leq 0.01$) are shown in bold. IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

	P Value	Odds Ratio
Overall Survival		
Female/male transplantation	0.297	0.475
CMV positivity	0.453	0.217
HLA-mismatch	0.211	0.543
Acute GVHD III-IV	**<0.0001**	32.734
Patient *IL6* -174 G,G	0.101	0.979

Table 4.9 Multivariate analysis for overall survival. Significant associations (P<0.05) are shown in bold. CMV, cytomegalovirus; HLA, human leukocyte antigen; GVHD, graft-versus-host disease; IL6, interleukin-6

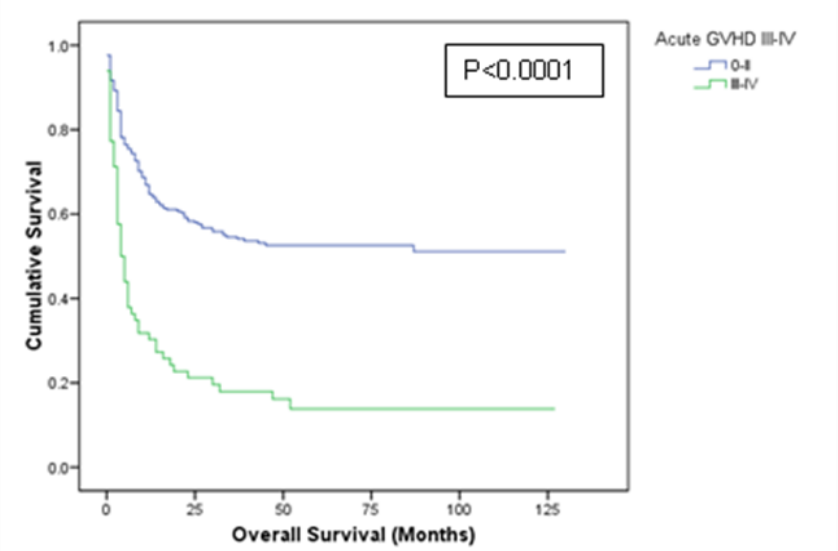

Figure 4.1 Overall Survival analysis using Kaplan-Meier curves and the Log-Rank test. A significant correlation was demonstrated between severe acute GVHD (grades III-IV) and decreased overall survival. P, P value; GVHD, graft-versus-host disease

4.9 Correlation of Non-HLA Polymorphisms with Relapse Incidence

Relapse was defined as the reoccurrence of an original disease following a period of treatment. As aplastic anaemia is a non-malignant disease, patients transplanted for this were not included in any analyses correlating non-HLA polymorphisms with relapse. Consequently, 8 patient/donor transplant pairs were excluded from this analysis and associations between the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms and relapse incidence were examined in 105 unrelated donor HSCT patients. Following univariate analysis, no correlations were demonstrated in either of the patient or donor groups (Table 4.10).

To determine whether the absence of any significant associations was related to the unrelated donor HSCT cohort employed, established clinical risk factors for disease relapse were analysed. Both animal and human studies have demonstrated that acute and chronic GVHD lower the incidence of relapse via the GVL effect (Weiden *et al.* 1979; Weiden *et al.* 1981; Horowitz *et al.* 1990), as a result the absence of these conditions constitute considerable risk factors. Consequently, acute and chronic GVHD was examined in our cohort. The absence of both factors was found to be significantly associated with an increased risk of relapse (no acute GVHD P=0.002, no chronic GVHD P=0.010). Thus, the unrelated donor HSCT cohort employed in this study was not responsible for the lack of associations between the non-HLA polymorphisms examined and the incidence of relapse.

	Relapse Incidence	
	P value	**Odds Ratio**
Patient		
IL2 -330 G	0.146	0.674
IL4 -590 T	0.389	0.250
IL6 -174 G,G	0.367	0.348
IL10 -592 A	0.233	1.004
IL10 -1082 G	0.370	0.308
IL1RN intron 2, VNTR 2	0.295	0.624
TNFAd intron 3 (GA)n 3,3	0.173	0.931
TNFRSF1B -196 R,R	0.455	0.199
IFNG intron 1 (CA)n 3,3	0.580	0.031
VDR intron 8 A,A	0.383	0.512
VDR exon 9 C	0.240	0.933
ESR1 intron 1 G	0.201	0.985
ESR1 intron 1 C	0.134	0.724
Donor		
IL2 -330 G	0.401	0.202
IL4 -590 T	0.296	0.605
IL6 -174 G,G	0.571	0.008
IL10 -592 A	0.528	0.160
IL10 -1082 G	0.548	0.008
IL1RN intron 2, VNTR 2	0.371	0.283
TNFAd intron 3 (GA)n 3,3	0.585	0.001
TNFRSF1B -196 R,R	0.267	0.694
IFNG intron 1 (CA)n 3,3	0.401	0.202
VDR intron 8 A,A	0.431	0.196
VDR exon 9 C	0.441	0.158
ESR1 intron 1 G	0.616	0.006
ESR1 intron 1 C	0.620	0.005

Table 4.10 Univariate analysis for relapse incidence in the unrelated donor HSCT cohort (n=105 patient/donor pairs). No significant associations were demonstrated in either the patient or the donor group. IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

4.10 Correlation of Non-HLA Polymorphisms with TRM

TRM was defined as death following transplantation, from all causes other than disease relapse. The relationship between this outcome and the *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFAd* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T polymorphisms was examined in 249 unrelated donor HSCT patients. Following univariate analysis, no correlations were demonstrated in either of the patient or donor groups (Table 4.11).

As with relapse incidence, a clinical risk factor for TRM was analysed in order to confirm that the absence of any associations was not related to our unrelated donor HSCT cohort. Gender mismatched transplantation is a well recognised risk, with TRM being significantly increased in male recipients of female donor stem cells (Zwaan *et al.* 1989; Gratwohl *et al.* 2001). Consequently, female to male transplantation was examined in this study. A significant link with TRM was observed (P=0.001). Thus, our inability to demonstrate any associations between the non-HLA polymorphisms examined and TRM in this study was not cohort related.

	TRM	
	P value	**Odds Ratio**
Patient		
IL2 -330 G	0.531	0.023
IL4 -590 T	0.175	0.563
IL6 -174 G,G	0.250	0.859
IL10 -592 A	0.156	0.647
IL10 -1082 G	0.100	0.691
IL1RN intron 2, VNTR 2	0.253	0.860
TNFAd intron 3 (GA)n 3,3	0.147	0.734
TNFRSF1B -196 R,R	0.531	0.023
IFNG intron 1 (CA)n 3,3	0.476	0.191
VDR intron 8 A,A	0.414	0.231
VDR exon 9 C	0.373	0.347
ESR1 intron 1 G	0.216	0.829
ESR1 intron 1 C	0.346	0.575
Donor		
IL2 -330 G	0.546	0.022
IL4 -590 T	0.567	0.019
IL6 -174 G,G	0.583	0.010
IL10 -592 A	0.243	0.716
IL10 -1082 G	0.359	0.868
IL1RN intron 2, VNTR 2	0.654	0.002
TNFAd intron 3 (GA)n 3,3	0.115	0.973
TNFRSF1B -196 R,R	0.141	0.685
IFNG intron 1 (CA)n 3,3	0.335	0.989
VDR intron 8 A,A	0.564	0.013
VDR exon 9 C	0.530	0.738
ESR1 intron 1 G	0.489	0.086
ESR1 intron 1 C	0.309	0.516

Table 4.11 Univariate analysis for TRM in the unrelated donor HSCT cohort (n=249 patient/donor pairs). No significant associations were demonstrated in either the patient or the donor group. IL2, interleukin-2; IL4, interleukin-4; IL6, interleukin-6; IL10, interleukin-10; IL1RN, interleukin-1 receptor antagonist; TNFA, tumour necrosis factor α; IFNG, interferon γ; VDR, vitamin D receptor; ESR1, oestrogen receptor

4.11 Discussion

Polymorphisms in the genes of IL-2 (-330 T/G), IL-4 (-590 C/T), IL-6 (-174 G/C), IL-10 (-592 A/C, -1082 G/A), IL-1Ra (intron 2 VNTR), TNFα (d intron 3 (GA)n), TNFRII (-196 M/R), IFNγ (intron 1 (CA)n), vitamin D receptor (intron 8 A/C, exon 9 T/C) and oestrogen receptor (intron 1 A/G, intron 1 C/T) have been shown to have a substantial influence on HSCT outcome, associating with both GVHD and survival after HLA-matched sibling HSCT (Middleton *et al.* 1998; Cavet *et al.* 1999; Cavet *et al.* 2001; Cullup H 2001; Socie *et al.* 2001; Middleton *et al.* 2002; Rocha V 2002; Lin *et al.* 2003; Middleton *et al.* 2003; Stark *et al.* 2003; Mlynarczewska *et al.* 2004; Bogunia-Kubik *et al.* 2005b; Karabon *et al.* 2005; Rocha *et al.* 2009). Consequently, these non-HLA polymorphisms were correlated with HSCT outcome in our unrelated donor transplant cohort. While no associations were observed with overall survival, relapse incidence and TRM, significant correlations were demonstrated with the development of acute and chronic GVHD.

Polymorphism analysis demonstrated significant associations between *VDR* intron 8 A/C and *IFNG* intron 1 (CA)n and acute GVHD in this study. Transplant patients possessing the *VDR* intron 8 A,A or the *IFNG* intron 1 (CA)n 3,3 genotypes were found to be much more susceptible to the development of severe acute GVHD (grades III-IV) than those without these genotypes. These associations were observed in the whole HSCT cohort (n=362, P=0.015 OR=5.786 and P=0.037 OR=4.071 respectively), a smaller validation cohort that consisted of the 10/10 HLA-matched unrelated donor HSCT patient/donor pairs (n=248, P=0.008 OR=6.979 and P=0.011 OR=5.801 respectively) and when established clinical risk factors for acute GVHD were entered into the analysis (P=0.005 OR=7.991 and P=0.007 OR=5.507 respectively). Although somewhat reduced, these associations also retained their significance (trend) following multiple comparison corrections (Bonferroni adjustment: P=0.065 and P=0.091 respectively). It therefore seems likely that the *VDR* intron 8 A/C and *IFNG* intron 1 (CA)n polymorphisms are independent risk factor for severe acute GVHD (grades III-IV) following unrelated donor HSCT.

The observed association with the *IFNG* intron 1 (CA)n 3,3 genotype has also been demonstrated by our research group in a cohort of HLA-matched sibling transplants (Cavet *et al.* 2001). As the 3,3 genotype has previously been linked to lower *in vitro*

IFNγ production following mitogen stimulation (Pravica *et al.* 1999), an association with GVHD was unexpected. However, it may be explained if IFNγ had a negative feedback regulatory role, as seen in some mouse models of GVHD. Weekly injections of IFNγ in a subacute murine model of GVHD prevents the condition and increases survival, whereas mice receiving stem cells from IFNγ knockout donors have been shown to develop accelerated lethal GVHD (Brok *et al.* 1997). An alternative explanation is that *in vivo* stimulation by HSCT conditioning regimens may have a different relationship to the *IFNG* intron 1 (CA)n polymorphism than *in vitro* stimulation by mitogens, as the network of cytokine interactions initiating acute GVHD is much more complex than direct *in vitro* T-cell activation (Cavet *et al.* 2001). A more recent study correlating the *IFNG* inton 1 (CA)n 3,3 genotype with an increased susceptibility to viral reactivation following HSCT may offer another explanation for the association with acute GVHD (Bogunia-Kubik *et al.* 2005a). As IFNγ plays a key role in anti-microbial and anti-viral immunity, the low IFNγ expression associated with *IFNG* intron 1 (CA)n 3,3 genotype may result in a decreased ability to fight and abolish infections and an increased susceptibility to pathogens, particularly viruses. It is therefore possible that the immune response produced against such infections could exacerbate GVHD (Bogunia-Kubik *et al.* 2005a). The correlation of skin and bowel manifestation of GVHD with herpes virus (CMV) reactivation seems to supports this theory (Yoshikawa *et al.* 2001; Holler 2002).

Patient carriage of the *VDR* intron 8 A,A genotype was also shown to associate with an increased susceptibility to severe acute GVHD (grades III-IV) in this study. The finding conflicts with that of Middleton *et al* (2002) and Bogunia-Kubik *et al.* (2008), who have reported an inverse association between the *VDR* intron 8 A,A genotype and the development of severe acute GVHD (grades III-IV). The reason for the conflicting data is currently unknown, but may reflect differences in cohort size; the current study examined the *VDR* intron 8 A/C polymorphism in 362 HSCT patients and donors, whereas Middleton *et al* (2002) and Bogunia-Kubik *et al.* (2008) employed cohorts of only 80 and 123 respectively. However, as the findings of the latter studies were demonstrated in HLA-matched sibling HSCT cohorts, it is also possible that the conflicting data reflects fundamental differences between HLA-matched sibling and unrelated donor transplantation. This is further supported by the observation that very few of the associations previously reported in the HLA-matched sibling setting have

been demonstrated in this study. In comparison to HLA-matched sibling HSCT, unrelated donor transplantation is associated with a greater degree of HLA disparity, has an increased risk of GVHD and other complications and is typically treated with higher intensity conditioning regimens and prophylaxis strategies (de Lima and Champlin 2001; Petersdorf *et al.* 2001); consequently there is a substantial difference in transplant biology between the two HSCT types. As a result, it is extremely unlikely that all genetic associations will exist in both transplant settings. In terms of future studies, it would therefore be sensible to analyse non-HLA polymorphisms in independent HLA-matched sibling and unrelated donor HSCT cohorts. Differences in transplant biology does not however explain the inability of this study to replicate the associations previously reported for the *IL2* -330 T/G, *ILRN* intron 2, *IL10* -592 A/C, *IL10* -1082 G/A, *TNFAd* intron 3 (GA)n and *TNFRSF1B* -196 M/R polymorphisms in the unrelated donor HSCT setting (Ishikawa *et al.* 2002; MacMillan *et al.* 2003a; MacMillan *et al.* 2003b; Kean *et al.* 2004; Bettens *et al.* 2006). Differences in the size, degree of heterogeneity and the ethnicity of the populations examined may be possible explanations.

Patient treatment could be another explanation for conflicting data, as the type of conditioning and prophylaxis used depends greatly on the transplant type, the age and condition of the patient and the stage of the disease at the time of HSCT (Morris and Hill 2007), consequently protocols employed differ from study to study. Due to the immunosuppressive rather than damaging nature of low intensity regimens, patients conditioned with these milder strategies are likely to produce completely different non-HLA polymorphism data, particularly in terms of GVHD, to those conditioned by myeloablation. The therapeutic effects of transplant drugs may also have a substantial impact on the results of genetic association studies. In this current study 61% of the transplant patients received stem cell grafts that had been treated with ATG or Campath-1H. Both of these drugs reduce the incidence of post-transplant GVHD by depleting T cells (Chakrabarti *et al.* 2004; Van Lint *et al.* 2006). The loss of this cell type is likely to have had a considerable affect on cytokine production during transplantation and subsequently on the genetic associations observed. The administration of steroids such as methylprednisone and prednisolone may also have a considerable effect on genetic association studies, as the anti-inflammatory actions of these drugs reduce the severity and even abolish GVHD.

The non-HLA polymorphism analysis performed in this study also demonstrated a significant association between *IL6* -174 G/C and chronic GVHD. Transplant patients possessing the *IL6* -174 G,G genotype were more at risk of developing the condition than those with the G,C or C,C genotypes. This association was again observed in the whole HSCT cohort (n=362, P=0.010 OR=5.634), the smaller validation cohort that consisted of the 10/10 HLA-matched unrelated donor HSCT patient/donor pairs (n=248, P=0.018 OR=5.799) and when established clinical risk factors for chronic GVHD were entered into the analysis (P=0.007 OR=6.562). Although substantially reduced, this association also retained its significance (trend) following multiple comparison corrections (Bonferroni adjustment, P=0.091). It therefore seems likely that the *IL6* -174 G/C polymorphism is an independent risk factor for chronic GVHD following unrelated donor HSCT.

Several studies have been undertaken to assess the role of IL-6 in chronic GVHD and the evidence appears to be consistent. IL-6 serum levels have been shown to be higher post-transplant in patients with chronic GVHD than in those without the condition (Imamura *et al.* 1994) (Barak *et al.* 1995) and the onset of extensive chronic GVHD has been shown to coincide with an increase in IL-6 mRNA expression (Tanaka *et al.* 1996). The involvement of this cytokine in chronic GVHD is also supported by polymorphism data. In HLA-matched sibling studies the relative risk for the condition has been shown to be significantly higher for patients with the *IL6* -174 G,G genotype (Cavet *et al.* 2001; Socie *et al.* 2001). The association demonstrated for *IL6* -174 G,G in this study therefore agrees with the previously published results and provides further support for the role of IL-6 in chronic GVHD. Although only a trend, the link between *IL6* -174 G,G and decreased overall survival demonstrated following univariate analysis in this study, also seems to substantiate the association between the *IL6* -174 G,G genotype and chronic GVHD; as the condition is still the major cause of late mortality following HSCT (Horwitz and Sullivan 2006).

In unrelated donor HSCT the HLA system is of great importance, as complete matching between patients and their unrelated donors is extremely low and the risk of GVHD and post-transplant mortality increases dramatically with single mismatches at HLA-A, -B, -C, -DRB1 or -DPB1 (Sasazuki T 1998; Flomberg *et al.* 2004; Chalandon *et al.* 2006; Lee *et al.* 2007; Shaw *et al.* 2007). It is therefore essential that HLA disparity is accounted for in genetic association studies performed in this setting. As no standard

method currently exists, previously published studies have employed different approaches, Ishikawa *et al* (2002) examined the *TNFA* and *TNFRSF1B* polymorphisms in HLA-A, -B and –DRB1 matched cases only, whilst Bettens *et al* (2006) analysed the *TNFA* and *IL10* polymorphisms initially in a cohort containing matched and mismatched transplants and then in a 10/10 HLA-matched sub-group. Other studies, however, have not accounted for HLA disparity at all (MacMillan *et al.* 2003a; MacMillan *et al.* 2003b; Remberger *et al.* 2003; Kean *et al.* 2004). These differences in methodology may therefore be another explanation for conflicting data. HLA disparity was accounted for in this study using a similar approach to that of Bettens *et al* (2006). As the associations demonstrated between the *VDR* intron 8 A/C, *IFNG* intron 1 (CA)n and *IL6* -174 G/C polymorphisms and GVHD were observed in the whole unrelated donor HSCT cohort and the 10/10 HLA-matched subset and were demonstrated to be independent of HLA-mismatching in all multivariate analyses, it is likely that the method employed adequately accounted for the effects of HLA disparity and the associations demonstrated in this study are real.

Although the data presented implicates the *VDR* intron 8 A/C, *IFNG* intron 1 (CA)n and *IL6* -174 G/C polymorphisms as independent risk factor for the development of GVHD, the unrelated donor HSCT cohort employed in this study was heterogeneous and due to number constraints all potential confounding variable could not be accounted for. It would therefore be of interest to examine the non-HLA polymorphisms in a larger independent cohort, as this would provide evidence of reproducibility, which is often lacking in disease association studies (Colhoun *et al.* 2003), and would allow the heterogeneous nature of HSCT populations to be addressed more effectively. Larger cohorts would permit the inclusion of a wider range of transplant parameters, such as stem cell source, underlying disease and stage of disease and would allow the thorough investigation of non-HLA polymorphisms in more homogeneous disease, treatment and transplant specific groups. Genetic factors identified and confirmed in large studies have implications for the inclusion, alongside clinical risk factors in a prognostic index for the prediction of GVHD prior to transplantation. Successful prediction would allow the identification of at risk patients, improve donor selection and allow post-transplant therapies to be tailored to an individual. Such an analysis would be extremely advantageous; particularly in the unrelated donor HSCT setting, were the risk of GVHD is greatly increased and several potential donors are often identified (Anasetti *et al.* 1995).

Chapter 5. The Role of IL-13 in GVHD: Genotyping and Functional Studies

IL-13 is a 10 KDa immunoregulatory cytokine that was first described in 1989 as P600, a protein cloned from a murine Th-2 cell-line (Brown *et al.* 1989). It is an unglycosylated protein product of 132 amino acids that is secreted predominantly by activated Th2 $CD4^+$ T cells (Brown *et al.* 1989; de Vries 1996). However, B cells, mast cells, basophils, NK cells and DCs also produce this cytokine (Burd *et al.* 1995; Gibbs *et al.* 1996; Li *et al.* 1996; de Saint-Vis *et al.* 1998; Hoshino *et al.* 1999). IL-13 mediates its effects through the type II IL-4 receptor that is expressed on a broad range of haematopoietic and non-haematopoietic cells types; except for T cells (Zurawski and de Vries 1994; Bochner *et al.* 1995; Murata *et al.* 1998; Li *et al.* 1999; Wills-Karp 2001). This receptor is composed of an IL-4 receptor chain (IL-4Rα) and an IL-13 receptor chain (IL-13Rα1) and can bind both of these cytokines (Aman *et al.* 1996; Hilton *et al.* 1996). Accordingly, IL-13 has biological activities very similar to those of IL-4.

The *IL13* gene has been mapped to chromosome 5q31-33 within the 'Th2-cytokine cluster'. It is located 12kb upstream of the gene encoding *IL4* and in close proximity to the *IL3, IL5, IL9* and *CSF2* genes (McKenzie *et al.* 1993). The 3950bp *IL13* gene is comprised of 4 exons and 3 introns and has been demonstrated to be highly polymorphic. 31 SNPs have been identified to date (Tarazona-Santos and Tishkoff 2005). An arginine (G)→glutamine (A) transition in exon 4 at position +2044 is however the most common, occurring in approximately 25% of the population. It is the only non-synonymous polymorphism within the *IL13* gene and results in the replacement of an arginine residue with a glutamine at position 130 (Heinzmann *et al.* 2000; Vladich *et al.* 2005). Mutational and nuclear magnetic resonance studies have shown *IL13* +2044 G/A to be located in the C-terminus of the IL-13 molecule, an area which has been implicated in interaction with the IL-13 receptor chain (Horie *et al.* 1997; Lentsch *et al.* 1997; Madhankumar *et al.* 2002). Thus, this polymorphism maps to a region which is potentially critical for the functional properties of the cytokine (Vercelli 2002). *IL13* +2044 G/A has been widely studied for disease associations, with the A allele being correlated with inflammatory and allergy phenotypes, including asthma (Heinzmann *et al.* 2000; Arima *et al.* 2002; Heinzmann *et al.* 2003; Vladich *et*

al. 2005), atopy (He *et al.* 2003), atopic dermatitis (Liu *et al.* 2000; Tsunemi *et al.* 2002) and food sensitization (Zitnik *et al.* 2009).

To date no studies examining the *IL13* +2044 G/A polymorphism in the HSCT setting have been published. However, IL-13 expression has been correlated with transplant outcome. Elevated IL-13 production by allo-reactive donor T cells has been demonstrated to associate with acute GVHD in humans (Jordan *et al.* 2004), whilst high IL-13 expression has been shown to attenuate the condition in murine models (Hildebrandt *et al.* 2007). In order to establish if IL-13 plays a role in the pathogenesis of GVHD, the *IL13* +2044 G/A polymorphism and *in vitro* and *in vivo* IL-13 expression will be examined in a HSCT cohort.

5.1 Study Aims

i) To establish and compare the allele and genotype frequencies of the *IL13* +2044 G/A polymorphism in a Newcastle control population with a HSCT cohort.

ii) To correlate HSCT outcome, in terms of acute GVHD incidence and severity, chronic GVHD incidence, overall survival, relapse incidence and TRM with the *IL13* +2044 G/A polymorphism by univariate and multivariate statistical analyses.

iii) To examine the role of *in vitro* and *in vivo* IL-13 expression in acute and chronic GVHD in a cohort of Newcastle HSCT patients.

iv) To determine whether IL-13 expression varies with the *IL13* +2044 G/A polymorphism in a cohort of Newcastle HSCT patients and donors.

5.2 Statistical Analysis

IL13 +2044 allele and genotype frequencies calculated for the HSCT cohort were compared to those of Newcastle controls using the chi-square (χ^2) and Fisher's exact test where appropriate. Expected and observed *IL13* +2044 genotype frequencies for both cohorts were assessed for Hardy-Weinberg equilibrium using the chi-square (χ^2) test. The *IL13* +2044 G/A polymorphism was correlated with acute and chronic GVHD using the chi-square (χ^2) test and forward stepwise logistic regression. Overall survival was analysed using Kaplan-Meier curves and the Log-Rank test, followed by Cox regression. The incidence of relapse and TRM were analysed using cumulative incidence curves and the Log-Rank test (competing risk analysis).

The distribution of IL-13 levels in MLC supernatants and mitogen stimulated supernatants were tested for normality using the Kolmogorov-Smirnov test. IL-13 expression in these sample cohorts did not fulfil the criteria for a Gaussian (normal) distribution. Consequently, the data were analysed using non-parametric methods. The Mann-Whitney U test was employed when comparing two groups and the Kruskal-Wallis test was utilised when three or more groups were present. Correlation calculations were performed using the Spearman's Rank Correlation test.

The sample sizes for all comparisons made with respect to IL-13 expression in serum samples were small (n=30), thus a normality test was not required and the data was assumed to have a non-Gaussian distribution. The relationship between IL-13 expression and acute or chronic GVHD was therefore examined using a non-parametric method; Mann-Whitney U test.

5.3 Experimental Design

5.3.1 *Inclusion and Exclusion Criteria*

IL13 Genotyping Study

All patients and their donors recruited as part of a European Commission FP5 biotechnology project (Eurobank QLRI-CT-2000-00010, PI Professor A M Dickinson), that were transplanted between November 1983 and December 2006 and had DNA and 1 year's clinical follow-up available, were included in the analyses. The study was given JLEC approval (Appendix B) and informed consent was obtained from all patients and donors. Exclusion criteria were as follows:

i) Transplantation not performed
ii) Incomplete HSCT outcome data
iii) Incomplete HLA typing data
iv) Below the age of 16 at the time of transplant
v) Transplantation for an immunological disease
vi) Transplantation with a source other than PBSC or bone marrow
vii) Two or more transplants performed

Once the inclusion and exclusion criteria had been taken into account, the HSCT cohort employed in the genotyping study had been reduced from 721 patient/donor pairs to 600.

IL-13 Functional Study

All patients and their donors recruited as part of a European Commission FP5 biotechnology project (Eurobank QLRI-CT-2000-00010, PI Professor A M Dickinson), that were transplanted between November 1983 and December 2006; with *IL13* +2044 G/A genotyping data plus MLC supernatants, GVHR grades and 1 year's clinical follow-up available, were included in the analyses. The study was given JLEC approval (Appendix B) and informed consent was obtained from all patients and donors. Exclusion criteria were the same as those employed for the genotyping study.

Once the inclusion and exclusion criteria had been taken into account, the cohort utilised in the functional study consisted of 93 Newcastle HSCT patient/donor pairs.

5.3.2 *Control Measures*

For all samples, successful *IL13* PCR amplification was confirmed by agarose gel electrophoresis prior to PCR-RFLP analysis (section 3.6.2). Accurate ascertainment of *IL13* +2044 G/A alleles was ensured by the inclusion of both homozygous and heterozygous control samples of known genotype in all PCR reactions, PCR-RFLP analyses and electrophoresis gels (section 3.6.6). Genotypes were determined with reference to these known samples and verified by an independent observer. In equivocal cases, samples were re-analysed (less than 5% of the cohort). All *IL13* +2044 G/A genotypes were determined whilst blinded to clinical outcome. Correct allele determination was also ensured by the inclusion of negative controls, appropriate DNA size markers and by re-typing 25% of each cohort tested.

In all MLC cultures and mitogen stimulations, IL-13 expression was determined in the experimental sample and its corresponding control. The specific controls employed for each method are described in sections 3.9 and 3.11. In order to demonstrate that any variation in *in vivo* IL-13 expression in HSCT patients was transplant or outcome related rather than a naturally occurring phenomenon, IL-13 serum levels in patients and controls were compared prior to any correlation with clinical outcome. All ELISAs and CBAs performed (sections 3.13 and 3.14) included an appropriate standard dilution series and a negative control. ELISA samples and standards were tested in duplicate and the mean value for each set was used for quantification. Samples were re-assayed if the values for the two replicates varied by more than 15%. All functional studies were performed whilst blinded to clinical outcome.

5.3.3 *Method of Analysis*

Prior to any correlation with clinical outcome, the accuracy of *IL13* +2044 G/A determination in the HSCT cohort was assessed by the comparison of calculated allele and genotype frequencies with those of a Newcastle control group and previously

published data. In order to rule out selection bias and genotype deviation, patient, donor and control genotype frequencies were also tested for Hardy-Weinberg equilibrium.

HSCT patient and donor *IL13* +2044 G/A alleles were then individually correlated with the incidence and severity of acute GVHD, incidence of chronic GVHD and overall survival using univariate analysis methods (chi-square test (χ^2), Kaplan-Meier curves and Log-Rank test). Alleles with P values $\leq$0.1 were each tested for independence against identified confounding clinical factors using multivariate analysis methods (forward stepwise logistic and Cox regression). In order to retain adequate power, only the strongest factors, up to a maximum of 5, were entered into all multivariate analyses. P values <0.05 were considered statistically significant and values between 0.05 and 0.1 were considered indicative of a trend.

IL13 +2044 G/A alleles were also correlated with relapse incidence and TRM. As both of these outcomes are affected by other clinical events, competing risk analysis methods were required. These methods allow all possible associations to be observed by excluding the relevant competing event (relapse or TRM). Univariate analysis (cumulative incidence curves and Log-Rank test) alone was performed in this study (section 4.3.3). Again, P values <0.05 were considered statistically significant and values between 0.05 and 0.1 were considered indicative of a trend.

HLA disparity and unrelated donor transplants are well established risk factors in HSCT, particularly for GVHD; where the risk of the condition increases in parallel with the number of mismatched HLA loci (Beatty *et al.* 1985; Petersdorf *et al.* 1998; Sasazuki T 1998). As our cohort included both HLA-matched sibling and unrelated donor transplants, these factors could have had a substantial impact on outcome in this study. Thus, prior to any analyses with the *IL13* +2044 G/A polymorphism, HLA disparity and transplant type were tested for associations with GVHD. Both HLA-mismatched transplants and unrelated donor transplants were found to correlate with a higher incidence of acute and chronic GVHD in this study (acute GVHD P=0.073 and P=0.067 respectively; chronic GVHD P=0.098 and P=0.077 respectively). The most accurate and reliable means of accounting for such associations involves stratification of the cohort. However, this was not possible for HLA disparity, as the mismatched group would be too small to analyse (n=102). Instead, HLA-mismatch was entered into all multivariate analyses as a confounding factor. Cohort stratification was possible for

transplant type. Consequently, *IL13* +2044 G/A alleles were correlated with HSCT outcome in the whole cohort (n=600) and then in HLA-matched sibling (n=342) and unrelated donor transplant (n=258) sub-groups. Due to the heterogeneity of the cohort, stratification should also be considered for clinical factors such as underlying disease, disease stage (early/advanced), conditioning regimen (low intensity/myeloablative), prophylaxis intensity (low/high) and graft manipulation (T cell replete/T cell deplete). However, due to number constraints and as these factors had no impact on HSCT outcome in this study, stratification according to these factors was not performed.

In an attempt to address the concerns about the proliferation of genetic association studies, especially those in which results have not been replicated (Colhoun *et al.* 2003), any significant correlation observed in this study was verified in a smaller second cohort (n=300). This cohort consisted of 50% of the original HSCT population selected using the random assignment function in SPSS 15.0. This cohort was not independent and therefore not ideal; however, it was the most appropriate means of verification available. Time constraints did not permit the formation of a completely independent population of adequate size (power). Sample collection for the creation of such a verification cohort is currently being undertaken.

The role of IL-13 in acute and chronic GVHD was further investigated via functional analysis of IL-13 expression. *In vitro* expression was correlated with the incidence and severity of acute GVHD; in both the clinical setting and in an *in vitro* model, and with chronic GVHD. While *in vivo* IL-13 expression was correlated with clinical acute and chronic GVHD alone. The impact of the *IL13* +2044 G/A polymorphism on IL-13 expression was also examined. Although the primary HSCT outcome investigated in this study was GVHD, IL-13 expression was also correlated with overall survival, disease relapse and TRM where possible. In all analyses, P values <0.05 were considered statistically significant and values between 0.05 and 0.1 were considered indicative of a trend.

The HSCT cohort employed in this functional study also contained HLA-matched sibling and unrelated donor transplants. However, due to the small nature of the population (n=93), cohort stratification was not possible. Instead, the three HLA-mismatched unrelated donor transplants in the original HSCT cohort were excluded from the study and IL-13 expression was examined in the remaining mixed population

(n=90; 62 HLA-matched sibling, 28 unrelated donor). There was no significant difference in the incidence of acute or chronic GVHD between the HLA-matched sibling and the unrelated donor transplants in this cohort (P=0.927 and P=0.842 respectively). Sample size also prevented the stratification of this heterogeneous cohort according to underlying disease, disease stage (early/advanced), conditioning regimen (low intensity/myeloablative), prophylaxis intensity (low/high) and graft manipulation (T cell replete/T cell deplete).

5.4 Correlation of the *IL13* +2044 G/A Polymorphism with HSCT Outcome

5.4.1 *Study Population*

HSCT Cohort

Genomic DNA was obtained from 600 allogeneic HSCT patients and their donors for genotyping of the *IL13* +2044 G/A polymorphism. This was an adult cohort (≥16 at the time of transplant) comprising 342 HLA-matched sibling transplants and 258 unrelated donor transplants. HLA matching between patients and donors was performed by high resolution DNA-based typing for HLA-A, B, C, DRB1 and DQB1. Complete matching at all 5 loci was observed in 498 (83%) transplant pairs.

Cohort characteristics:
The 600 HSCT patients included in this cohort were transplanted between November 1983 and December 2006 at clinical centres in Austria, England, France, Germany and Spain. All patients and donors were recruited and consented as part of the previously described 'Eurobank Project' (section 5.3.1).

Acute GVHD was deemed assessable in those alive ≥30 days post-transplant and developed in 392 patients. 208 patients had no acute GVHD (grade 0), 116 grade I, 165 grade II, 67 grade III and 44 had grade IV. Acute GVHD grades III and IV were considered severe. Chronic GVHD was assessable from 100 days post-transplant in 463 patients. 250 of these patients developed the condition. All transplant patients were prepared for HSCT with low intensity (n=221) or myeloablative conditioning (n=370) regimens and 34% (n=202) were transplanted with T cell depleted stem cell grafts. 462 patients were given high intensity GVHD prophylaxis (CsA plus MTX or MM), whilst 138 received CsA monotherapy. The survival rate for this cohort was 49% (n=297) and causes of death included disease relapse, infection and GVHD. Table 5.1 shows the demographic characteristics of the HSCT cohort examined in this aspect of the study.

Table 5.1 Characteristics of the HSCT cohort (n=600).
CMV, cytomegalovirus; CsA, cyclosporine A; MTX, methotrexate; MM, mycophenolate mofetil; TCD, T cell depletion

TRANSPLANT PATIENTS		
Median Age		40 years (range 16-67)
		Number in each group
Gender		
	Male	359
	Female	241
	Female to Male HSCT	130
CMV Status		
	Positive	309
	Negative	291
Underlying Disease		
	Acute Lymphoblastic Leukaemia	78
	Acute Myeloid Leukaemia	196
	Chronic Lymphocytic Leukaemia	11
	Chronic Myeloid Leukaemia	129
	Hodgkin disease	14
	Non-Hodgkin Lymphoma	73
	Myeloproliferative Disorders	44
	Plasma Cell Neoplasm	39
	Aplastic Anaemia	16
Conditioning Therapy		
	Low intensity	221
	Myeloablative	379
GVHD Prophylaxis		
	CsA	138
	CsA and MTX	298
	CsA and MM	164
	TCD	202
TRANSPLANT DONORS		
Median Age		38 years (range 19-62)
		Number in each group
Gender		
	Male	345
	Female	255
CMV Status		
	Positive	281
	Negative	319

5.4.2 *Allele and Genotype Frequencies*

IL13 +2044 G/A allele and genotype frequencies were determined in the HSCT cohort for both patients and donors. Allele frequencies were calculated as 0.80 and 0.20 for the G and A alleles respectively in the two groups. These values did not differ significantly from those determined for the Newcastle control cohort (P=0.704; Table 3.3, section 3.6.2) and European controls published in the literature (P=1.000; Table 3.3, section 3.6.2) (Heinzmann *et al.* 2003). This polymorphism is therefore not a consequence of disease, but occurs naturally within all populations

All three possible *IL13* +2044 G/A genotype combinations were observed in the patient and donor groups and the genotype frequencies determined were similar to those calculated for the control cohort (patient, P=0.965; donor, P=0.911). Potential deviation from Hardy-Weinberg equilibrium was examined for using the chi-square (χ^2) test. No significant difference between the observed and calculated expected values was demonstrated for the patients, donors or controls (P=0.772, P=0.883 and P=1.000 respectively; Table 5.2). Thus, the genotype frequencies for all three groups were in Hardy-Weinberg equilibrium; there was no selection bias and genotype deviation was absent. Furthermore, the genotype frequencies generated in this study were comparable to those published in the literature (Heinzmann *et al.* 2003; Maier *et al.* 2006; Sadeghnejad *et al.* 2008).

	Newcastle Controls (n=100)		**HSCT Patients (n=600)**		**HSCT Donors (n=600)**	
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**
***IL13* +2044**						
GG	0.66	0.66	0.65	0.65	0.64	0.64
GA	0.30	0.30	0.30	0.32	0.31	0.32
AA	0.04	0.04	0.05	0.03	0.05	0.04

Table 5.2 Hardy-Weinberg equilibrium analysis of *IL13* +2044 G/A genotype frequencies in the Newcastle controls and the HSCT patients and donors. HSCT, haematopoietic stem cell transplantation; IL13, interleukin-13

5.4.3 *Correlation of Clinical Factors with Acute and Chronic GVHD*

It is well established that certain clinical factors have a considerable impact on HSCT outcome, particularly GVHD (section 1.3.4) (Beatty *et al.* 1985; Atkinson *et al.* 1990; Gratwohl *et al.* 1998; Nichols *et al.* 2002; Hahn *et al.* 2008). These factors are therefore potential confounding variables that need to be accounted for when genetic association studies are performed in the HSCT setting. As a result of this, the effects of clinical factors on the development of acute and chronic GVHD in our HSCT cohort were assessed prior to *IL13* +2044 G/A polymorphism analysis.

Eight clinical factors were tested for associations with acute and chronic GVHD. These were patient and donor age at HSCT (over 40 years), gender mismatch transplantation (female to male), CMV status (patient and/or donor positivity), HLA matching, conditioning regimen (low intensity/myeloablative), prophylaxis intensity (low/high) and graft manipulation (T cell replete/T cell deplete). The results of the univariate analyses are shown in Table 5.3. No significant associations were demonstrated with donor age over 40 years, CMV positivity, myeloablative conditioning, low intensity prophylaxis and T cell depletion. Thus, these factors had no impact on HSCT outcome in this study and as a result were not included in any further analyses. Significant correlations were however demonstrated between GVHD and patient age over 40 years, female to male transplantation and HLA-mismatch. These three clinical factors were therefore identified as confounding variables and needed to be accounted for when associations between the *IL13* +2044 G/A polymorphism and HSCT outcome were examined using multivariate analysis.

	Acute GVHD		Chronic GVHD	
	P	**OR**	**P**	**OR**
Clinical Factors				
Patient age >40 years	**0.013**	6.132	0.107	1.393
Donor age >40 years	0.362	0.183	0.299	0.353
Female/male transplantation	**<0.0001**	10.536	**0.001**	7.560
CMV positivity	0.108	1.311	0.112	1.151
HLA-mismatch	**0.073**	1.992	**0.098**	1.713
Myeloablative conditioning	0.452	0.114	0.446	0.135
Low intensity prophylaxis	0.211	0.371	0.337	0.213
T cell depletion	0.304	0.245	0.529	0.079

Table 5.3 Univariate analysis demonstrating the associations between clinical factors and acute and chronic GVHD. P values showing a significant association (≤0.1) are shown in bold. CMV, cytomegalovirus; HLA, human leukocyte antigen; GVHD, graft-versus-host disease; P, P value; OR, odds ratio

5.4.4 *Correlation of the IL13 +2044 G/A Polymorphism with Acute and Chronic GVHD*

In all published studies to date, the A allele of the *IL13* +2044 G/A polymorphism has been linked with disease susceptibility. Consequently, only the A allele was examined for associations with HSCT outcome in this study. The very low frequency of the AA genotype in our HSCT cohort (0.05% in both patients and donors) prevented disease association analysis with this genotype.

Associations between the *IL13* +2044 A allele and the incidence and severity of acute GVHD were examined in the entire cohort of 600 HSCT patients. Associations between this allele and the incidence of chronic GVHD were analysed in 463 patients alive >100 days post-HSCT. The results of the univariate analyses are shown in Table 5.4. No associations were observed between the A allele and overall acute GVHD (grades I-IV) or clinical acute GVHD (grades II-IV). A strong correlation was however demonstrated between the A allele and the development of severe acute GVHD (grades III-IV). HSCT patients possessing the *IL13* +2044 A allele were more susceptible to higher grades of acute GVHD than patients with the G allele (22% versus 16% respectively, P=0.028 OR=3.977). A significant association was also demonstrated between the A allele and chronic GVHD. Patients receiving stem cell transplants from

donors with the *IL13* +2044 A were more at risk of developing chronic GVHD than patients with *IL13* +2044 G donors (59% versus 48% respectively, P=0.010 OR=5.808).

	Acute GVHD Grade							
	I-IV		II-IV		III-IV		Chronic GVHD	
	P	**OR**	**P**	**OR**	**P**	**OR**	**P**	**OR**
***IL13* +2044 A**								
Patient	0.171	0.900	0.362	0.183	**0.028**	3.977	0.498	0.008
Donor	0.304	0.345	0.404	0.101	0.201	0.701	**0.010**	5.808

Table 5.4 Univariate analysis demonstrating the associations between the *IL13* +2044 A allele and acute and chronic GVHD. P values demonstrating an association (≤0.1) are shown in bold. IL13, interleukin-13; GVHD, graft-versus-host disease; P, P value; OR, odds ratio

The associations identified between the *IL13* +2044 A allele and acute (grades III-IV) and chronic GVHD were tested for independence against confounding clinical factors using multivariate analysis. The clinical factors entered into the analyses where those shown in section 5.4.3 to have a significant impact on the development of GVHD in this cohort. The factors included were: patient age over 40 years, female to male transplantation and HLA-mismatch. Acute GVHD grades I-IV and PBSC transplants were also included in the multivariate analysis for chronic GVHD, as both are considerable risk factors for the development of the condition (Atkinson *et al.* 1990; Bensinger *et al.* 2001). The results of the multivariate analyses are shown in Table 5.5.

Following multivariate analyses, the previously demonstrated associations between patient *IL13* +2044 A and severe acute GVHD (grades III-IV) and donor *IL13* +2044 A and chronic GVHD retained their significance (P=0.023 OR=2.260 and P=0.013 OR=6.848 respectively). Thus, the observed associations are independent of established clinical risk factors for HSCT outcome.

Several clinical factors were also shown to be significantly associated with GVHD following multivariate analyses. Patient age over 40 years, female to male transplantation and HLA-mismatch correlated with acute GVHD III-IV (P=0.048

OR=1.225, P=0.045 OR=1.884 and P=0.047 OR=1.453 respectively), whilst female to male transplantation, patient age over 40 years, PBSC transplantation and acute GVHD grades I-IV were linked to chronic GVHD (P=0.014 OR=6.447, P=0.049 OR=3.375, P=0.002 OR=12.646 and P<0.0001 OR=36.202 respectively). These findings acted as a further control in this study, by validating the cohort analysed. As all five factors are well recognised clinical risks in HSCT (Atkinson *et al.* 1990; Gratwohl *et al.* 1998; Bensinger *et al.* 2001), demonstrated associations with GVHD would be expected in an unbiased cohort.

	P Value	Odds Ratio
Acute GVHD III-IV		
Patient age >40	**0.048**	1.225
Female/male transplantation	**0.045**	1.884
HLA-mismatch	**0.047**	1.453
Patient *IL13* +2044 A allele	**0.023**	2.260
Chronic GVHD		
Patient age >40	**0.049**	3.375
Female/male transplant	**0.014**	6.447
PBSC Transplants	**0.002**	12.646
Acute GVHD I-IV	**<0.0001**	36.202
Donor *IL13* +2044 A allele	**0.013**	6.848

Table 5.5 Multivariate analysis for severe acute (grades III-IV) and chronic GVHD. Significant associations (P<0.05) are shown in bold. GVHD, graft-versus-host disease; HLA, human leukocyte antigen; IL13; interleukin-13; PBSC, peripheral blood stem cell

In an attempt to replicate the genetic associations demonstrated, the *IL13* +2044 A allele was correlated with acute (grades III-IV) and chronic GVHD in a second cohort. This population consisted of a randomly selected subset of the original HSCT cohort (n=300). The A allele was again significantly linked with the development of severe acute (grades III-IV, patient) and chronic GVHD (donor) (P=0.018 OR=6.762 and P=0.011 OR=10.107 respectively). Consequently, it is extremely likely that these associations are real and not merely a result of chance.

The relationship between the *IL13* +2044 G/A alleles and GVHD was also examined in separate cohorts of HLA-matched sibling (n=342) and unrelated donor (n=258) transplants. Following univariate analyses, the previously described associations between patient *IL13* +2044 A and severe acute GVHD (III-IV) and donor *IL13* +2044 A and chronic GVHD were demonstrated in both transplant groups. The observed associations for unrelated donor transplantation were, however, almost twice as strong as those for HLA-matched sibling HSCT (acute GVHD III-IV, P=0.024 OR=3.722 and P=0.042 OR=1.794 respectively; chronic GVHD, P=0.015 OR=9.851 and P=0.034 OR=5.063 respectively).

To determine the significance of transplant type, the demonstrated associations between *IL13* +2044 A and acute and chronic GVHD in both the HLA-matched sibling and unrelated donor HSCT cohorts were then tested against each other using multivariate analysis. The three strongest confounding clinical factors for each outcome (acute III-IV or chronic GVHD) were also included. The results of the multivariate analyses are shown in Table 5.6.

Following multivariate analyses, the associations between patient *IL13* +2044 A and severe acute GVHD (III-IV) and donor *IL13* +2044 A and chronic GVHD were still observable for both transplant types. In the unrelated donor cohort, significance was retained; in fact both associations had gained in strength (acute GVHD III-IV, P=0.020 OR=5.451; chronic GVHD, P=0.011 OR=11.087). In the HLA-matched sibling cohort, however, the significance for both associations had been substantially reduced (acute GVHD III-IV, P=0.091 OR=0.798; chronic GVHD, P=0.076 OR=0.703). The data therefore seems to suggest that even though it has an impact on GVHD in both HLA-matched sibling and unrelated donor HSCT, the *IL13* +2044 G/A polymorphism is of greater importance in the latter setting.

	P Value	Odds Ratio
Acute GVHD III-IV		
Patient age >40	0.107	0.572
Female/male transplantation	**0.015**	7.899
HLA-mismatch	**0.049**	1.210
P *IL13* +2044 A allele in sibling HSCT	0.091	0.798
P *IL13* +2044 A allele in unrelated HSCT	**0.020**	5.451
Chronic GVHD		
Female/male transplant	0.069	0.854
PBSC Transplants	**0.047**	1.131
Acute GVHD I-IV	**0.007**	15.331
D *IL13* +2044 A allele in sibling HSCT	0.076	0.703
D *IL13* +2044 A allele in unrelated HSCT	**0.011**	11.087

Table 5.6 Multivariate analysis of the *IL13* +2044 G/A polymorphism in HLA-matched sibling versus unrelated donor transplants. Significant associations (P<0.05) are shown in bold. GVHD, graft-versus-host disease; HLA, human leukocyte antigen; P, patient; IL13; interleukin-13; HSCT, haematopoietic stem cell transplantation; PBSC, peripheral blood stem cell; D, donor

5.4.5 *Correlation of the IL13 +2044 G/A Polymorphism with Overall Survival*

Overall survival was defined as the total amount of time (in months) each patient survived post-HSCT and was measured from the date of transplantation to the date of death or most recent clinic appointment. The relationship between the *IL13* +2044 A allele and this outcome was examined in the entire cohort of 600 HSCT patients. Following univariate analysis, no correlations were demonstrated in either of the patient or donor groups (P=0.743 and P=0.868 respectively, Figure 5.1 A and B respectively). Due to the lack of associations at this stage, no further analyses were performed.

To determine whether the absence of any significant associations was related to the HSCT cohort employed, clinical factors with already established links to post-transplant mortality were analysed. The factors included were severe acute GVHD (III-IV) and disease relapse. Correlations with overall survival were demonstrated; both factors were found to be significantly linked to increased mortality in our cohort (P<0.0001 and P=0.001 respectively, Figure 5.1 C and D). These findings agree with those already published in the literature (Vogelsang *et al.* 1988; Gratwohl *et al.* 1998; Doney *et al.*

2003). Thus, our inability to demonstrate any associations with the *IL13* +2044 A allele was not cohort related, but was an observation of the apparent lack of associations between this allele and overall survival in our study.

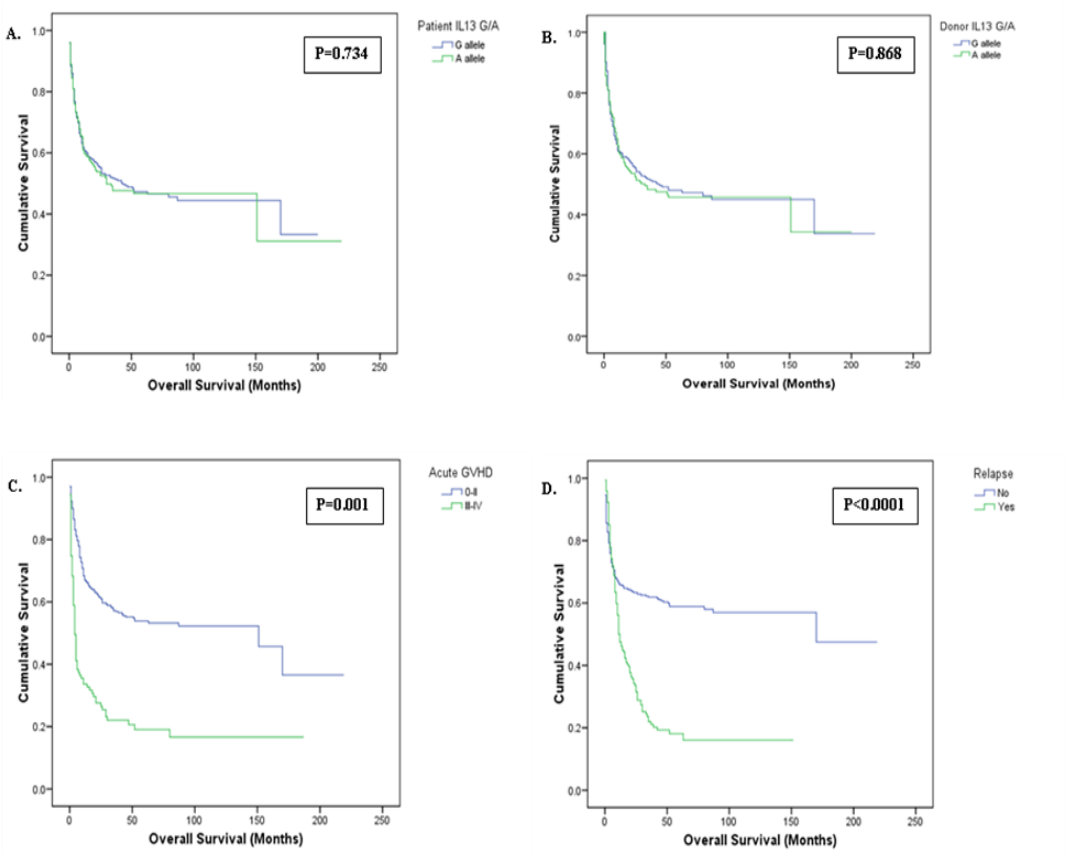

Figure 5.1 Overall survival analysis using Kaplan-Meier curves and the Log-Rank test. Correlation with patient (**A.**) and donor (**B.**) *IL13* +2044 G/A, acute GVHD grades III-IV (**C.**) and relapse (**D.**). P, P value; IL13, interleukin-13; GVHD, graft-versus-host disease

5.4.6 *Correlation of the IL13 +2044 G/A Polymorphism with Relapse Incidence and TRM*

Relapse was defined as the reoccurrence of an original disease following a period of treatment. As aplastic anaemia is a non-malignant disease, patients transplanted for this were not included in any analyses correlating the *IL13* +2044 G/A polymorphisms with relapse. Consequently, 16 patient/donor transplant pairs were excluded from this analysis and associations between the *IL13* +2044 A allele and relapse incidence were examined in 156 HSCT patients. The incidence of relapse was calculated for the *IL13* +2044 G and A alleles at 12, 24, 36, 48 and 60 months post-transplant. Our findings demonstrate that disease relapse was lower at all five time points for the patients and donors with the A allele than those with the G allele (Table 5.7). However, the difference did not reach significance in either group (patient, P=0.336; donor, P=0.453). The small nature of the relapse population investigated (n=172) may have prevented these findings reaching significance. Thus, the examination of *IL13* +2044 G/A alleles in a larger cohort of relapsed patients would be beneficial.

	Cumulative Incidence (95% CL)				
***IL13* +2044**	**12M**	**24M**	**36M**	**48M**	**60M**
Patient					
A Allele	0.205 (0.167-0.250)	0.253 (0.212-0.301)	0.278 (0.235-0.328)	0.283 (0.239-0.333)	0.290 (0.240-0.340)
G Allele	0.267 (0.217-0.333)	0.306 (0.261-0.386)	0.371 (0.310-0.447)	0.388 (0.324-0.465)	0.397 (0.332-0.476)
Donor					
A Allele	0.194 (0.157-0.239)	0.257 (0.215-0.306)	0.281 (0.237-0.331)	0.289 (0.245-0.341)	0.295 (0.249-0.347)
G Allele	0.234 (0.183-0.299)	0.279 (0.225-0.345)	0.312 (0.253-0.384)	0.319 (0.260-0.391)	0.332 (0.282-0.411)

Table 5.7 Competing risk analysis for the *IL13* +2044 G/A polymorphism and relapse incidence. IL13, interleukin-13; 95% CL, 95% confidence limits (lower-upper); M, months

TRM was defined as death following transplantation, from all causes other than disease relapse. The relationship between this outcome and the *IL13* +2044 A allele was examined in 179 HSCT patients. No significant difference was observed in the incidence of TRM at 12, 24, 36, 48 and 60 months post-HSCT between the G and A alleles in either the patient or donor groups (P=0.993 and P=0.950 respectively, Table 5.8).

As with overall survival, a clinical risk factor for TRM was analysed in order to confirm that the absence of any associations was not cohort related. Gender mismatched (female to male) transplantation is a well recognised risk for TRM (Zwaan *et al.* 1989; Gratwohl *et al.* 2001), consequently, this factor was examined for associations in this study. A strong link with TRM was demonstrated (P<0.001), thus, our cohort was not responsible for the lack of associations with the *IL13* +2044 A allele.

	Cumulative Incidence (95% CL)				
***IL13* +2044**	**12M**	**24M**	**36M**	**48M**	**60M**
Patient					
A Allele	0.246 (0.193-0.314)	0.269 (0.213-0.338)	0.275 (0.219-0.345)	0.284 (0.226-0.357)	0.295 (0.237-0.368)
G Allele	0.244 (0.204-0.291)	0.258 (0.217-0.306)	0.271 (0.229-0.319)	0.287 (0.244-0.338)	0.303 (0.257-0.356)
Donor					
A Allele	0.249 (0.196-0.314)	0.265 (0.211-0.333)	0.275 (0.233-0.356)	0.284 (0.226-0.335)	0.293 (0.248-0.345)
G Allele	0.233 (0.182-0.298)	0.250 (0.209-0.298)	0.268 (0.226-0.317)	0.286 (0.238-0.339)	0.290 (0.232-0.351)

Table 5.8 Competing risk analysis for the *IL13* +2044 G/A polymorphism and TRM.
IL13, interleukin-13; 95% CL, 95% confidence limits (lower-upper); M, months

5.5 Examination of the Functional Effects of IL-13 in GVHD

5.5.1 *Study Populations*

HSCT Cohort

The functional effects of IL-13 in GVHD were studied in a cohort of 90 allogeneic HSCT patients and their donors. This was an adult cohort (≥16 at the time of transplant) comprising 62 HLA-matched sibling transplants and 28 unrelated donor transplants. HLA matching between patients and donors was performed by high resolution DNA-based typing for HLA-A, B, C, DRB1 and DQB1. Complete matching at the 5 loci was observed for all transplant pairs.

Cohort characteristics:
The 90 HSCT patients included in this cohort were transplanted between November 1983 and December 2006 at the Royal Victoria Infirmary, Newcastle upon Tyne, UK. All patients and donors were recruited and consented as part of the previously described 'Eurobank Project' (section 5.3.1).

Acute GVHD was assessable in those alive ≥30 days post-transplant and developed in 53 patients. 37 patients had no acute GVHD (grade 0), 17 grade I, 24 grade II, 8 grade III and 4 had grade IV. Chronic GVHD was assessable from 100 days post-transplant in 71 patients. 41 of these patients developed the condition. All transplant patients were prepared for HSCT with low intensity (n=49) or myeloablative conditioning (n=41) regimens and 56% (n=50) were transplanted with T cell depleted stem cell grafts. 46 patients were given high intensity GVHD prophylaxis (CsA plus MTX), whilst 44 received CsA monotherapy. The survival rate for this cohort was 52% (n=47) and causes of death included relapse, infection and GVHD. Table 5.9 shows the demographic characteristics of the HSCT cohort examined in this aspect of the study.

Table 5.9 Characteristics of the HSCT cohort (n=90).
CMV, cytomegalovirus; CsA, cyclosporine A; MTX, methotrexate; TCD, T cell depletion

TRANSPLANT PATIENTS

Median Age		38 years (range 19-57)
		Number in each group
Gender		
	Male	55
	Female	35
	Female to Male HSCT	25
CMV Status		
	Positive	42
	Negative	48
Underlying Disease		
	Acute Lymphoblastic Leukaemia	14
	Acute Myeloid Leukaemia	28
	Chronic Myeloid Leukaemia	15
	Hodgkin disease	7
	Non-Hodgkin Lymphoma	13
	Plasma Cell Neoplasm	7
	Aplastic Anaemia	2
	Other	5
Conditioning Therapy		
	Low intensity	49
	Myeloablative	41
GVHD Prophylaxis		
	CsA	44
	CsA and MTX	46
	TCD	50

TRANSPLANT DONORS

Median Age		37 years (range 16-58)
		Number in each group
Gender		
	Male	51
	Female	39
CMV Status		
	Positive	37
	Negative	51
	Unknown	2

Control Cohort

18 Newcastle laboratory staff volunteers were employed as healthy controls when IL-13 expression *in vivo* was examined. The volunteer group comprised 5 males and 13 females, with a median age of 39 years (range 27-52).

5.5.2 *IL-13 Expression in the In Vitro Human Skin Explant Model of Acute GVHD*

In this aspect of the study allogeneic MLCs were used to mimic the allo-response produced following HSCT and the human skin explant assay was utilised as an *in vitro* model of acute GVHD (sections 3.9 and 3.10). IL-13 expression during these assays was examined in order to establish the roles, if any that IL-13 plays in the development of acute GVHD.

The cohort employed comprised of 90 HSCT patients with MLC supernatants and GVHR grades. The severity of GVHR ranged from I-IV; 17 human skin explants were found to give a histopathological grade of I, 12 grade II, 57 grade III and 4 human skin explants were found to give a grade IV. IL-13 quantification was achieved using the Human IL-13 Development ELISA kit described in section 3.13.

Comparison of IL-13 Expression in Autologous and Allogeneic MLC Supernatants

To determine if IL-13 expression in allogeneic MLC supernatants was a consequence of an allo-response between donor and patient cells, IL-13 levels in autologous (patient cells only as stimulator and responder) and allogeneic MLC supernatants were compared. IL-13 levels were found to be significantly higher in the latter samples. In some cases, the IL-13 levels observed in the allogeneic supernatants were over 5 times greater than those in the autologous MLCs. The mean (± standard error of the mean (SEM)) IL-13 level (pg/ml) in the allogeneic MLC supernatants was 101.89 ± 15.11 compared to only 46.19 ± 2.94 in the autologous supernatants (P=0.040, Figure 5.2). Thus, the IL-13 expression observed in the allogeneic MLC supernatant samples was a consequence of allo-recognition between donor and patient cells.

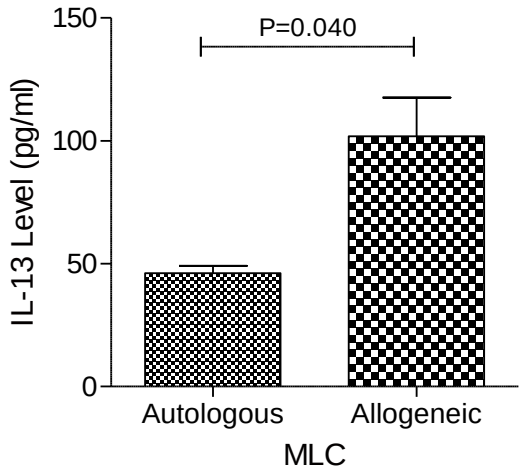

Figure 5.2 Comparison of IL-13 expression (pg/ml) in autologous (n=90) and allogeneic (n=90) MLC supernatants. The mean (± SEM) level of IL-13 was significantly higher in the supernatants from allogeneic MLCs (P=0.040). IL-13, interleukin-13; P, P value; MLC, mixed lymphocyte culture

The Relationship between IL-13 Expression in MLC Supernatants and GVHR Grade

Comparison of IL-13 levels in allogeneic MLC supernatants with human skin explant GVHR grades demonstrated an increase in IL-13 expression with increasing GVHR severity (Figure 5.3). The mean (± SEM) IL-13 level (pg/ml) for GVHR grades II-IV was 117.30 ± 18.45 compared to only 34.80 ± 19.26 for grade I (P=0.002, Figure 5.4A). Moreover, the mean (± SEM) IL-13 level for GVHR grades III-IV was 125.70 ± 21.59 compared to only 53.44 ± 15.96 for grades I-II (P=0.008, Figure 5.4B).

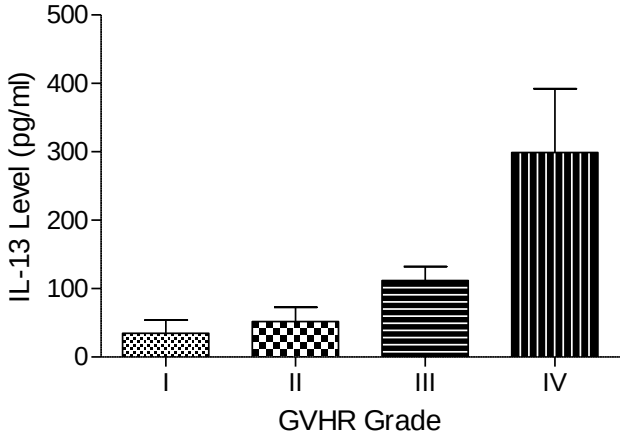

Figure 5.3 Relationship between GVHR grade in the human skin explant model and IL-13 expression (pg/ml) in allogeneic MLC supernatants. IL-13 expression increases with increasing severity of GVHR. IL-13, interleukin-13; GVHR, graft-versus-host reaction

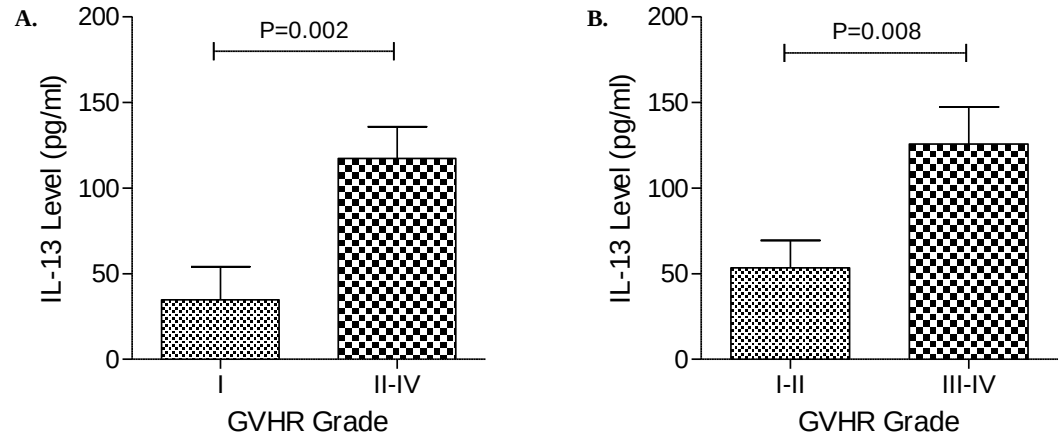

Figure 5.4 Correlation between GVHR grade in the human skin explant model and IL-13 expression (pg/ml) in allogeneic MLC supernatants. **A.** The mean (± SEM) IL-13 level was significantly higher for GVHR grades II-IV (n=73, P=0.002). **B.** The mean (± SEM) IL-13 level was significantly higher for GVHR grades III-IV (n=61, P=0.008). IL-13, interleukin-13; P, P value; GVHR, graft-versus-host reaction

Cytokine Profile of MLC Supernatants

To establish if the IL-13 expression observed was affected by other cytokines implicated in the pathogenesis of acute GVHD, the cytokine profile of the allogeneic MLC supernatants was determined. Cytokine quantification was achieved using the Human Soluble Protein CBA kit described in section 3.14. The cytokines measured were IL-2, IL-4, IL5, IL-10, TNFα and IFNγ. The mean (± SEM) levels (pg/ml) were 18.40 ± 1.50, 2.46 ± 0.92, 11.02 ± 2.88, 24.52 ± 4.38, 97.50 ± 16.50, 49.61 ± 11.49 and 56.44 ± 10.66 for IL-2, IL-4, IL-5, IL-10, IL-13, TNFα and IFNγ respectively (Figure 5.5). IL-13 expression was demonstrated to be significantly higher than IL-2, IL-4, IL-5 and IL-10 in the MLC supernatants ($P<0.0001$). The levels were also higher when compared to those of TNFα and IFNγ, but for these cytokines the difference did not reach significance (P=0.070).

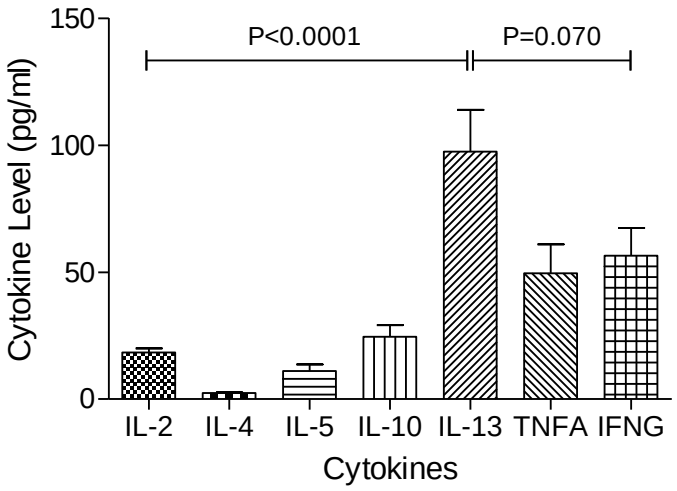

Figure 5.5 Cytokine profile of allogeneic MLC supernatants. The mean (± SEM) IL-13 level (pg/ml) was significantly higher than the mean (± SEM) levels of IL-2, IL-4, IL-5 and IL-10 (P<0.0001). IL-13 expression was also higher than that of TNFα and IFNγ, although the difference did not reach significance (P=0.070). P, P value; IL, interleukin; TNF, tumour necrosis factor alpha; IFNG, interferon gamma

To ensure that the IL-13 expression observed in the allogeneic MLC supernatants was independent of IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ expression, the Spearman's Rank Correlation test was also performed. This test was used to determine the individual relationship between IL-13 and each of the other cytokines examined. Following the analyses, no significant correlation could be demonstrated between the levels of IL-13 and the levels of IL-2 (P=0112 r=0.219 r^2=0.048), IL-4 (P=0.231 r=0.139 r^2=0.019), IL-5 (P=0150 r=0.178 r^2=0.031), IL-10 (P=0.472 r=0.098 r^2=0.009), TNFα (P=0.155, r=0.156, r^2=0.024) or IFNγ (P=0.342, r=0.106, r^2=0.011) in the allogeneic MLC supernatant samples. Thus, the IL-13 expression observed was independent of all other cytokines tested.

The Relationship between IL-13 Expression in MLC Supernatants and GVHD

IL-13 expression in MLC supernatants was also correlated with acute and chronic GVHD in the clinical setting. In contrast to the findings of the human skin explant model, where IL-13 levels increased with GVHR severity; when correlated with clinical acute GVHD, IL-13 expression was found to decrease with GVHD severity (Figure 5.6A). In addition, mean (± SEM) IL-13 levels (pg/ml) were found to be lower in overall acute GVHD (grades I-IV; mean (± SEM) IL-13 level: grade 0, 109.60 ± 28.06 grades I-IV, 98.02 ± 20.53), clinical acute GVHD (grades II-IV; mean (± SEM) IL-13 level: grades 0-I, 115.20 ±281 grades II-IV, 90.76 ± 19.63) and severe acute GVHD (grades III-IV, mean (± SEM) IL-13 level: grades 0-II, 108.40 ± 18.70 grades III-IV, 65.66 ± 25.96) (Figure 5.6B-C). However, none of these observed associations reached significance (P=0.677, P=0.905 and P=0.394 respectively).

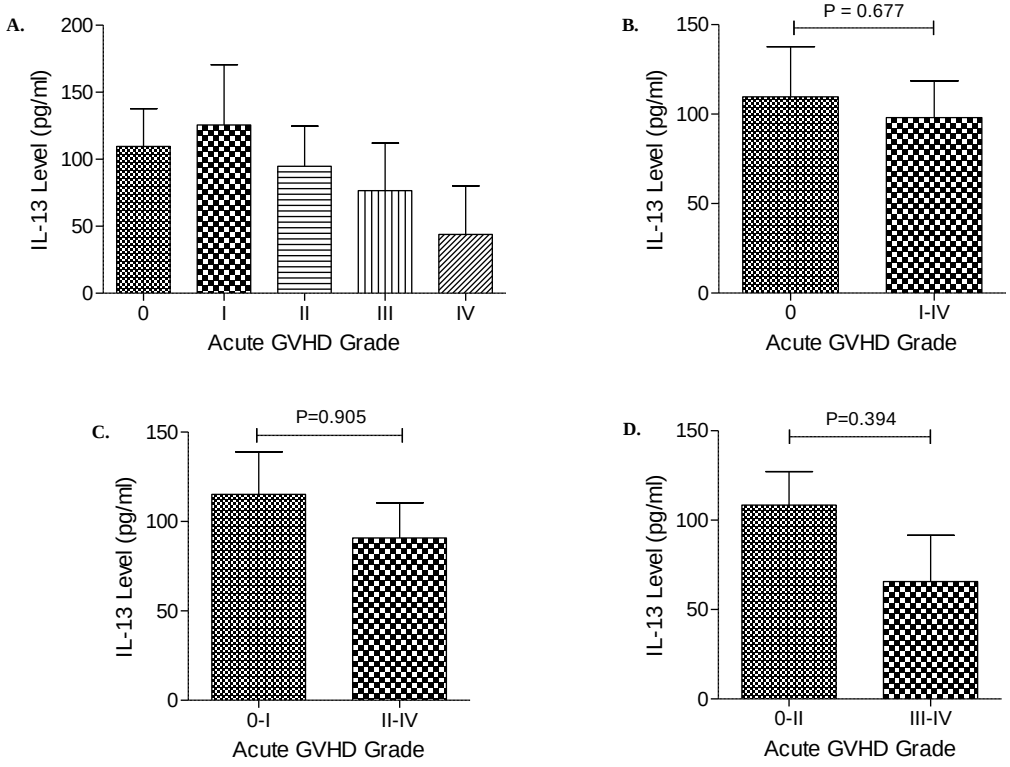

Figure 5.6 Correlation between IL-13 expression (pg/ml) in MLC supernatants and clinical acute GVHD. **A.** IL-13 levels decrease with increasing GVHD severity. **B.** The mean (± SEM) IL-13 level was lower for overall acute GVHD (grades I-IV (n=53), P=0.677). **C.** The mean (± SEM) IL-13 level was lower for clinically significant acute GVHD (grades II-IV (n=36), P=0.905). **D.** The mean IL-13 level was lower for severe acute GVHD (grades III-IV (n=12), P=0.394). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

In terms of chronic GVHD, IL-13 expression was found to be lower for HSCT patients with the condition than those without it. The mean (± SEM) IL-13 level (pg/ml) for patients with chronic GVHD was only 85.57 ± 21.25 compared to 101.10 ± 30.85 for unaffected individuals (Figure 5.7). Again, the difference did not reach significance (P=0.681).

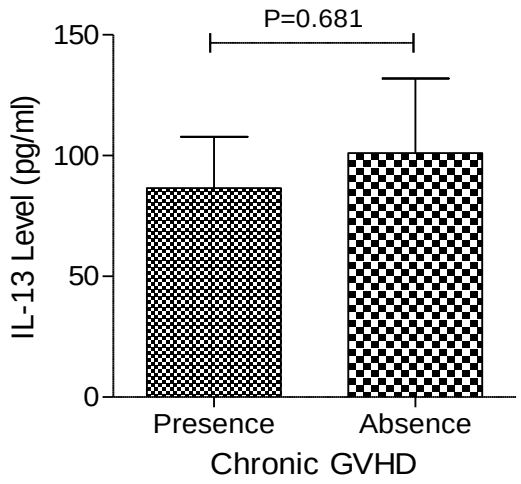

Figure 5.7 Correlation between IL-13 expression (pg/ml) in MLC supernatants and Chronic GVHD. The mean (± SEM) IL-13 level was lower for patients with chronic GVHD (n=41) than those without the condition (n=30, P=0.681). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

IL-13 expression in MLC supernatants was also correlated with overall survival, relapse incidence and TRM in our HSCT cohort. No significant associations were observed with any of these clinical outcomes (P=0.361, P=0.404 and P=0.319 respectively).

5.5.3 *IL-13 Serum Expression and Acute and Chronic GVHD*

Serum samples were obtained from HSCT patients at eight time points across the transplant period (section 3.12). The time points employed for sampling were day -7 (pre-HSCT, taken prior to the administration of any conditioning regimens), day 0 (taken immediately prior to stem cell infusion), day 7, day 14, day 28, 3 months, 6 months and 12 months. These time points were chosen to allow intensive monitoring of peri-transplant IL-13 expression with respect to both acute and chronic GVHD. Serum samples were also obtained from 18 laboratory staff volunteers at the same eight time points. These individuals were employed as controls in this aspect of the study (section 5.6.1).

The cohort examined comprised of 30 of the original 90 HSCT patients with complete sets of serum samples (8 samples) and clinical data on acute and chronic GVHD. Acute GVHD was assessable in all 30 patients and developed in 24. The severity of GVHD ranged from grades 0-III; 6 patients developed no GVHD, 10 grade I, 10 grade II and 4 grade III. Chronic GVHD was assessable in 20 patients and developed in 12. IL-13 quantification was achieved using the Human IL-13 Low Level ELISA Kit described in section 3.13.

Comparison of IL-13 Serum Expression in HSCT Patients and Healthy Controls

To determine the effects of HSCT on IL-13 expression, IL-13 serum levels in patients and healthy controls were compared. IL-13 expression was found to be significantly higher in the HSCT cohort ($P<0.0001$ at all time points, Figure 5.8). Mean (± SEM) patient IL-13 levels (pg/ml) were over 5 times greater than those of the healthy controls at all time points examined. Age and gender were found to have no effect on IL-13 in either group. Mean (± SEM) IL-13 serum levels in the healthy controls remained relatively constant (0.86 ± 0.22 ,0.77 ± 0.21, 0.89 ± 0.21, 0.91 ± 0.20, 0.90 ± 0.22, 0.86 ± 0.22, 0.87 ± 0.21 and 0.85 ± 0.21 pg/ml respectively), thus, the variability observed in patient IL-13 expression (5.85 ± 0.63, 8.50 ± 0.89, 5.46 ± 0.70, 8.42 ± 1.04, 8.09 ± 0.98, 9.37 ± 0.73, 5.83 ± 0.41 and 5.06 ± 0.35 pg/ml respectively) is likely to be transplant and/or treatment related rather than a naturally occurring phenomenon. The mean (± SEM) IL-13 expression level observed in the transplant patients on day -7 was not however transplant or treatment related; as all serum samples utilised at this time point

were obtained on the day of hospital admission and prior to the administration of any conditioning, transplant or treatment modalities (pre-transplant), it is more likely that the higher expression observed was patient or disease related. The IL-13 serum levels demonstrated for the transplant patients on day -7 ranged from 1.07-13.82pg/ml. As no clear cause was obvious, the high degree of variability observed at this time point was of great interest and warranted further investigation. As previously published studies have correlated pre-transplant release of inflammatory cytokines, such as TNFα and IL-10 with HSCT outcome (Holler *et al.* 1995; Holler *et al.* 2000), it is possible that similar associations exist with IL-13. Consequently, the prognostic significance of IL-13 serum expression prior to transplantation will be examined in the following chapter.

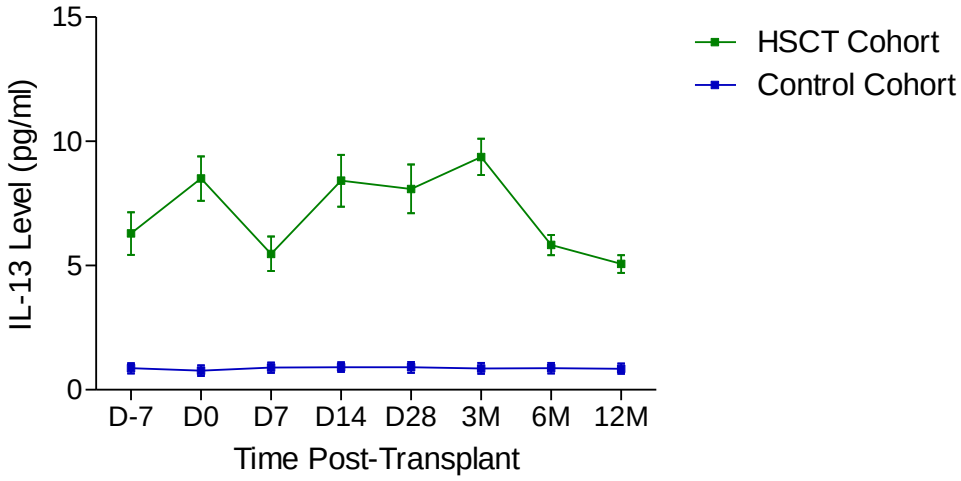

Figure 5.8 Comparison of IL-13 serum expression (pg/ml) in HSCT patients (n=30) and healthy volunteers (n=18). Mean (± SEM) IL-13 expression was significantly higher in the HSCT patients at day -7, day 0, day 7, day 14, day 28, 3 months, 6 months and 12 months (P<0.0001). IL-13, interleukin-13; HSCT, haematopoietic stem cell transplantation; D, day; M, months

The Relationship between IL-13 Serum Expression and Acute GVHD

IL-13 serum expression was analysed between day -7 and 3 months amongst HSCT patients who did and did not develop GVHD grades I-III post-transplant; the traditional window for acute GVHD (Figure 5.9). IL-13 levels in the GVHD (grades I-III) patients were found to be higher than those without the disease (grade 0) at all time points. Despite this, the difference between the two groups only reached significance at day 28 and 3 months (P=0.043 and P=0.037 respectively). Mean (± SEM) IL-13 levels (pg/ml) at these time points were 9.24 ± 1.30 and 9.76 ± 0.69 respectively for patients with acute GVHD (grades I-III) compared to 6.02 ± 1.08 and 6.43 ± 1.23 respectively for those with no acute GVHD.

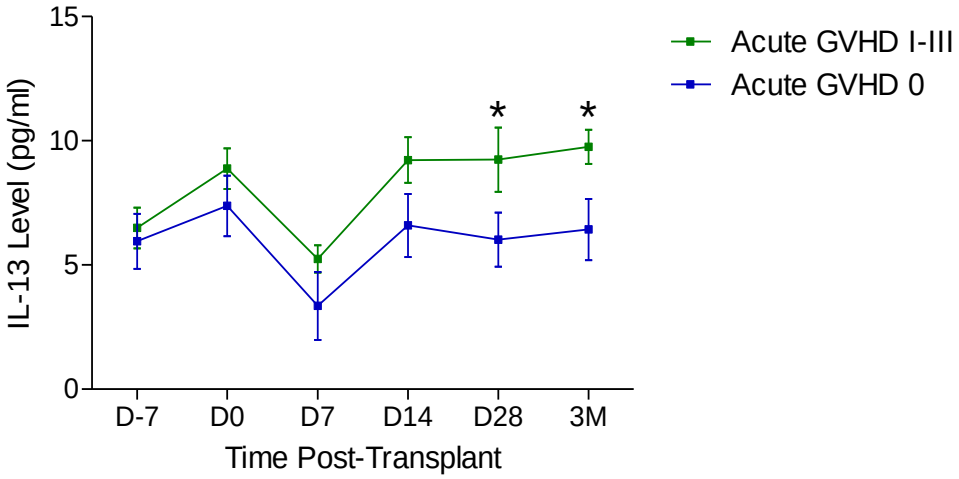

Figure 5.9 Comparison of IL-13 serum expression (pg/ml) in HSCT patients with grades I-III acute GVHD (n=24) and those with no acute GVHD (n=6). Mean (± SEM) IL-13 expression was higher in the HSCT patients with grades I-III acute GVHD at day -7, day 0, day7, day14, day 28 and 3 months. An asterisk denotes a significant difference between the two groups at the designated time point (P=0.043 and P=0.37 respectively). IL-13, interleukin-13; GVHD, graft-versus-host disease; D, day; M, months

IL-13 serum expression was also analysed amongst HSCT patients who did and did not develop clinical acute GVHD (grades II-III) between day -7 and 3 months post-transplant (Figure 5.10). Again, IL-13 levels were found to be higher between day 0 and 3 months in the patients with clinical acute GVHD (grades II-III) compared to those with minimal or no disease (grade 0-I), although, the difference between the two groups

was considerably reduced and consequently did not reach significance at any point. In contrast, pre-transplant levels (day -7) were found to be higher in the patients with grades 0-I GVHD compared to those with grades II-III. However, the difference between the two groups at this time point was minimal. Mean (± SEM) pre-transplant IL-13 levels (pg/ml) for patients with acute GVHD grades 0-I was 6.08 ± 0.93 compared to 5.58 ± 0.86 for those with grades II-III.

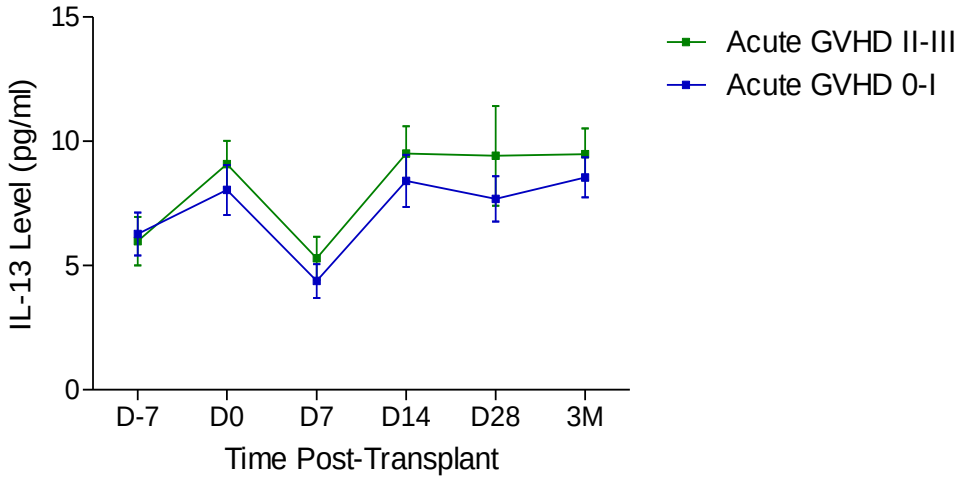

Figure 5.10 Comparison of IL-13 serum expression (pg/ml) in HSCT patients with grades II-III acute GVHD (n=14) and those with minimal or no acute GVHD (n=16). Mean (± SEM) IL-13 expression was higher in the HSCT patients with grades II-III acute GVHD at all time points except day -7. IL-13, interleukin-13; GVHD, graft-versus-host disease; D, day; M, months

Correlations could not be made between IL-13 serum expression and severe acute GVHD (grades III-IV) in this study. No patients in our HSCT cohort developed grade IV acute GVHD and only 4 developed grade III.

The Relationship between IL-13 Serum Expression and Chronic GVHD

IL-13 serum expression was compared amongst HSCT patients who did and did not develop chronic GVHD between 3 and 12 months post-transplant; the traditional window for this condition. IL-13 levels were found to be higher at 3 months, 6 months and 12 months for the patients with chronic GVHD (Figure 5.11A-C). However, the difference between the two groups only reached significance for the latter two time points (P=0.008 and P=0.016 respectively). Mean (± SEM) IL-13 levels (pg/ml) at 6 and 12 months were 6.93 ± 0.41 and 6.00 ± 0.38 respectively for the patients with chronic GVHD compared to 4.17 ± 0.27 and 3.66 ± 0.18 respectively for those without chronic GVHD.

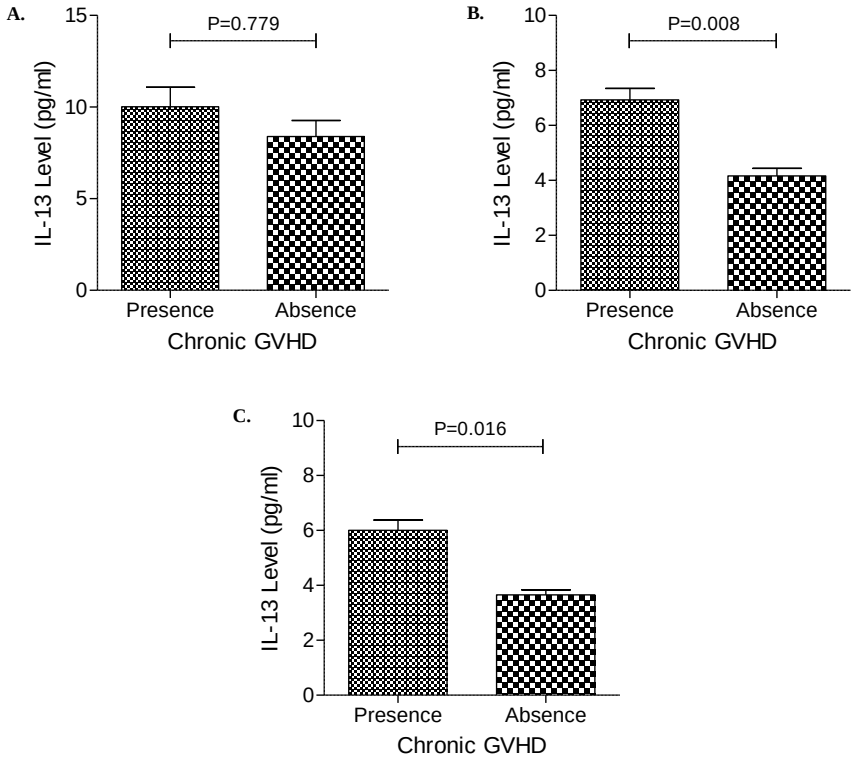

Figure 5.11 Comparison of IL-13 serum expression (pg/ml) in HSCT patients with chronic GVHD (n=12) and those without chronic GVHD (n=8). **A.** Mean (± SEM) IL-13 expression was slightly higher at 3 months post-transplant in the patients with chronic GVHD (P=0.779). **B & C.** Mean (± SEM) IL-13 expression was significantly higher in chronic GVHD patients at 6 months and 12 months post-transplant (P=0.008 and P=0.016 respectively). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

The Relationship between Serum IL-13 Expression and Other HSCT Outcomes

IL-13 serum expression was also correlated with overall survival and relapse incidence in this study. No significant associations were observed between IL-13 levels and these two clinical outcomes at any of the time points analysed (data not shown). IL-13 expression could not be correlated with TRM, as all the observed deaths in our cohort were relapse related.

5.5.4 *The Relationship between IL-13 Expression and the IL13 +2044 G/A Polymorphism*

Patient and donor PHA stimulated PBMC supernatants (section 3.11) were used to determine if the elevated IL-13 expression observed in patients with acute and chronic GVHD (section 5.6.3) was attributable to the *IL13* +2044 G/A polymorphism (section 5.5.4). The previously examined serum cohort (section 5.6.3) was not employed in this aspect of the study due to its small nature (n=30). All PBMCs employed in the mitogen stimulation assays were obtained before patients received any preparative treatments and prior to transplantation, thus IL-13 levels in these samples were not affected by conditioning regimens, the transplant process or GVHD prophylaxis drugs.

The cohort examined comprised 60 of the original 90 HSCT patients with PHA stimulated supernatants for both patients and donors, *IL13* +2044 G/A genotypes and clinical data on acute and chronic GVHD. Acute GVHD was assessable in all 60 patients and developed in 38. The severity of GVHD ranged from grade 0-IV; 20 patients developed no GVHD, 9 grade I, 23 grade II, 5 grade III and 1 patient developed grade IV acute GVHD. Chronic GVHD was assessable in 51 patients and developed in 33. IL-13 quantification was achieved using the Human IL-13 Development ELISA kit described in section 3.13.

Comparison of IL-13 Expression in Supernatant from PHA Stimulated and Unstimulated PBMCs

Prior to any correlation with the *IL13* +2044 G/A polymorphism, IL-13 release from PHA stimulated PBMCs was compared to spontaneous IL-13 release from unstimulated PBMCs (medium alone control samples). This was to ensure that IL-13 expression was

a consequence of PHA activation. Expression was found to be significantly higher in the stimulated samples. In some cases, IL-13 release from PHA stimulated PBMCs was over 20 times greater than that from unstimulated cells. The mean (± SEM) IL-13 level (pg/ml) in PHA stimulated supernatants was 1292.00 ± 112.10 compared to only 129.10 ± 20.45 in unstimulated supernatants (P<0.0001).

Correlation between IL-13 Expression and Patient and Donor *IL13* +2044 G/A Alleles

Correlation of IL-13 levels in PHA stimulated supernatants with patient *IL13* +2044 G/A demonstrated that even though IL-13 levels were slightly higher for patients with *IL13* +2044 A, there was no significant difference in IL-13 expression between the G and A alleles (P=0.171, Figure 5.12A). The mean (± SEM) IL-13 level (pg/ml) for patient *IL13* +2044 A was 1312.00 ± 227.40 compared to 1174.00 ± 213.50 for patient *IL13* +2044 G. Similar findings were also observed for donor *IL13* +2044 G/A. Donors with *IL13* +2044 A were shown to have slightly higher IL-13 levels than those with *IL13* +2044 G, however, the difference between the two groups did not reach significance (P=0.479, Figure 5.12B). The mean (± SEM) IL-13 level (pg/ml) for donor *IL13* +2044 A was 1384.00 ± 231.20 compared to 1242.00 ± 189.90 for donor *IL13* +2044 G. It therefore seems unlikely that the *IL13* +2044 G/A polymorphism was responsible for the higher IL-13 expression observed in the HSCT patients with acute and chronic GVHD.

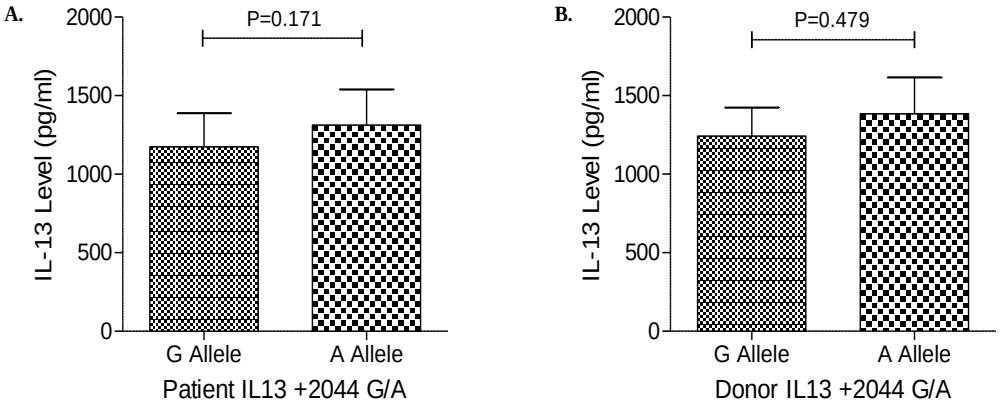

Figure 5.13 Correlation between IL-13 expression (pg/ml) in PHA stimulated supernatants and patient and donor *IL13* +2044 G/A alleles. Mean (± SEM) IL-13 expression was higher for both patients (**A.** n=60**)** and donors (**B.** n=60) with the *IL13* +2044 A allele (P=0.171 and 0.479 respectively). IL-13, interleukin-13; P, P value

In the genetic aspect of this study, patients with the *IL13* +2044 A allele were shown to be more susceptible to acute GVHD (section 5.5.4); similar findings were demonstrated in this HSCT cohort. 62% of the patients with *IL13* +2044 A developed clinical acute GVHD (grade II-IV) post-transplant, whereas only 46% of those with the G allele developed the condition (P=0.010 OR=7.991). Furthermore, 14% of the *IL13* +2044 A patients had severe acute GVHD (grade III-IV) compared to only 8% of the *IL13* +2044 G patients (P=0.037 OR=3.219). The genetic study also demonstrated that the HSCT patients receiving stem cell transplants from donors with the *IL13* +2044 A allele were more susceptible to chronic GVHD. In this cohort, 67% of the patients with *IL13* +2044 A donors developed chronic GVHD post-transplant, whilst only 59% of the patients with *IL13* +2044 G donors developed the condition (P=0.026 OR=5.016).

5.6 Discussion

Polymorphisms in the gene encoding *IL13* have been widely studied for associations with disease susceptibility. *IL13* +2044 G/A is of particular interest because it occurs in approximately 25% of the general population and results in an amino acid substitution (Vladich *et al.* 2005). Consequently, association studies have mainly been focused on this polymorphism. Asthma and allergy phenotypes have been thoroughly investigated due to the role that IL-13 plays in these biological pathways. In asthma, the *IL13* +2044 A allele has been strongly linked with disease occurrence. Heinzmann *et al* demonstrated an association between *IL13* +2044 A and allergic and non-allergic asthma in British and Japanese subjects (Heinzmann *et al.* 2000). The association was later confirmed by Arima *et al* (2002) and Heinzmann *et al* (2003) in children with bronchial asthma. *IL13* +2044 G/A has also been linked with elevated serum IL-13 levels in asthmatics. *IL13* +2044 A homozygous individuals have been shown to have significantly higher serum IL-13 levels than G homozygous individuals (Heinzmann *et al.* 2000; Arima *et al.* 2002).

The *IL13* +2044 G/A polymorphism has also been linked with IgE, a crucial pathogenic component of allergy (Punnonen *et al.* 1993; Minton 2008). The *IL13* +2044 A allele has been correlated with elevated total serum IgE levels, a characteristic seen in a number of allergic phenotypes (Graves *et al.* 2000; Liu *et al.* 2000; Heinzmann *et al.* 2003; Wang *et al.* 2003; Kabesch *et al.* 2006; Maier *et al.* 2006; Hunningshake *et al.* 2007). This allele has also been shown to strongly associate with atopy (He *et al.* 2003), atopic dermatitis (Liu *et al.* 2000; Tsunemi *et al.* 2002), food sensitization (Zitnik *et al.* 2009) and a grouped phenotype that includes eosinophilia, elevated IgE and positive skin tests (DeMeo *et al.* 2002). In terms of transplantation, no studies associating *IL13* polymorphisms and disease susceptibility have been published to date. Elevated IL-13 levels have however, been linked to chronic lung allograft rejection and acute GVHD (Jordan *et al.* 2004; Keane *et al.* 2007).

Polymorphism analysis of *IL13* +2044 G/A in this study has demonstrated a significant association with the development of acute GVHD following HSCT. Transplant patients possessing the A allele were more susceptible to severe acute GVHD (grades III-IV) than those with the G allele. This association was observed in the whole HSCT cohort (n=600, P=0.028 OR=3.977) and a smaller validation cohort that consisted of 50% of

the original transplant population selected at random (n=300, P=0.018 OR=6.762). The association also retained its significance when established clinical risk factors for acute GVHD were entered into the analysis (P=0.023 OR=2.260) and when the cohort was stratified into HLA-matched sibling and unrelated donor transplants (P=0.042 OR=1.794 and P=0.024 OR=3.722 respectively). It therefore seems likely that the *IL13* +2044 G/A polymorphism is an independent risk factor for severe acute GVHD following HLA-matched sibling and unrelated donor HSCT.

Our hypothesis from the outset was that, as acute GVHD is considered as a Th1 disease (Antin and Ferrara 1992; Ferrara 1998), IL-13; a Th2 cytokine, would have anti-inflammatory actions and therefore the *IL13* +2044 G/A polymorphism would be beneficial to the patient. However, the results were surprising and seem to challenge the notion that acute GVHD is purely a Th1 cytokine driven response. As IL-13 up-regulates MHC class II expression on monocytes, macrophages and B cells (Punnonen *et al.* 1993; Zurawski and de Vries 1994; de Vries 1998), it is possible that this cytokine could augment acute GVHD through enhanced antigen presentation to host reactive donor T cells. IL-13 may also contribute to the pathogenesis of acute GVHD through the activation and recruitment of immune cells, such as eosinophils, mast cells, monocytes and B cells (Hershey 2003).

The development of chronic GVHD was also shown to be significantly associated with the *IL13* +2044 G/A polymorphism in this study. Patients receiving stem cells transplants from donors with the *IL13* +2044 A were more susceptible to chronic GVHD than patients with *IL13* +2044 G donors. This association was observed in the whole HSCT cohort (n=600, P=0.010 OR=5.808), the smaller validation cohort (n=300, P=0.011 OR=10.107) and when established clinical risk factors for chronic GVHD were included in the analysis (P=0.013 OR=6.848). The association also retained its significance when the cohort was stratified into HLA-matched sibling and unrelated donor transplants (P=0.034 OR=5.063 and P=0.015 OR=9.851 respectively). It is therefore likely that the *IL13* +2044 G/A polymorphism is also an independent risk factor for chronic GVHD following HLA-matched sibling and unrelated donor HSCT.

Unlike acute GVHD, the pathophysiology of chronic GVHD remains largely unclear (Joseph *et al.* 2008). However, animal and human studies have demonstrated the presence of Th2 CD4+ allo-reactive T cells, a predominance of Th2 cytokines and B

cell rather than T cell activation in chronic GVHD (Allen *et al.* 1993; Tanaka *et al.* 1996; Slayback *et al.* 2000; Horwitz and Sullivan 2006). In addition, the elevation of Th2 cytokines has been shown to be consistent with the clinical manifestations of the condition (Ratanatharathorn *et al.* 2001). It therefore seems completely feasible that IL-13, a Th2 cytokine, could play a role in the development of chronic GVHD, a Th2 disease. Furthermore, as IL-13 is involved in the activation and proliferation of B cells (Punnonen *et al.* 1993; Chomarat and Banchereau 1998), a role for this cytokine in the development of the autoimmune component of chronic GVHD is plausible.

Polymorphism analysis of *IL13* +2044 G/A with respect to transplant type, also demonstrated significant associations between the *IL13* +2044 A allele and the development of acute and chronic GVHD. In both HLA-matched sibling and unrelated donor transplants, patients with *IL13* +2044 A were more susceptible to severe acute GVHD (grades III-IV) and those with *IL13* +2044 A transplant donors were more likely to develop chronic GVHD (acute GVHD III-IV, P=0.042 OR=1.794 and P=0.024 OR=3.722 respectively; chronic GVHD, P=0.034 OR=5.063 and P=0.015 OR=9.851 respectively), however the associations were much more significant in the latter group. This was further supported when HLA-matched sibling and unrelated donor transplants were tested against each other using multivariate analysis. The demonstrated associations between patient *IL13* +2044 A and acute GVHD (III-IV) and donor *IL13* +2044 A and chronic GVHD were again observable in both groups (acute GVHD III-IV: P=0.091 OR=0.798, P=0.020 OR=5,451; chronic GVHD: P=0.076 OR=0.703, P=0.011 OR=11.087 respectively), however, they were found to be significantly stronger in the unrelated donor HSCT cohort. Although this data requires replication, the stronger associations demonstrated in the unrelated donor population seem to suggest that the *IL13* +2044 G/A polymorphism is of greater importance in this transplant setting. The variation in association strengths observed for the HLA-matched sibling and unrelated donor cohorts is likely to result from differences in the biology (unrelated donor HSCT is typically associated with a greater degree of HLA disparity, enhanced allo-recognition and a heightened immune response compared to HLA-matched sibling transplantation) and clinical protocols of the two transplant types. As discussed previously (section 4.11), these differences also highlight the need for non-HLA polymorphisms to be analysed independently in both transplant populations, as significant associations identified in one may not necessarily be reflective of the other.

The origins of the genetic associations demonstrated further substantiate the findings of this study. In all groups analysed, patient *IL13* +2044 A correlated with acute GVHD and donor *IL13* +2044 A correlated with chronic GVHD, both of which fit with the biology of HSCT and the time-scale for GVHD development post-transplant. Cells typically switch from patient to donor origin between 3-6 months post-HSCT, the time period traditionally used to differentiate between acute and chronic GVHD.

Polymorphism analysis of *IL13* +2044 G/A failed to demonstrate any significant associations with overall survival or TRM in this study. Although the associations did not reach significance, the incidence of disease relapse was however shown to be lower at 12, 24, 36, 48 and 60 months post-transplant for patients and donors with the *IL13* +2044 A allele (patient, P=0.336; donor, P=0.453). As the relapsed population analysed comprised of only 172 HSCT patients, the small nature of this cohort may have prevented these findings reaching significance (type II error). It would therefore be of interest to examine *IL13* +2044 G/A in a larger cohort of relapsed patients. Sample collection for this European study is ongoing and should facilitate such an investigation in the future. Confirmation of these findings would however, further support the observed associations between *IL13* +2044 A and GVHD in our HSCT cohort. Animal and human studies have demonstrated that acute and chronic GVHD lower the incidence of relapse via the GVL effect (Weiden *et al.* 1979; Weiden *et al.* 1981; Horowitz *et al.* 1990). Host reactive donor T cells that produce GVHD are also capable of destroying residual malignant cells, thereby reducing the possibility of the disease reoccurring (Kolb 2008). The potency of the GVL effect is illustrated by the ability of DLI to induce a durable remission in patients with relapsed leukaemia following transplantation (Kolb *et al.* 1995; Collins *et al.* 1997). Thus, if patients with the *IL13* +2044 A allele are more susceptible to acute and chronic GVHD it is possible that they will get less relapse through this mechanism.

Currently, there is no solid link in the literature between the *IL13* +2044 G/A polymorphism and altered IL-13 expression. However, higher serum IL-13 levels in asthmatic individuals with the *IL13* +2044 A,A genotype have been reported in two independent studies (Heinzmann *et al.* 2000; Arima *et al.* 2002). Considering these findings, in combination with the genetic associations already demonstrated in this study, it would seem biologically plausible that polymorphic variation in the *IL13* gene could influence IL-13 levels and the subsequent development of GVHD.

At present, IL-13 functional studies in the HSCT setting are extremely limited. Only a single study examining IL-13 expression in relation to GVHD has been published to date. In this study, Jordan *et al* (2004) demonstrated a significant association between high IL-13 production by donor T cells in allogeneic MLCs and the development of severe acute GVHD following HSCT. In this current study, IL-13 expression in allogeneic MLCs was correlated with the severity of acute GVHD using an *in vitro* model (the human skin explants model of acute GVHD). IL-13 expression by donor cells was found to increase with increasing GVHR severity (Figure 5.3) and the highest IL-13 levels were observed in the patients with the most severe GVHR grades (grades III-IV; P=0.008, Figure 5.4). Thus, our findings agree with those of Jordan *et al* (2004) and seem to suggest that IL-13 production by donor T cells during the allo-response is accountable for at least some of the pathology associated with severe acute GVHD. However, the findings of these studies differ greatly to those of a recent murine study, where IL-13 was shown to abrogate acute GVHD by down-regulation of TNFα production (Hildebrandt *et al.* 2007). Hildebrandt *et al* (2007) suggested that since allo-reactive T cells are a significant source of IL-13, the observed correlation between IL-13 expression in MLCs and GVHD simply reflected an overall allo-activation state of the donor T cells rather than a direct link between IL-13 and GVHD severity.

IL-13 expression in allogeneic MLCs was also correlated with clinical acute and chronic GVHD in this study. No significant associations were observed with either form of GVHD; however, the relationship between IL-13 expression and acute GVHD was seemingly opposite to that of the human skin explant model; IL-13 levels were found to decrease with increasing acute GVHD grades (Figure 5.6). The most likely explanation for this discrepancy is that the human skin explant model is a clean system. This *in vitro* model does not account for GVHD prophylaxis drugs that are routinely employed in the clinical setting to reduce the incidence and severity of acute GVHD. Wang *et al* (2006) have demonstrated similar discrepancies, with human skin explant GVHR grades correlating significantly with clinical acute GVHD in HLA-matched sibling transplant patients treated with CsA alone, but not those treated with CsA + MTX. In this current study, as a result of the administration of high intensity GVHD prophylaxis post-transplant (CsA ± MTX or MM), 54 of the 61 patients with GVHR grades III-IV in the human skin explant model developed only grades 0-II acute GVHD in the clinical setting. Consequently, the high *in vitro* production of IL-13 was not reflective of the markedly reduced acute GVHD observed *in vivo*. Thus, the development of an *in vitro*

model of acute GVHD that includes the administration of GVHD prophylaxis drugs would be more representative of HSCT *in vivo* and may therefore be a more useful research tool.

As previously described, Jordan *et al* (2004) demonstrated a significant association between high IL-13 production in allogeneic MLCs and the development of severe acute GVHD in the clinical setting. This study, however, failed to reproduce the finding. Instead our results showed an inverse relationship between high IL-13 expression and acute GVHD severity. Differences in methodology may account for this discrepancy. In the present study, raw IL-13 expression data from 90 allogeneic MLCs was correlated with acute GVHD, whereas Jordan *et al* correlated positive and negative IL-13 expression in 24 allogeneic MLCs with the condition. In addition to the noticeable difference in sample size, the transplant cohort in the latter study was divided into positive and negative IL-13 producers using a seemingly arbitrary cut-off value of 3 standard deviations above the lowest level of detection (Jordan *et al.* 2004). Manipulation of the data in such a manner, especially considering the small nature of the cohort examined, may have introduced some degree of error into the study. It is arguable that our approach, which relies on the direct comparison of IL-13 levels with clinical acute GVHD grades, is more accurate and sensitive to subtle differences in IL-13 expression. Despite this, due to the small nature of both studies the findings should be interpreted with caution, until either is confirmed in a larger allogeneic MLC cohort.

In vivo IL-13 expression was also examined in this study. Initially, IL-13 serum levels in HSCT patients and healthy controls were compared. The significantly higher levels observed in the transplant patients at all eight time points (day -7, day 0, day 7, day 14, day 28, 3 months, 6 months and 12 months) investigated ($P<0.0001$, Figure 5.8) indicate that HSCT has a substantial affect on *in vivo* IL-13 expression, while, the extremely low IL-13 serum levels demonstrated in the healthy controls seem to suggest that IL-13 release is predominantly from activated cells. As IL-13 levels in the healthy controls remained relatively constant, it is likely that the variability observed in patient IL-13 expression was transplant, treatment or outcome related rather than a naturally occurring phenomenon. Noticeable decreases in IL-13 were observed on day 7, 6 months and 12 months post-transplant in all 30 HSCT patients and most probably reflected the effects of the transplant procedure on patient and/or donor cell numbers. The administration of steroids, such as methylprednisone and prednisolone, can also

cause a similar reduction in cytokine expression by the suppression of immune (T) cells. However, as the decreases in IL-13 expression were observed in both patients with and without GVHD, the cause is unlikely to be steroid related. A relatively sharp increased in IL-13 between day -7 (pre-transplant) and day 0 was also observed in all 30 HSCT patients. This rise coincided with the period of conditioning prior to HSCT. As conditioning regimens used to prepare the patient for transplantation cause tissue damage and activate host cells, the increase in IL-13 serum levels between these time points may reflect the release of this cytokine from activated T and other immune cells in response to the damage. As IL-13 can up-regulate MHC expression (Punnonen *et al.* 1993; Chomarat and Banchereau 1998), it is possible that the IL-13 produced during the conditioning period contributes to the first phase of the acute GVHD mechanism. The higher expression observed between day -7 and day 0 in the patients with acute GVHD compared to those without the condition (Figures 5.9 and 5.10) seem to support this hypothesis. However, it is also possible that the IL-13 expression observed between the two time points is merely a by-product from activated cells; particularly T cells and the higher IL-13 serum levels associated with acute GVHD are simply reflective of the heightened activation state in these patients.

In vivo IL-13 expression was also examined with respect to HSCT outcomes. Correlations could not be made with TRM in this study, as all observed deaths were relapse related. No associations were demonstrated with overall survival and relapse incidence. However, elevated IL-13 serum expression was observed in HSCT patients with GVHD. Transplant patients with overall acute GVHD (grades I-III) had higher IL-13 serum levels than those with no acute GVHD at all time points tested (day -7, day 0, day 7, day 14, day 28 and 3 months; Figure 5.9). Furthermore, HSCT patients with chronic GVHD had considerably higher IL-13 serum levels than those without the disease (Figure 5.11). Although this data should be interpreted with some caution due to the small nature of the HSCT population examined (n=30), the findings of this *in vivo* expression study seem to suggest a role for IL-13 in both acute and chronic GVHD. As IL-13 levels were higher at all time points across the transplant period it is unlikely that the expression of this cytokine is related solely to the onset of acute or chronic GVHD; a role in the maintenance of these conditions is more likely. As IL-13-mediated Th2 effector functions are capable of producing the inflammation associated with asthma (Grunig *et al.* 1998; Wills-Karp *et al.* 1998; Walter *et al.* 2001), it is possible that IL-13 secreted from donor T cells during GVHD contributes similarly to this inflammatory

response. Considering the associations already demonstrated between the *IL13* +2044 A allele and acute and chronic GVHD, it is possible that this allele is involved in the up-regulation of IL-13 observed in GVHD.

Possession of the *IL13* +2044 A allele was demonstrated to associate with the development of severe acute (grades III-IV) and chronic GVHD in the HSCT cohort utilised in the functional study (P=0.037 OR=3.219 and P=0.026 OR=5.016 respectively). However, altered IL-13 expression could not be correlated with the *IL13* +2044 G/A polymorphism in this cohort; there was no significant difference in the IL-13 levels of *IL13* +2044 A or G individuals. It therefore seems extremely likely that the *IL13* +2044 G/A polymorphism is linked with acute and chronic GVHD susceptibility, although, it is unlikely that the *IL13* +2044 A allele is directly responsible for the elevated IL-13 levels observed in patients with these conditions. The increased stability of *IL13* +2044 A encoded IL-13 molecules in serum and plasma (Arima *et al.* 2002) is a more plausible explanation for the slightly higher IL-13 levels observed in the individuals with the *IL13* +2044 A allele (Figure 5.12).

Given that the location of *IL13* +2044 G/A is in exon 4, a gene coding region; it is more likely that the polymorphism will affect the structure and/or function of IL-13 rather than its expression. In the single published study examining the structural and functional significance of *IL13* +2044 G/A, the G→A acid substitution caused by the polymorphism was shown to occur in α helix D, a region that is critical for IL-13/IL-13 receptor interactions and IL-13-mediated signalling (Vladich *et al.* 2005). *IL13* +2044 A encoded IL-13 was also shown to be much more active than wild-type IL-13 at triggering the IL-13 signalling pathway (IL-4Rα-Stat6 pathway) and inducing IL-13-mediated effector functions, such as CD23 expression and IgE synthesis (Vladich *et al.* 2005). Moreover, *IL13* +2044 A encoded IL-13 was found to be neutralized less effectively by IL-13Rα2, a soluble receptor that normally restrains the activity of wild-type IL-13 *in vivo* (Vladich et al. 2005). Thus, the findings of this functional study seem to suggest that the *IL13* +2044 A allele increases the biological activity of IL-13. As this cytokine is a potent effector of Th2-mediated responses it is therefore possible that *IL13* +2044 A mediates its effects on GVHD through augmentation of these responses.

Despite the associations demonstrated between *IL13* +2044 A and acute and chronic GVHD in this study, it is highly unlikely that a single polymorphic allele is solely responsible for any role IL-13 plays in the development of GVHD. It is more likely that the effects of IL-13 are also mediated by other polymorphisms within *IL13* or other genes in close proximity. One such polymorphism is *IL13* -1112 C/T. This promoter polymorphism has been found to be in strong linkage disequilibrium with *IL13* +2044 G/A in several populations (Tarazona-Santos and Tishkoff 2005) and the *IL13* -1112 T allele has been shown to increase IL-13 production through enhanced *IL13* transcription (van der Pouw Kraan *et al.* 1999; Cameron *et al.* 2006). Although the individual effects of *IL13* +2044 G/A and *IL13* -1112 C/T are relatively modest, any increase in IL-13 activity caused by *IL13* +2044 A combined with the enhanced *IL13* transcription associated with *IL13* -1112 T may synergize to amplify IL-13-dependent events (van der Pouw Kraan *et al.* 1999; Vladich *et al.* 2005). Thus, individuals with both *IL13* +2044 A and *IL13* -1112 T are expected to produce large amounts of an over-active form of IL-13 (Vercelli 2008). It would therefore be of great importance to examine the *IL13* -1112/+2044 T-A haplotype in the HSCT setting. The investigation of other functional polymorphisms in linkage disequilibrium with *IL13* +2044 G/A would also be of interest.

The genetic associations demonstrated with severe acute and chronic GVHD in this study provide strong evidence that the *IL13* +2044 G/A polymorphism is an independent risk factor for the development of these conditions following both HLA-matched sibling and unrelated donor HSCT. Thus, it is extremely likely that *IL13* +2044 G/A will provide key pre-transplant information and may strengthen the predictive value of a prognostic index for GVHD when included alongside other significant clinical and genetic parameters. Although this polymorphism was examined in a relatively large cohort (n=600), the confirmation of our findings in an even larger independent transplant population would be of greater significance, as this would provide evidence of reproducibility and would allow the heterogeneous nature of HSCT cohorts to be accounted for more effectively (section 4.11). The future analysis of polymorphisms in linkage disequilibrium with *IL13* +2044 G/A is also of great importance. As the modest influence that individual polymorphisms have on GVHD may become much more significant when considered in combination.

The functional significance of the *IL13* +2044 G/A polymorphism in GVHD is however, less clear. The higher IL-13 expression observed in patients with acute and chronic GVHD seems to implicate IL-13 in the pathogenesis of these conditions, but this altered expression could not be directly attributed to *IL13* +2044 G/A. IL-13 production by allo-reactive donor T cells during GVHD and/or increased *IL13* gene transcription by linked polymorphisms are more likely to be responsible. Published functional data seems to suggest that *IL13* +2044 A increases the activity and stability of IL-13 rather than its expression. Thus, carriers of this allele will have enhanced IL-13-mediated Th2 effector functions. Further functional studies are required to confirm this heightened activity, however, verification of the finding would suggest specific blocking of IL-13 as a possible future GVHD treatment for patients with *IL13* +2044 A.

Chapter 6. Prognostic Significance of Elevated IL-13 Serum Levels Prior to HSCT

It is well established that the production of inflammatory cytokines following HSCT strongly associates with the development of transplant complications, particularly GVHD (Antin and Ferrara 1992; Allen *et al.* 1993; Via and Finkelman 1993; Hempel *et al.* 1997; Liem *et al.* 1998; Remberger *et al.* 2003; Schot *et al.* 2003; Visentainer *et al.* 2003). However, the release of these cytokines prior to transplantation may also correlate with HSCT outcome. Studies have shown elevated TNFα production during the course of pre-transplant conditioning to be highly predictive of acute GVHD (Holler *et al.* 1995), whilst increased production of IL-10 at the time of hospital admission has been found to predict an uneventful course, with the absence of acute GVHD (Holler *et al.* 2000). Such findings seem to suggest that cytokine monitoring prior to transplantation may be a means of gaining valuable prognostic information, that in the future may allow the identification of high risk patients and the early detection of major complications in these individuals.

Considering the high degree of variability observed in the pre-transplant (day -7) IL-13 serum levels obtained for the Newcastle HSCT patients examined in the previous chapter, it is possible that altered expression of this cytokine prior to transplantation may also inform on outcome following HSCT. As a result, this current study aims to establish the prognostic significance, if any, of IL-13 serum expression prior to transplantation.

6.1 Study Aims

v) To establish and compare pre-transplant IL-13 serum levels in Newcastle and Regensburg HSCT patients.

vi) To compare HSCT outcome, in terms of acute GVHD incidence and severity, chronic GVHD incidence, relapse incidence and overall survival with pre-transplant IL-13 serum levels in Newcastle and Regensburg HSCT patients.

vii) To determine the effects of clinical and patient parameters on pre-transplant IL-13 serum levels.

6.2 Statistical Analysis

As the two HSCT cohorts examined in this study were small (Newcastle n=30, Regensburg n=36), normality tests were not performed and all correlations were examined using non-parametric methods. The Mann-Whitney U test was employed when comparing two groups and the Kruskal-Wallis test was utilised when three or more groups were present. Data analysed with respect to age, gender and CMV status were tested using Fisher's exact test. In all cases, P values <0.05 were considered statistically significant and values between 0.05 and 0.1 were indicative of a trend.

6.3 Experimental Design

6.3.1 Inclusion and Exclusion Criteria

All patients recruited as part of a European Commission FP5 biotechnology project (Eurobank QLRI-CT-2000-00010, PI Professor A M Dickinson), that were transplanted between November 1983 and December 2006 and had pre-transplant serum samples and 1 year's clinical follow-up available, were included in the analyses. The study was given JLEC approval (Appendix B) and informed consent was obtained from all patients. Exclusion criteria were as follows:

i) Transplantation not performed
ii) Incomplete HSCT outcome data
iii) Incomplete HLA typing data
iv) Below the age of 16 at the time of transplant
v) Transplantation for an immunological disease
vi) Transplantation with a source other than PBSC or bone marrow
vii) Two or more transplants performed

Once the inclusion and exclusion criteria had been taken into account, the Newcastle, UK (cohort 1) and Regensburg, German (cohort 2) HSCT populations examined in this study consisted of 30 and 36 patients respectively.

6.3.2 Control Measures

To exclude treatment related effects, all serum samples employed were obtained at the time of hospital admission and prior to the administration of any conditioning regimens. IL-13 quantification was achieved using the Human IL-13 Low Level ELISA Kit described in section 3.13. All ELISAs performed included an appropriate standard dilution series and a negative control. Samples and standards were tested in duplicate and the mean value for each set was used for quantification. Samples were re-assayed if the values for the two replicates varied by more than 15%. The quantification of IL-13 in all serum samples was performed whilst blinded to clinical outcome.

6.3.3 Method of Analysis

In an attempt to prevent spurious results, pre-transplant IL-13 serum levels were examined in two independent HSCT cohorts. In order to demonstrate that selection bias was absent, the IL-13 levels determined for each of these cohorts were compared prior to any correlations with HSCT outcome. The prognostic significance of IL-13 was then established by examining for associations between pre-transplant IL-13 levels and the incidence and severity of acute GVHD, incidence of chronic GVHD, relapse incidence, and overall survival. Correlations with TRM could not be carried out in this study, as only 5 patients died from causes other than relapse. The affect of clinical and patient factors, including age, gender, CMV status, underlying disease, disease stage at the time of HSCT and *IL13* +2044 G/A alleles on IL-13 expression was also established.

The two HSCT cohorts employed in this study were mixed for transplant type (HLA-matched sibling/unrelated donor), underlying disease, disease stage (early/advanced), conditioning regimen (low intensity/myeloablative), prophylaxis intensity (low/high) and graft manipulation (T cell replete/T cell deplete); all of which have the potential to influence HSCT outcome. The most accurate and reliable means of accounting for this heterogeneity is cohort stratification. However, due to the small nature of both populations, this approach could not be performed.

6.4 Study Populations

6.4.1 *Cohort 1*

Newcastle HSCT Patients

Pre-transplant IL-13 serum levels were examined in 30 allogeneic HSCT patients. This was an adult cohort (≥16 at the time of transplant) comprising of 26 HLA-matched sibling HSCT patients and 6 unrelated donor transplant patients. HLA matching was performed by high resolution DNA-based typing for HLA-A, B, C, DRB1 and DQB1. Complete matching at all 5 loci was observed in all transplant pairs.

Cohort characteristics:
The 30 HSCT patients included in this cohort were transplanted between November 1983 and December 2006 at the Royal Victoria Infirmary, Newcastle upon Tyne, UK. All patients and donors were recruited and consented as part of the previously described 'Eurobank Project' (section 6.3.1).

Acute GVHD was assessable in all 30 patients and developed in 24. The severity of GVHD ranged from 0-III; 6 patients developed no GVHD, 10 grade I, 10 grade II and 4 grade III. Chronic GVHD was assessable in 20 patients and developed in 12 cases. All transplant patients were prepared for HSCT with low intensity (n=18) or myeloablative conditioning (n=12) regimens and 57% (n=17) were transplanted with T cell depleted stem cell grafts. The survival rate for this cohort was 77% (n=23). The cause of death (n=7) in all cases was disease relapse. Table 6.1 shows the demographic characteristics of the HSCT patients included in this study.

Table 6.1 Characteristics of the Newcastle HSCT Patients (cohort 1 n=30). CMV, cytomegalovirus; TCD, T cell depletion

TRANSPLANT PATIENTS

Median Age		40 years (range 19-57)
		Number in each group
Gender		
	Male	21
	Female	9
CMV Status		
	Positive	16
	Negative	14
Underlying Disease		
	Acute Lymphoblastic Leukaemia	3
	Acute Myeloid Leukaemia	9
	Chronic Myeloid Leukaemia	3
	Hodgkin disease	5
	Non-Hodgkin Lymphoma	4
	Plasma Cell Neoplasm	3
	Aplastic Anaemia	1
	Myelodysplastic Syndrome	2
Conditioning Therapy		
	Low intensity	18
	Myeloablative	12
	TCD	17
HSCT Outcome		
	Acute GVHD	24
	Chronic GVHD	12
	Relapse	10
	Survival	23

6.4.2 *Cohort 2*

Regensburg HSCT Patients

Pre-transplant IL-13 serum levels were examined in 36 allogeneic HSCT patients. This was an adult cohort (≥16 at the time of transplant) comprising of 15 HLA-matched sibling HSCT patients and 21 unrelated donor transplant patients. HLA matching was performed by high resolution DNA-based typing for HLA-A, B, C, DRB1 and DQB1. Complete matching at all 5 loci was observed in all transplant pairs.

Cohort characteristics:
The 36 HSCT patients included in this cohort were transplanted between November 1983 and December 2006 at the University Medical Centre, Regensburg, Germany. All patients and donors were recruited and consented as part of the previously described 'Eurobank Project' (section 6.3.1).

Acute GVHD was assessable in all 36 patients and developed in 20. The severity of GVHD ranged from 0-IV; 16 patients developed no GVHD, 11 grade I, 4 grade II, 4 grade III and 1 grade IV. Chronic GVHD was assessable in 30 patients and developed in 15 cases. All transplant patients were prepared for HSCT with low intensity (n=29) or myeloablative conditioning (n=7) regimens and 61% (n=22) were transplanted with T cell depleted stem cell grafts. The survival rate for this cohort was 75% (n=27) and causes of death (n=9) included disease relapse, infection and GVHD. Table 6.2 shows the demographic characteristics of the HSCT patients included in this study.

Table 6.2 Characteristics of the Regensburg HSCT Patients (cohort 2 n=36). CMV, cytomegalovirus; TCD, T cell depletion

TRANSPLANT PATIENTS

Median Age		42 years (range 20-67)
		Number in each group
Gender		
	Male	21
	Female	15
CMV Status		
	Positive	19
	Negative	17
Underlying Disease		
	Acute Lymphoblastic Leukaemia	3
	Acute Myeloid Leukaemia	21
	Chronic Myeloid Leukaemia	2
	Hodgkin disease	3
	Non-Hodgkin Lymphoma	3
	Plasma Cell Neoplasm	2
	Aplastic Anaemia	1
	Myelodysplastic Syndrome	1
Conditioning Therapy		
	Low intensity	29
	Myeloablative	7
	TCD	22
HSCT Outcome		
	Acute GVHD	20
	Chronic GVHD	15
	Relapse	9
	Survival	27

6.5 Comparison of Pre-transplant IL-13 Serum Expression in Two Independent HSCT Cohorts

Prior to any correlations with HSCT outcome, pre-transplant IL-13 serum levels for the two independent cohorts (cohort 1 and 2) examined in this study were compared. Although IL-13 expression in general was found to be slightly lower for cohort 2, there was no significant difference in the two populations (P=0.175, Figure 6.1). The mean (± SEM) IL-13 level (pg/ml) in cohort 1 was 5.85 ± 0.63 compared to 4.84 ± 0.43 in cohort 2. In addition, low, intermediate and high IL-13 producers were observed in both cohorts. These data therefore demonstrate that selection bias was absent and the prognostic significance of pre-transplant IL-13 expression could be determined in both cohorts.

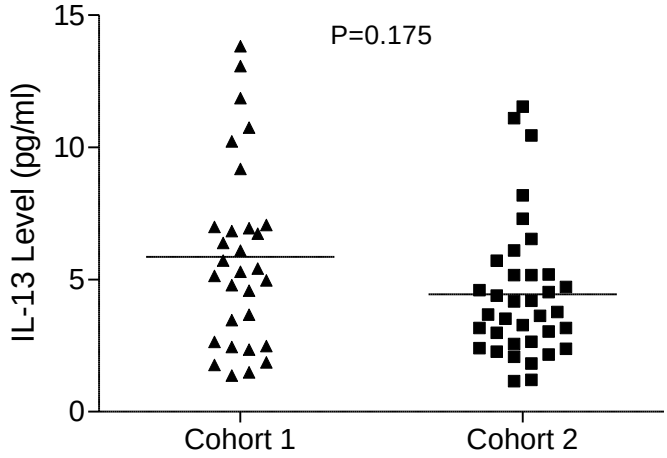

Figure 6.1 Comparison of pre-transplant IL-13 expression (pg/ml) in two independent populations of HSCT patients. No significant difference was observed in the mean (± SEM) IL-13 serum level for cohort 1 (n=30) and cohort 2 (n=36, P=0.175). IL-13, interleukin-13; P, P value

6.6 Correlation of Pre-Transplant IL-13 Serum Expression with HSCT Outcome

6.6.1 *Correlation of IL-13 Serum Expression with Acute GVHD*

Pre-transplant IL-13 serum levels were correlated with overall acute GVHD in cohort 1 and 2. No significant difference was observed between IL-13 levels in patients with or without the condition after transplantation (cohort 1 P=0.979; cohort 2 P=0.689, Figure 6.2). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 5.90 ± 0.71 and 4.33 ±0.47 respectively for patients with overall acute GVHD (grades I-IV) compared to 5.66 ± 1.50 and 4.75 ± 0.95 respectively for those with no acute GVHD.

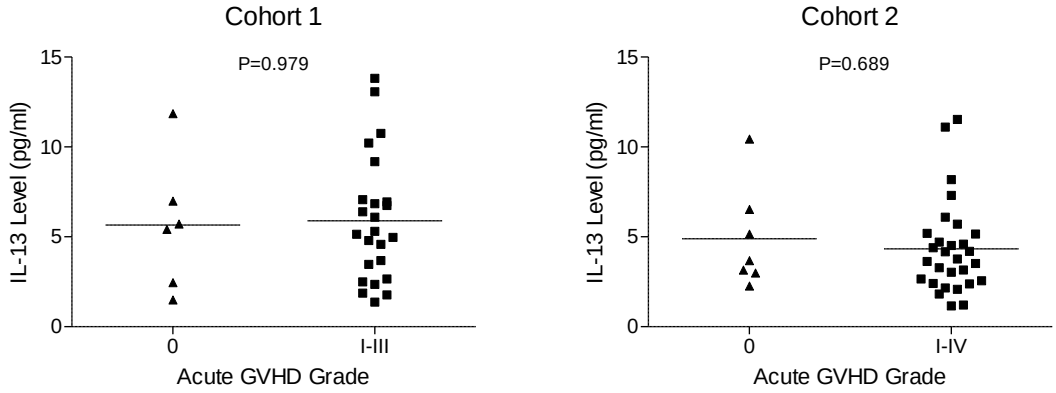

Figure 6.2 Correlation of pre-transplant IL-13 serum expression (pg/ml) with overall acute GVHD (grades I-IV) in two independent HSCT cohorts. No significant difference was demonstrated between the mean (± SEM) IL-13 level for patients with and without overall acute GVHD (grades I-IV) in cohort 1 or cohort 2 (P=0.979 and P=0.689 respectively). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

Pre-transplant IL-13 serum levels were also correlated with clinical acute GVHD (grades II-IV) in cohort 1 and cohort 2. Again, no significant difference was observed between IL-13 levels in patients with or without the condition after transplantation (cohort 1 P=0.819; cohort 2 P=0.661, Figure 6.3). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 5.58 ± 0.86 and 4.96 ± 1.04 respectively for patients with clinical acute GVHD (grades II-IV) compared to 6.08 ± 0.93 and 4.27 ± 0.47 respectively for those with minimal or no acute GVHD (grades 0-I).

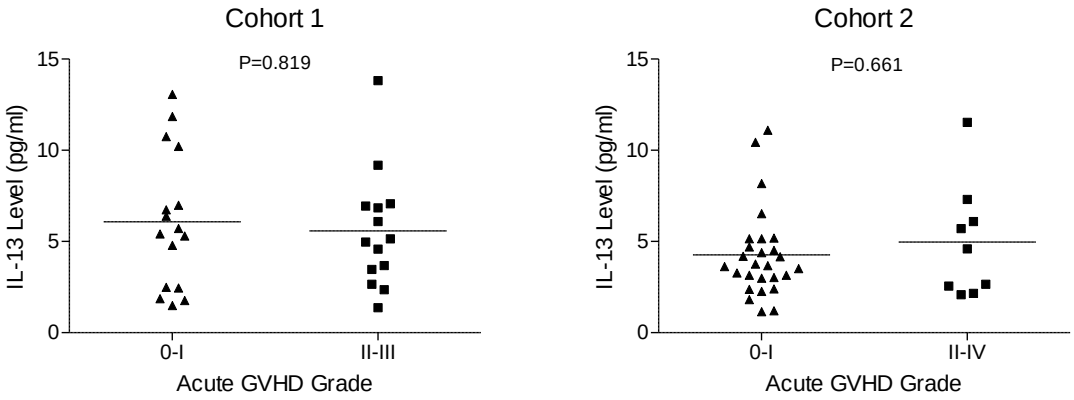

Figure 6.3 Correlation of pre-transplant IL-13 serum expression (pg/ml) with clinical acute GVHD (grades II-IV) in two independent HSCT cohorts. No significant difference was observed between the mean (± SEM) IL-13 level for patients with and without clinical acute GVHD (grades II-IV) in cohort 1 or cohort 2 (P=0.819 and P=0.661 respectively). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

Finally, pre-transplant IL-13 serum levels were correlated with severe acute GVHD (grades III-IV) in cohort 1 and cohort 2. No significant difference was observed between IL-13 levels in patients with severe (grades III-IV) or mild (grades 0-II) forms of the condition after transplantation (cohort 1 P=0.342; cohort 2 P=0.468, Figure 6.4). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 4.30 ± 0.77 and 4.78 ± 1.01 respectively for patients with severe acute GVHD (grades III-IV) compared to 5.89 ± 0.71 and 4.25 ± 0.51 respectively for those with mild acute GVHD (grades 0-II).

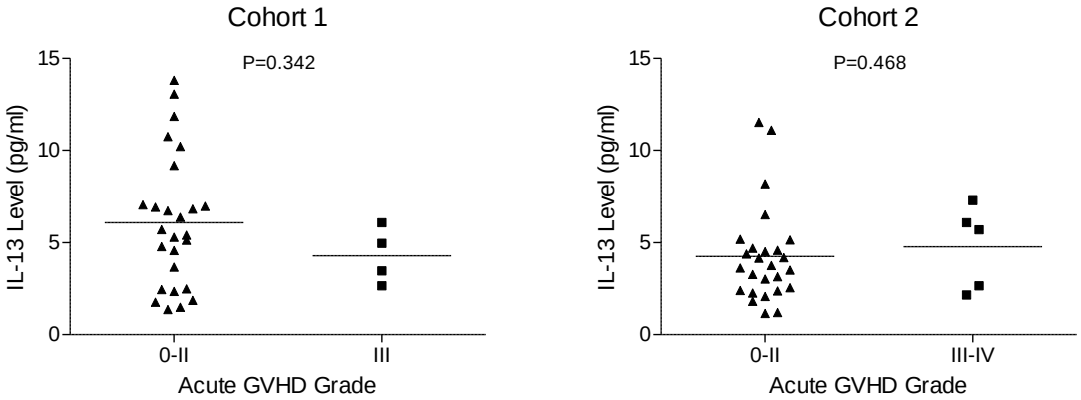

Figure 6.4 Correlation of pre-transplant IL-13 serum expression (pg/ml) with severe acute GVHD (grades III-IV) in two independent HSCT cohorts. No significant difference was observed between the mean (± SEM) IL-13 level for patients with and without severe acute GVHD (grades III=IV) in cohort 1 or cohort 2 (P=0.342 and P=0.468 respectively). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

6.6.2 *Correlation of IL-13 Serum Expression with Chronic GVHD*

Correlation of pre-transplant IL-13 serum levels with chronic GVHD following transplantation revealed that there was no significant difference between IL-13 levels in the patients with or without the condition (cohort 1 P=0.472; cohort 2 P=0.772, Figure 6.5). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 5.33 ± 0.80 and 4.55 ± 0.63 respectively for patients with chronic GVHD compared to 6.49 ± 1.17 and 4.37 ± 0.64 respectively for those without chronic GVHD.

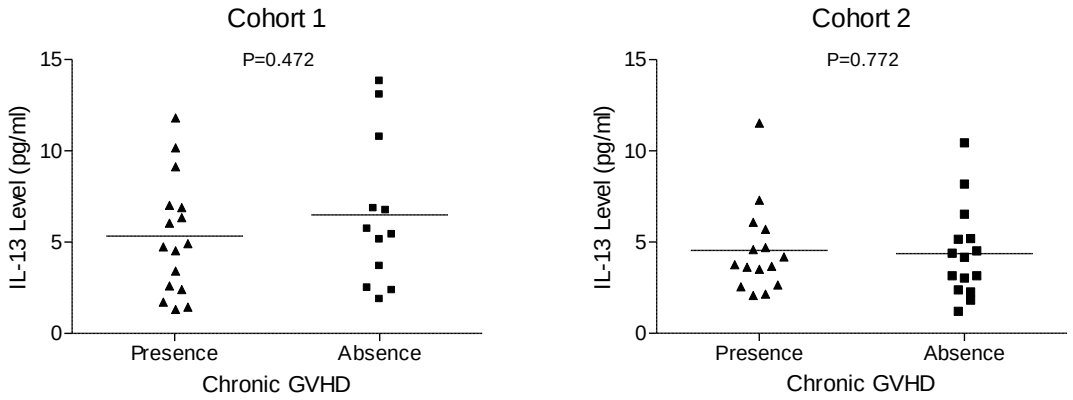

Figure 6.5 Correlation of pre-transplant IL-13 serum expression (pg/ml) with chronic GVHD in two independent HSCT cohorts. No significant difference was observed between the mean (± SEM) IL-13 level for patients with and without chronic GVHD in cohort 1 or cohort 2 (P=0.472 and P=0.772 respectively). IL-13, interleukin-13; P, P value; GVHD, graft-versus-host disease

6.6.3 *Correlation of IL-13 Serum Expression with Relapse Incidence*

Comparison of pre-transplant IL-13 serum levels with the incidence of relapse revealed a correlation in both cohort 1 and cohort 2. IL-13 levels were demonstrated to be higher for the patients who had disease relapse following HSCT (cohort 1 P=0.012; cohort 2 P<0.0001, Figure 6.6). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 8.61 ± 1.17 and 7.83 ± 0.88 respectively for relapsed patients compared to only 4.47 ± 0.53 and 3.31 ± 0.24 respectively for those with no relapse.

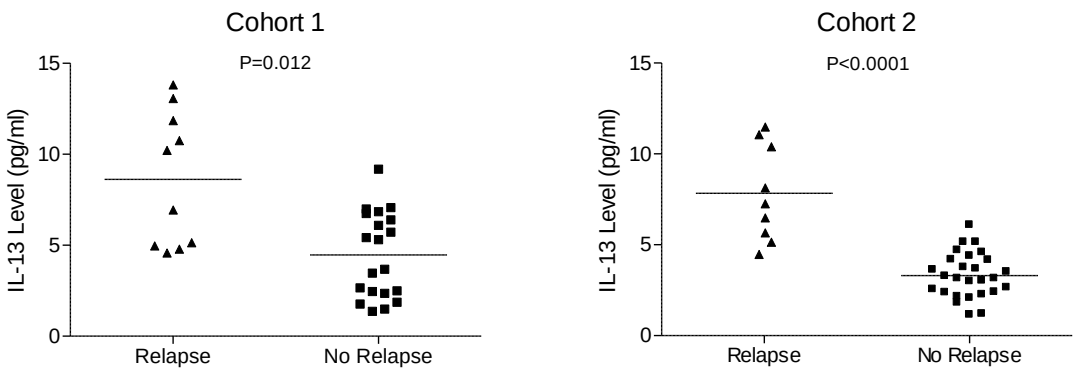

Figure 6.6 Correlation of pre-transplant IL-13 serum expression (pg/ml) with the incidence of relapse in two independent HSCT cohorts. The mean (± SEM) IL-13 level was significantly higher for patients with disease relapse following transplantation in cohort 1 and cohort 2 (P=0.012 and P<0.0001 respectively). IL-13, interleukin-13; P, P value

6.6.4 *Correlation of IL-13 Serum Expression with Post-Transplant Survival*

Correlation of pre-transplant IL-13 serum levels with post-transplant survival demonstrated an association with increased mortality in cohort 1. IL-13 levels were found to be higher for patients who died following transplantation (P=0.019, Figure 6.7). The mean (± SEM) IL-13 level (pg/ml) for patients alive after HSCT was only 4.91 ± 0.61 compared to 8.94 ± 1.27 for those who died. However, this association could not be replicated in cohort 2. No significant difference in IL-13 expression was observed between the patients who were alive or died post-transplant in this cohort (P=0.465, Figure 6.7). The mean (± SEM) IL-13 level (pg/ml) for patients alive after HSCT was 4.08 ± 0.39 compared to 5.51 ± 1.25 for those that died.

In terms of the cause of death, all 7 patients in cohort 1 died from disease relapse. In cohort 2, 4 patients died from disease relapse; the highest IL-13 producers, 3 died from infection and 2 patients died from GVHD.

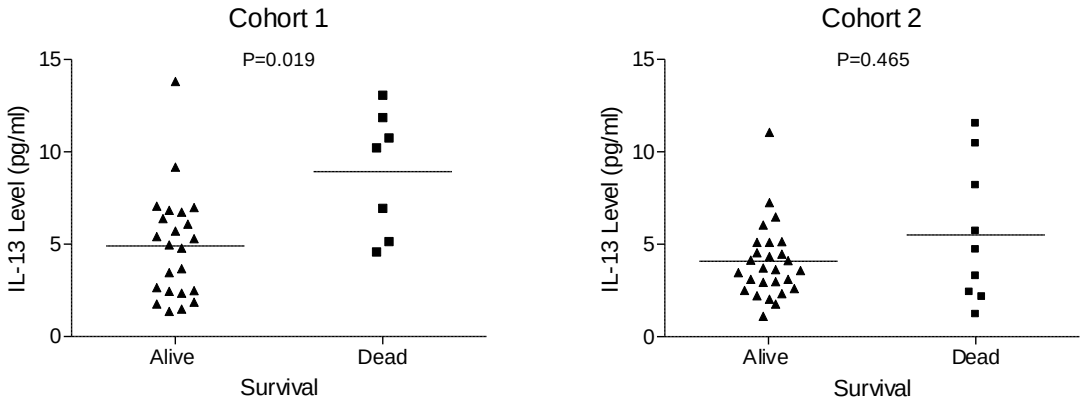

Figure 6.7 Correlation of pre-transplant IL-13 serum expression (pg/ml) with post-transplant survival in two independent HSCT cohorts. In cohort 1, the mean (± SEM) IL-13 level was significantly higher for patients who died following transplantation (P=0.019). In cohort 2, no significant difference was demonstrated in the mean (± SEM) IL-13 level for patients who were alive or died post-transplant (P=0.465). IL-13, interleukin-13; P, P value

6.7 Analysis of Risk Factors of Elevated IL-13 Serum Expression

A variety of clinical and patient parameters were analysed in order to identify factors associated with elevated IL-13 serum expression prior to transplantation. The parameters included in this aspect of the study were age, gender, CMV status, underlying disease, stage of disease at the time of HSCT and *IL13* +2044 G/A alleles.

6.7.1 *Patient Age*

As patient age over 40 years is a considerable clinical risk factor in HSCT (Gratwohl *et al.* 1998; Cavet *et al.* 2001), pre-transplant IL-13 serum levels were compared amongst patients aged ≤40 years and >40 years in both cohort 1 and cohort 2. No significant difference was demonstrated for IL-13 levels in either age group (cohort 1 P=0.125; cohort 2 P=0.610). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 6.86 ± 1.03 and 4.92 ± 0.76 respectively for patients ≤40 years compared to 5.85 ± 0.66 and 4.06 ± 0.48 for patients >40 years.

6.7.2 *Patient Gender*

Pre-transplant IL-13 serum levels were compared amongst male and female HSCT patients. No significant correlation was demonstrated between the level of this cytokine and either gender (cohort 1 P=0.469; cohort 2 P=0.352). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 5.47 ± 0.70 and 4.08 ± 0.53 respectively for male HSCT patients compared to 6.73 ± 1.33 and 4.95 ± 0.72 respectively for female patients.

6.7.3 *Patient CMV status*

As CMV infection is a considerable clinical risk factor in HSCT (Nichols *et al.* 2002), pre-transplant IL-13 serum levels were compared amongst CMV positive and negative patients in both cohort 1 and cohort 2. No significant difference was demonstrated for IL-13 levels in either group (cohort 1 P=0.787; cohort 2 P=0.296). Mean (± SEM) IL-13 levels (pg/ml) in cohort 1 and 2 were 5.62 ± 0.77 and 5.07 ± 0.70 respectively for CMV positive patients compared to 6.12 ± 1.05 and 4.74 ± 0.43 respectively for CMV negative patients.

6.7.4 *Underlying Disease and Stage at the Time of HSCT*

Both HSCT cohorts examined in this study were heterogeneous for disease. Diagnoses included acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myeloid leukaemia (CML), Hodgkin disease (HD), plasma cell neoplasm (PCN), aplastic anaemia (AA), myelodysplastic syndrome (MDS) and non-Hodgkin lymphoma (NHL, section 6.4). As the biology of each of these haematological diseases differs greatly, disease specific IL-13 expression was examined in this study. As expected, pre-transplant IL-13 serum levels were found to vary for each disease, however, IL-13 levels for patients with HD were demonstrated to be significantly higher than those with any other disease diagnoses (cohort 1 P=0.004; cohort 2 P=0.015, Figure 6.8). In both cohorts, the IL-13 levels observed for HD patients were over twice as high as the levels observed for most of the other diseases. Mean (± SEM) IL-13 levels (pg/ml) for the patients with HD, AML, ALL, CML, PCN, AA, MDS and NHL were 11.94 ± 0.68, 6.12 ± 0.61, 2.10 ± 0.28, 4.48 ± 1.11, 3.94 ± 0.75, 1.49, 6.06 ± 0.34

and 3.88 ± 1.16 respectively in cohort 1 and 11.02 ± 0.32, 4.39 ± 0.36, 3.31 ± 0.92, 4.14 ± 0.38, 1.51 ± 0.31, 1.16, 2.56 and 3.21 ± 0.59 respectively in cohort 2.

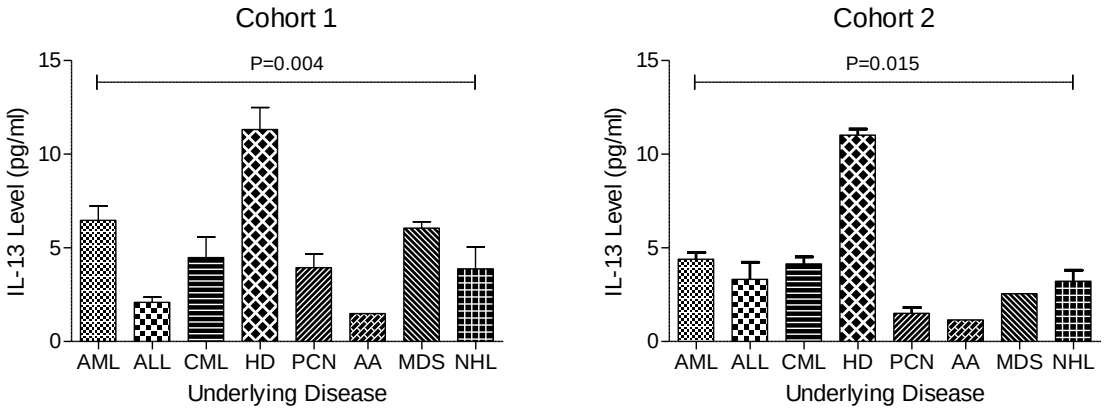

Figure 6.8 Correlation of pre-transplant IL-13 serum expression (pg/ml) with underlying disease in two independent HSCT cohorts. The mean (± SEM) IL-13 level was significantly higher for the patients with Hodgkin disease in both cohort 1 and cohort 2 (P=0.004 and P=0.015 respectively). IL-13, interleukin-13; P, P value; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; HD, Hodgkin disease; PCN, plasma cell neoplasm; AA, aplastic anaemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma

Considering the correlation between relapse incidence and pre-transplant IL-13 levels already demonstrated in this study (section 6.6.3), disease specific IL-13 expression was also examined in the patients who relapsed post-transplant. In cohort 1, disease relapse was observed in 10 HSCT patients; 2 had AML, 1 had CML, 5 had HD and 2 had PCN. In cohort 2, disease relapse occurred in 9 patients; 5 had AML, 1 had CML and 3 had HD. Again, pre-transplant IL-13 serum expression was found to be significantly higher for the patients with HD (cohort 1 P=0.042; cohort 2 P=0.040, Figure 6.9). In cohort 1, the mean (± SEM) IL-13 level (pg/ml) for the HD patients was 11.94 ± 0.68 compared to only 6.04 ± 0.90, 4.97 and 4.69 ± 0.11 for those with AML, CML and PCN respectively. In cohort 2, the mean (± SEM) IL-13 level for the HD patients was 11.02 ± 0.32 compared to only 6.58 ± 0.54 and 4.52 for those with AML and CML respectively.

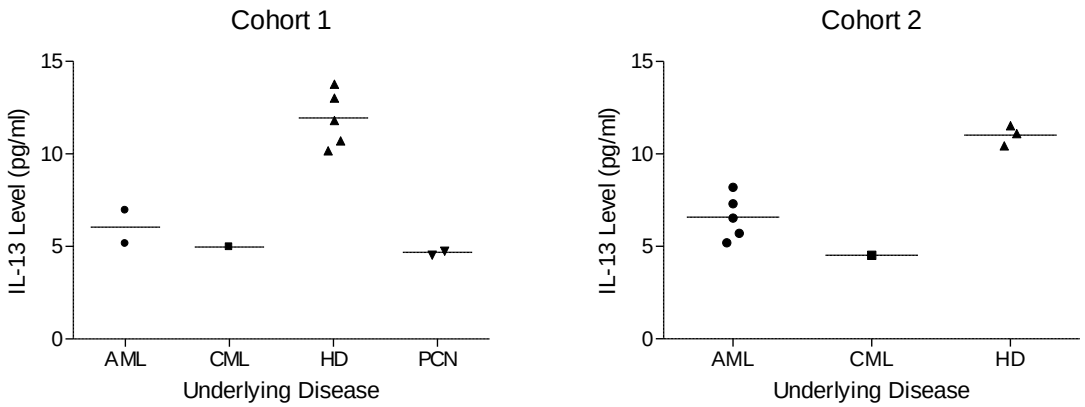

Figure 6.9 Correlation of pre-transplant IL-13 serum expression (pg/ml) with underlying disease in two independent relapse cohorts. Pre-transplant IL-13 expression was demonstrated to be significantly higher for the patients with Hodgkin disease (cohort 1 P=0.042, cohort 2 P=0.040). IL-13, interleukin-13; P, P value; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; HD, Hodgkin disease; PCN, plasma cell neoplasm

The affect of disease stage on pre-transplant IL-13 expression was also examined in this study. IL-13 serum levels were compared amongst patients with early (first chronic phase, first complete remission or plateau 1) and advanced (accelerated phase, second or third complete remission or plateau 2) disease at the time of HSCT. No significant difference was demonstrated for IL-13 levels in either group (cohort 1 P=0.197; cohort 2 P=0.183, Figure 6.10), although, the mean (± SEM) expression (pg/ml) for the patients with advanced disease in cohort 1 and 2 was 7.59 ± 1.36 and 5.04 ± 0.68 respectively compared to only 4.85 ± 0.51 and 3.77 ± 0.47 respectively for the patients with early disease. The elevated mean (± SEM) IL-13 expression observed for the advanced disease groups was not truly representative, but rather was a consequence (falsely skewed) of the higher pre-transplant IL-13 serum levels associated with the patients with HD (cohort 1 n=5; cohort 2 n=3).

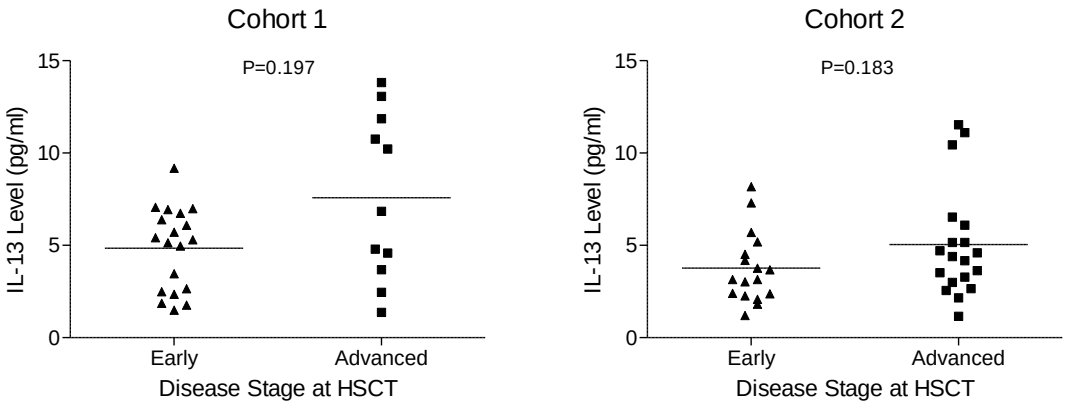

Figure 6.10 Correlation of pre-transplant IL-13 serum expression (pg/ml) with disease stage at the time of HSCT in two independent HSCT cohorts. No significant difference was observed in the mean (± SEM) IL-13 level for patients with early or advanced stage disease at the time of HSCT in cohort 1 or cohort 2 (P=0.196 and P=0.183 respectively). IL-13, interleukin-13; P, P value

6.7.5 *IL13 +2044 G/A Polymorphism*

Pre-transplant IL-13 serum levels were correlated with the allelic variants of the previously examined *IL13* +2044 G/A polymorphism (chapter 5, section 5.5). Although in both cohorts, mean (± SEM) IL-13 levels for the patients with the *IL13* +2044 A allele were found to be higher than those with the *IL13* +2044 G allele, the difference between the two groups did not reach significance (cohort 1 P=0.619; cohort 2 P=0.296, Figure 6.11). Mean (± SEM) IL-13 levels (pg/ml) for cohort 1 and 2 were 6.61 ± 1.12 and 5.23 ± 0.95 respectively for the patients with *IL13* +2044 A compared to only 5.09 ± 0.56 and 3.88 ± 0.38 respectively for those with *IL13* +2044 G. The highest pre-transplant IL-13 levels observed for both cohorts, were however, for the 8 HD patients included in this study (cohort 1 n=5; cohort 2 n=3); all of which possessed the *IL13* +2044 A allele.

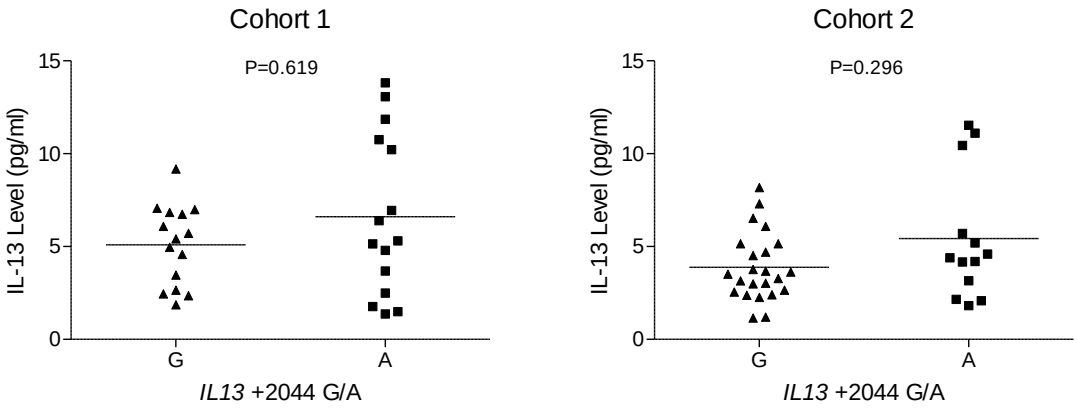

Figure 6.11 Correlation of pre-transplant IL-13 serum expression (pg/ml) with the *IL13* +2044 G/A polymorphism in two independent HSCT cohorts. No significant difference was observed in the mean (± SEM) IL-13 level for patients with the A or G allele in cohort 1 or cohort 2 (P=0.196 and P=0.183 respectively). IL-13, interleukin-13; P, P value

6.8 Discussion

In an attempt to prevent spurious results, pre-transplant IL-13 serum levels were examined in two independent patient cohorts; Newcastle HSCT patients (cohort 1) and Regensburg HSCT patients (cohort 2). As this was not a case-controlled study, the two patient populations examined were compared prior to any correlations with HSCT outcome. In terms of patient demographics, both populations were similar for size (cohort 1 n=30, cohort 2 n=36), age (median age: cohort 1, 40(19-57); cohort 2, 42(20-67)) and gender (cohort 1 M=21 F=9, cohort 2 M=21 F=15). In addition, all patients had haematological diseases and all samples were collected at the time of hospital admission and prior to the administration of any preparative treatments. With respect to IL-13 levels, although expression in general was found to be slightly lower for cohort 2, there was no significant difference in the mean (± SEM) IL-13 level observed for the two cohorts (P=0.175). Furthermore, expression levels were representative of the whole population, with high, intermediate and low IL-13 producers being observed in both groups. Thus, the two HSCT cohorts employed in this study were unbiased and comparable.

The mean (± SEM) IL-13 serum levels (pg/ml) observed in cohort 1 and cohort 2 were 5.85 ± 0.63 and 4.84 ± 0.43 respectively. These levels were considerably higher than the mean (± SEM) IL-13 serum levels determined previously (section 5.6.3) for a cohort of healthy Newcastle controls (range 0.77 – 0.91 pg/ml). Thus, the elevated expression observed in cohort 1 and 2 was related to the patient and/or the underlying disease rather than being naturally occurring. This elevated expression was however relatively unexpected; IL-13 release in HSCT is thought to be predominantly from activated cells (Jordan *et al.* 2004; Hildebrandt *et al.* 2007), although at this time point (pre-transplant) the transplant patients had not yet received any form of intervention to have caused such activation. Only a small number of ELISA-based studies have been published for IL-13 detection in serum and the expression observed in these studies is variable. The mean IL-13 serum level amongst an Iranian control population in a study of ischemic heart disease was 7.31pg/ml (Jafarzadeh *et al.* 2009), whilst the mean level for controls in an American burns study was only 0.9pg/ml (Gauglitz *et al.* 2008). In another American study, IL-13 serum levels were reported to be undetectable for the control population and at least 30pg/ml for the disease population (Fiumara *et al.* 2001). Although, differences in ELISA-based studies are often caused by inter-assay variation, it is also

possible that IL-13 serum levels are affected by ethnic differences; which would account for the variation in expression between cohort 1 and 2 in this current study.

The examination of pre-transplant IL-13 serum levels with respect to HSCT outcome failed to demonstrate any significant associations with overall acute GVHD (grades I-IV; cohort 1 P=0.979, cohort 2 P=0.689), clinical acute GVHD (grades II-IV; cohort 1 P=0.819, cohort 2 P=0.661), severe acute GVHD (grades III-IV; cohort 1 P=0.342, cohort 2 P=0.468) or chronic GVHD (cohort 1 P=0.472; cohort 2 P=0.772, Figure 6.2, 6.3, 6.4 and 6.5). Even though elevated IL-13 serum levels were observed over the transplant period in patients with acute and chronic GVHD in the previous chapter, the pre-transplant levels demonstrated for both groups were very similar; thus the lack of associations in this study were not totally unexpected. If allo-reactive donor T cells are the primary source of IL-13 in HSCT, as proposed (Jordan *et al.* 2004; Hildebrandt *et al.* 2007), then any link that may exist between IL-13 and GVHD is only likely to become apparent once the stem cell graft had been infused and donor T cells have become activated.

A significant association was however demonstrated between pre-transplant IL-13 serum levels and the incidence of relapse. Patients who had disease relapse following HSCT were shown to have elevated IL-13 serum levels prior to transplantation (cohort 1 P=0.012; cohort 2 P<0.0001, Figure 6.6). Although sample populations were small, demonstration of this finding in both HSCT cohorts examined seems to suggest that pre-transplant IL-13 serum expression is predictive of relapse post-transplant in this study. The use of this cytokine as a marker of relapse, however, currently seems questionable. The considerable overlap between the lowest IL-13 levels observed for the relapsed patients and the highest levels observed for the non-relapsed patients in this study indicates a lack of sensitivity for relapse at the lowest extreme. It is possible, however, that the measurement of pre-transplant IL-13 serum levels in a larger HSCT cohort may reduce the standard error and increase the potential of this cytokine as a predictive marker.

The elevated expression observed in the relapsed patients may also reflect the role of IL-13 in this disease process, as the cytokine has been demonstrated to promote the growth and survival of tumour cells. Studies using murine models have proposed a novel pathway in which IL-13 down-regulates CD8+ cytotoxic T cell-mediated tumour

immunosurveillance (Terabe *et al.* 2000). IL-13 released from CD4+ natural killer T (NKT) cells in response to tumour antigens has been demonstrated to suppress the tumour killing actions of cytotoxic T cells through the IL-13 signalling pathway (IL-4Rα-Stat6 pathway) (Kacha *et al.* 2000; Ostrand-Rosenberg *et al.* 2000; Terabe *et al.* 2000). It still however, remains unclear how IL-13 actually represses cytotoxic T cells, since T cells do not express the IL-13 receptor (type II IL-4 receptor). A possible mechanism involves the release of TGF-β from intermediary cells. In tumour-bearing mice, IL-13 released by NKT cells has been shown to activate myeloid cells to produce TGF-β, which acts directly on CD8+ T cells to down-regulate tumour-specific cytotoxic T cell induction (Terabe *et al.* 2003).

Pre-transplant IL-13 serum levels were also shown to correlate with post-transplant survival. Patients who died following HSCT were shown to have elevated IL-13 serum levels prior to transplantation. However, this association was only demonstrated for cohort 1 (P=0.019, Figure 6.7). No significant difference in IL-13 expression was observed between the patients who were alive or died post-transplant in cohort 2 (P=0.465, Figure 6.7). Due to the extremely small number of deaths observed in both populations (cohort 1 n=7, cohort 2 n=9), these findings should be interpreted with caution until either is confirmed in a larger cohort. Nevertheless, considering that relapse was the cause of all deaths in cohort 1, it is highly likely that the association observed in this population merely reflects the correlation already described between pre-transplant IL-13 expression and post-transplant disease relapse (section 6.6.3).

A variety of clinical and patient parameters were also analysed in this study. No associations were demonstrated between pre-transplant IL-13 serum levels and age, gender or CMV status in either cohort examined (cohort 1 P=0.125, P=0.469 and P=0.787 respectively; cohort 2 P=0.610, P=0.352 and P=0.296 respectively). A significant correlation was however, observed with underlying disease. In both cohorts, pre-transplant IL-13 serum levels for patients with HD were shown to be substantially higher than the levels observed for patients with any other disease diagnoses (cohort 1 P=0.005; cohort 2 P=0.015, Figure 6.8). A similar finding has been described by Fiumara *et al* (2001). In a study examining IL-13 serum expression in HD and healthy volunteers, IL-13 levels of at least 30pg/ml were detected in patients with HD, whilst IL-13 expression was undetectable in all controls (Fiumara *et al.* 2001). Although the number of HD patients examined in this current study was extremely small (cohort 1

n=5, cohort 2 n=3), the disease specific IL-13 expression demonstrated echoes the role of IL-13 in the pathogenesis of HD. Hodgkin and Reed-Sternberg (H/RS) cells of HD have been demonstrated *in vitro* and *in vivo* to substantially express both IL-13 and IL-13 receptors (Kapp *et al.* 1999; Skinnider *et al.* 2001) and have been shown to use this cytokine as an autocrine growth factor to enhance their own survival (Skinnider *et al.* 2002a; Skinnider *et al.* 2002b). Blocking of IL-13 has also been demonstrated to induce apoptosis in H/RS cells *in vitro* (Kapp *et al.* 1999). This latter finding, not only emphasises the importance of IL-13 in the disease mechanism of HD, but implicates this cytokine as potential target for future treatment strategies.

HD specific IL-13 expression was confirmed when pre-transplant IL-13 serum levels were examined in the context of post-transplant disease relapse. The HD patients who relapsed following HSCT were shown to have significantly elevated IL-13 serum levels prior to transplantation (cohort 1 P=0.042; cohort 2 P=0.040, Figure 6.9). In fact, the IL-13 levels observed for relapsed HD were almost twice as high as the levels observed for any of the other relapsed diseases. Although the HD population examined in this study was extremely small (n=8; cohort 1=5 cohort 2=3), this finding seems to suggest that high IL-13 serum expression in HD patients prior to HSCT reflects a poor prognosis in terms of disease relapse. From the data presented, it is not clear however, if this elevated expression is simply a marker of post-transplant relapse or is somehow involved in the development of the outcome. It must also be considered however, that allogeneic transplantation for HD is often only performed when chemotherapy and autologous HSCT has failed and hence the demonstrated correlation between pre-transplant IL-13 expression and HD relapse in this study may merely reflect the advanced nature of the disease at the time of transplantation. Further investigations into the role of pre-transplant IL-13 in HD and post-transplant relapse in a larger patient population are therefore warranted. In addition, while no significant correlations could be made between pre-transplant IL-13 expression and the *IL13* +2044 G/A polymorphism when all patients were examined, the possession of the *IL13* +2044 A allele by all 8 HD patients seems to suggest that an association may also exist between this polymorphism and post-transplant HD relapse. As the *IL13* +2044 A allele is unlikely to influence cytokine expression, it is possible that the increased IL-13 activity proposed to associated with this allele (Vladich *et al.* 2005) plays a role HD relapse. Again, due to the extremely small nature of the HD subgroup examined in this study,

further investigations into the *IL13* +2044 G/A polymorphism in a larger patient population are warranted.

The examination of pre-transplant IL-13 serum levels with respect to disease stage (early/advanced) at HSCT failed to demonstrate an association in this current study (Figure 6.10). Although the mean (± SEM) expression levels observed for the patients with advanced disease were elevated, they were not demonstrated to be significantly higher than those for the patients with early stage disease. In a previously published study examining pre-transplant IL-10 levels, low expression of this cytokine prior to HSCT was found to correlate with advance disease stage. The explanation for this finding was a decreased capacity for cytokine production in macrophages derived from patients with advanced disease at the time of transplant. Exhaustion of immune regulation or an effect from more aggressive previous cytotoxic treatment were suggested causes (Holler *et al.* 2000). This is seemingly not the case for IL-13 in this study. However, on closer inspection of the data obtained, 6/11 and 16/19 of the patients with advanced disease in cohort 1 and 2 respectively had IL-13 levels that were comparable with those of the early stage groups. Thus, the elevated means (± SEM) associated with advanced disease in both patient cohorts were therefore solely attributable to the remaining 8 patients (cohort 1 n=5; cohort 2 n=3); all of which were transplanted for HD. In the context of this disease, an association between high IL-13 serum levels and advanced disease should be expected. Considering the role IL-13 plays in the disease mechanism of HD, serum levels of this cytokine will increase as the disease progresses.

The observed relationship between HD and IL-13 expression in this study, not only confirms the role of IL-13 in the disease, but brings the validity of the previously demonstrated correlation between pre-transplant IL-13 expression and post transplant relapse (section 6.6.3) into question. As the HD patients accounted for the highest IL-13 levels observed in the relapsed groups and these levels were significantly higher than those for all of the other patients, it is extremely likely that the elevated IL-13 expression associated with HD had a substantial influence on the mean (± SEM) IL-13 level detected for relapse in both HSCT cohorts. As a result, exclusion of the HD patients from the relapse analyses is likely to reduce the difference in IL-13 serum expression between the relapsed and non-relapsed patients considerably and result in the loss of the correlation. Although confirmation of this is required in a larger cohort, it

now seems unlikely that pre-transplant IL-13 expression is a predictive marker for post-transplant relapse. It is more likely that the data presented in this study simply reflects the significance of IL-13 in HD.

Chapter 7. Conclusions

The findings of this study clearly demonstrate that the genetic make-up of transplant patients and their donors can strongly influence GVHD development following HSCT. In the unrelated donor transplant setting, patient carriage of the *VDR* intron 8 A,A and *IFNG* intron 1 3,3 genotypes were associated with the development of severe acute GVHD (grades III-IV), whilst patient carriage of the *IL6* -174 G,G genotype was a risk for chronic GVHD. In both the HLA-matched sibling and unrelated donor settings, patient carriage of the *IL13* +2044 A allele was associated with an increased susceptibility to severe acute GVHD (grade III-IV) and donor carriage of this allele was associated with an increased risk of chronic GVHD. Although these associations require replication in a large independent validation cohort, they do provide further evidence for the employment of non-HLA polymorphisms pre-transplant as predictive markers of HSCT outcome; particularly GVHD development. The findings of this study, in combination with previously published data, also highlight the need for non-HLA polymorphisms to be analysed independently in both the HLA-matched sibling and unrelated donor transplant setting, as the differences in biology and clinical protocols employed significantly affect the genetic associations observed in each transplant type.

The functional significance of IL-13 and the *IL13* +2044 G/A polymorphism were also examined in this study. Elevated IL-13 serum levels across the transplant period were demonstrated in patients with both acute and chronic GVHD, suggesting a role for IL-13 in the pathogenesis of these conditions. However, this altered expression could not be directly attributed to *IL13* +2044 G/A. A published functional study seems to suggest that this polymorphism increases the biological activity of IL-13 rather than its expression (Vladich *et al.* 2005), although, further studies are necessary to confirm this. Elevated IL-13 serum levels were also demonstrated prior to transplantation. These levels could not be correlated with GVHD, but were shown to be disease specific; patients with HD had significantly higher pre-transplant IL-13 serum expression than patients with any other disease diagnoses. This finding substantiates published data demonstrating a role for IL-13 in the disease mechanism of HD (Kapp *et al.* 1999; Skinnider *et al.* 2001; Skinnider *et al.* 2002a; Skinnider *et al.* 2002b). Elevated IL-13 expression prior to transplantation was also shown to correlate with post-transplant relapse. Although our finding seems to implicate IL-13 as a predictive marker of this

outcome, it is likely that the elevated IL-13 expression associated with HD patients had a substantial influence on the correlation, thus further studies are warranted.

This study has also highlighted some of the problems associated with investigations in the field of HSCT. The number of transplant procedures undertaken, particularly at single centres, is relatively small; thus laboratory sample and clinical data accrual is slow. The collection of samples, including blood and skin biopsies is highly dependent on the consent and co-operation of the patient. Consequently, study numbers are reduced if the patient with-holds consent or does not attend clinic appointments. In addition, complete clinical information is often unavailable for patients eligible for inclusion in retrospective studies that were transplanted many years before the investigation was initiated; thus exclusion of these individuals reduces sample numbers even further.

As HSCT is a potential treatment for a wide range of malignant and non-malignant haematological diseases with a variety of options available to the transplant patient and their treating clinician, population heterogeneity is another problem encountered in the transplant setting. In addition to the underlying diagnosis of the patient, variations in transplant type, conditioning regimen and GVHD prophylaxis therapy have the potential to impact on HSCT studies. As a result only large registry-based studies allow the stratification of patients with respect to these variations (Gratwohl *et al.* 1998; van Beisen *et al.* 2003). In terms of genetic association studies such as this one, heterogeneity is extremely problematic, as the existence of multiple confounding variables is likely to prevent all possible disease associations from being observed. The extrapolation of data from different heterogeneous studies should therefore be examined with caution.

There is little doubt that the adoption of a limited number of conditioning regimens and GVHD prophylaxis therapies for transplant procedures would ease the interpretation of scientific studies. At the present time, however, this would be difficult to instigate. Despite extensive research and advances in the HSCT setting, clinical outcome is unfortunately still relatively poor. Thus, clinicians are keen to implement results from phase II studies as quickly as possible.

Despite the difficulties associated with HSCT studies, there is clearly a need for greater knowledge in the setting, as there is little doubt that acute and chronic GVHD remain a major cause of morbidity and mortality. The ability to predict these conditions would therefore be invaluable. The ideal predictive factor for GVHD would be assessable pre-transplant. This would permit the selection of the best possible donor if several were available, allow the patient to make more informed decisions about their therapeutic options and would possibly allow clinicians to modify conditioning and prophylaxis therapies in those patients at the lowest or highest risk of GVHD. The ideal predictive factor would also be highly sensitive and specific and unaffected by post-transplant factors, such as conditioning regimen induced-toxicity and infection. Although the *VDR* intron 8 A/C, *IFNG*, intron 1 (CA)n, *IL6* -174 G/C and *IL13* +2044 G/A polymorphisms were shown to be independent risk factors for GVHD in this study, it is unlikely that any of these alone will be the 'ideal predictive factor'. The identification of a single easily measurable factor that is highly predictive for GVHD is an attractive goal. However, given the complexity of GVHD and the existence of numerous clinical risk factors associated with the condition, achievement of this seems extremely doubtful. Much more likely, is the development of a prognostic index that consists of both clinical and genetic risk factors for GVHD.

The data presented in this investigation have provided the basis for future non-HLA polymorphism studies included as part of a multicentre European project investigating potential risk factors for GVHD. The study, co-ordinated in Haematological Sciences, Newcastle University and lead by Professor A M Dickinson is ongoing and will provide the opportunity to accrue a greater number of DNA samples, skin biopsies and serum samples from participating centres. This will allow potential GVHD risk factors to be examined in much larger transplant populations, will permit the stratification of HSCT cohorts into more informative homogeneous disease, treatment and transplant specific groups and will facilitate the creation of independent validation cohorts, that are required to address reproducibility problems (Colhoun *et al.* 2003). With regard to the non-HLA polymorphisms investigated in this study; *IL2* -330 T/G, *IL4* -590 C/T, *IL6* -174 G/C, *IL10* -592 A/C, *IL10* -1082 G/A, *IL1RN* intron 2 VNTR, *TNFA* intron 3 (GA)n, *TNFRSF1B* -196 M/R, *IFNG* intron 1 (CA)n, *VDR* intron 8 A/C, *VDR* exon 9 T/C, *ESR1* intron 1 A/G and *ESR1* intron 1 C/T, future aims will mainly focus on cohort expansion and association confirmation. However, analysis of these polymorphisms with respect to HSCT outcome in larger separate HLA-matched sibling and unrelated

donor transplant cohorts will also be performed. As previously mentioned, due to the differences in biology and clinical protocols employed, it is unlikely that exactly the same genetic associations will exist for both transplant settings.

Cohort expansion and association confirmation in the HLA-matched sibling and/or unrelated donor transplant setting would not only provide substantial evidence that any association observed was the result of a true genetic risk factor for HSCT outcome, but would also permit the development of the previously outlined prognostic index. Such an index would consist of the established European Group for Blood and Marrow Transplantation (EBMT) clinical risk score (Gratwohl *et al.* 1998) for all patients undergoing HSCT for a haematologic disorder (Gratwohl *et al.* 2009) combined with any identified genetic risk factor(s) using the approach adopted by Dickinson *et al* (2010). Using prediction error and concordance index statistics this group demonstrated that the integration of three genetic factors (*TNFRSFIB* -196 M/R, *IL10* -592/-819/-1082 haplotype and *IL1RN* intron 2 VNTR) into the EBMT clinical risk score (five clinical factors: patient age, stage of disease, time interval from diagnosis to transplantation, histocompatibility and gender mismatch (female to male) transplantation) significantly improved this predictive model in a cohort of CML patients and donors (Dickinson *et al.* 2010).

In addition to the development of a prognostic index, expansion of our existing transplant cohorts would also allow Genome Wide Association Studies (GWAS) to be performed. These studies employ high throughput genotyping technologies to analyse the maximum number of genomic polymorphisms in a single assay; current microarray-based platforms can parallel genotype almost 2 million markers dispersed throughout the human genome. The employment of GWAS technologies in the transplant setting would be extremely valuable, as it is highly unlikely that a single genetic factor or inflammatory mediators alone are responsible for the complex clinical picture associated with GVHD and any other HSCT outcome. However, a major drawback exists with the GWAS approach, the requirement for vast sample sizes; the achievement of which is likely to be problematic in the field of transplantation as sample accrual is notoriously slow.

Future studies with respect to IL-13, will again mainly be focused on the confirmation of the genetic associations demonstrated in this study. However, the *IL13* -1112/+2044

T-A haplotype will be also be examined in the context of GVHD, as further definition of linked polymorphisms is likely to increase the predictive power of this cytokine. The preliminary investigations into IL-13 expression prior to and across the transplant period will be ongoing. Continued serial serum sample collection is expanding the cohort considerably. Larger numbers will not only allow the heterogeneous nature of HSCT to be accounted for, but will permit the more accurate definition of the role of IL-13 in GVHD. Blocking experiments employing the human skin explants assay as a HSCT model will also be performed. Blocking of IL-13 using soluble receptors or receptor antagonists may down-regulate the heightened biological activity possibly associated with the *IL13* +2044 A allele and thus diminish GVHD. In addition, expansion of our existing HD population (n=8) will be undertaken, as larger numbers will allow the role of IL-13 and the *IL13* +2044 G/A polymorphism in both the disease mechanism of HD and post-transplant HD relapse to be explored more thoroughly.

The ultimate goal of this collaborative European project is the creation of a prognostic index for GVHD. This index will comprise the most informative clinical and genetic risk factors and will be employed pre-transplant to predict the risk of GVHD on an individual basis. The successful prediction of GVHD prior to transplantation would lead to several immediate and long term benefits, such as better donor selection, improved or tailored prophylaxis therapies, shorter hospital stays and most importantly, reduced GVHD morbidity and mortality. It is therefore essential that research into GVHD risk factors continues and encompasses all potential genetic candidates.

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Appendix A

List of Suppliers

Company Name (shortened)	Address of Supplier
AB	Applied Biosystems, Lingley House, Cheshire UK
Alpha Innotech	GRI Ltd, Queenborough Lane, Braintree, Essex UK
Axis-Shield	Axis-Shield Plc, Luna Place, The Technology Park, Dundee UK
BDH/Merck	Merck Ltd, North East Laboratory Supplies, Newton Aycliffe UK
BD	BD Biosciences, Between Town Road, Cowely, Oxford UK
Bioline	Bioline UK Ltd, The Edge Business Centre, London UK
Bio-Rad	Bio-Rad Laboratories Ltd, Maylands Avenue, Hemel Hempstead UK
Fisher Scientific	Thermo Fisher Scientific, Bishop Meadow Road, Leicestershire UK
Flow Laboratories	ICN Biomedicals, Wenman Road, Thame, Oxfordshire UK
Flowgen	Flowgen Instruments Ltd, Lichfield, Staffordshire UK
Gelman Sciences	Pall Gelman Laboratories, Havant Street, Portsmouth UK
Gibco	Invitrogen Life Technologies, Inchinnian Business Park, Paisley UK
Greiner	Greiner Bio-One Ltd, Stroudwater Business Park, Gloustershire UK
Inverness Medical	Inverness Medical Innovations, Bio-Stat House, Cheshire UK
Lonza	Lonza Sales Ltd, Muenchchensteiner Strasse, Basel Switzerland
MWG	MWG Eurofins Genetic Services Ltd, Worple Park, London UK
NBS Biologicals	NBS Biologicals Ltd, Edison House, Hertfordshire UK
NEB	New England Biolabs Inc, Knowl Place, Hertfordshire UK
Nunc	Thermo Fisher Scientific, Kamstrupvej, Roskilde Denmark
PAA	PAA Laboratories Ltd, Houndstone Business Park, Somerset UK
PeproTech	PeproTech EC Ltd, Margravine Road, London UK
Promega	Promega Ltd, Chilworth Research Centre, Southampton UK
Rathburn Chemicals	Rathburn Chemicals Ltd, Caberston Road, Peebleshire UK
RayBiotech	RayBiotech Inc, Parkway Lane, Norcross, Georgia USA

Roche	Roche Diagnostic Ltd, Bell Lane, Lewes, East Sussex UK
Sigma	Sigma-Aldrich Chemical Company, Fancy Road, Poole, Dorset UK
SLS	Scientific Laboratories Supplies Ltd, Elm Road, Tyne and Wear UK
ThermoLabsystems	Thermo Electron Corporation, The Ringway Centre, Basingstoke UK
VWR	VWR International Ltd, Magna Park, Leicestershire UK

Appendix B

Ethical Approval

Northumberland and Tyne & Wear NHS
Health Authority

LOCAL RESEARCH ETHICS COMMITTEE
(Newcastle and North Tyneside)
Newcastle General Hospital
Westgate Road
Newcastle
NE4 6BE

Your Ref: AMD/LS

18 February 2003

Professor A M Dickinson
Professor of Marrow Transplant Biology
Department of Haematology
Royal Victoria Infirmary

Dear Professor Dickinson

Predicting Outcome And Developing New Therapeutic Strategies For Haematological Stem Cell Transplant Recipients Using *In Vitro* Techniques
(Our Ref: 2002/306)

Thank you for your letter of 10 January which addresses the issues identified by the Ethics Committee when it considered your application in respect of this study.

In the light of your response I can now confirm the grant of ethical approval in respect of your research study application.

Yours sincerely

Professor P A Heasman
Chairman
Local Research Ethics Committee

dickinson2

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Newcastle and North Tyneside NHS
Health Authority

JOINT ETHICS COMMITTEE
Newcastle & North Tyneside Health Authority
University of Newcastle upon Tyne
University of Northumbria at Newcastle

Newcastle General Hospital
Westgate Road
Newcastle
NE4 6BE

Your Ref: AMD/LS

16 October 2001

Dr A M Dickinson
Senior Lecturer in Marrow Transplant Biology
Department of Haematology
Royal Victoria Infirmary

Dear Dr Dickinson

The Use Of An In Vitro Human Skin Explant Assay To Investigate Graft Versus Host Disease And In'Vitro Graft Versus Host Reactions
(Min Ref: 2001/213)

Thank you for your letter of 25 September which addresses the issues identified by the Joint Ethics Committee when it considered your application in respect of this study.

In the light of your response I can now confirm the grant of ethical approval in respect of your research study application.

Yours sincerely

Dr P A Heasman
Chairman
Joint Ethics Committee

dickinson5

Please note revised contact information

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Appendix C

Personal Publications and Communications

Publications:

JL Harrold, EA Leitch, GH Jackson and AM Dickinson. Prognostic significance of elevated interleukin-13 levels in patients prior to haematopoietic stem cell transplantation. *Bone Marrow Transplantation* 2009; **43** (Supplement 1): 141.

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Haematopoietic stem cell transplantation: can our genes predict clinical outcome?

Anne M. Dickinson*, Jane L. Harrold and Hannah Cullup

Haematopoietic stem cell transplantation (HSCT) is currently the only curative treatment for many patients with malignant and non-malignant haematological diseases. The success of HSCT is greatly reduced by the development of complications, which include graft-versus-host disease (GVHD), relapse and infection. Human leukocyte antigen (HLA) matching of patients and donors is essential, but does not completely prevent these complications; non-HLA genes may also have an impact upon transplant outcome. Polymorphisms within genes that are associated with an individual's capability to mount an immune response to alloantigen and infectious pathogens and/or response to drugs (pharmacogenomics) are all currently being studied for their association with HSCT outcome. This review summarises the potential role of non-HLA polymorphisms in predicting HSCT outcome, from studies on retrospective transplant cohorts of HLA-identical siblings and matched unrelated donors. The clinical relevance and interpretation of non-HLA genetics, and how these could be used alongside clinical risk factors in HSCT, are also discussed.

Matching at the human leukocyte antigen (HLA) locus is crucial to the prevention of severe graft-versus-host disease (GVHD) in both HLA-matched and matched unrelated donor (MUD) transplants. Failure to match patient and donor at HLA class I and class II alleles can lead to rejection and/or GVHD, depending on the allele mismatch (Ref. 1). Numerous studies of fully HLA-matched transplants compared with one- or two-antigen-mismatched transplants have shown that the degree of GVHD is directly proportional to the degree of HLA mismatch. However, GVHD still occurs in patients when their donors are fully matched at the HLA loci. Therefore, other genes, in addition to minor histocompatibility genes and killer-immunoglobulin-like receptor (*KIR*) genes, might also impact upon GVHD and overall survival. These non-HLA-encoded genes include those involved in the immune response, such as cytokines.

Polymorphisms within the 5′ or 3′ regulatory sequences of genes, such as single-nucleotide polymorphisms (SNPs) or microsatellites, can

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alter transcription factor binding, and hence the level of protein produced. Cytokine gene polymorphisms occur naturally within the normal population (Refs 2, 3) and can result in individuals producing higher or lower levels of cytokines. Their impact therefore affects both the quality and quantity of, for example, the cytokine or cytokine receptor, and in the transplant setting may influence the cytokine storm associated with GVHD. Cytokine gene polymorphisms (CGPs) have been investigated in a number of diseases where there is an immune pathology, such as autoimmune diseases, cancer and transplantation. Immunosuppressed, solid-organ transplant patients with a 'high-producer' tumour necrosis factor α (TNF-α) genotype and 'low-producer' interleukin 10 (IL-10) genotype are more likely to reject their graft (Ref. 4). These findings led to studies in the haematopoietic stem cell transplant (HSCT) field, with *TNFA* and *IL10* gene polymorphisms being the first to be investigated. In recent years, the number of different polymorphisms studied has increased. Molecules that are secreted by cells, those expressed on the cell surface and those within the cell – all of which may have an impact on other areas of the immune response – have been analysed. These molecules may be involved in various complications of HSCT other than GVHD, including relapse, survival and immunity to infection. Since T cells, cytokine release and the immunobiology associated with GVHD can also give rise to beneficial graft versus leukaemia (GVL) effects, CGPs involved in GVHD may have a concomitant effect on GVL. The biology of the transplant, including disease status and type of transplant, may also influence the effect of CGPs. Therefore, their clinical impact and subsequent potential use in therapeutic or selection algorithms may vary depending on the HSCT cohort under study.

Pathogenesis of graft-versus-host disease

The aetiology of GVHD has been investigated through both basic and clinical studies. Murine transplant models have facilitated the elucidation of the mechanisms involved in acute GVHD (aGVHD) and the roles of particular cell types and soluble mediators. Classically, aGVHD has been defined as occurring within the first 100 days post-transplant. Recent changes in conditioning regimens, such as the use of reduced-intensity conditioning (RIC), have altered this timeframe owing to the reduced impact of the regimens on toxicity and the immune system. After RIC, aGVHD commonly occurs post day 100. The overall grade of aGVHD ranges from grade I to IV in severity and predicts the clinical course of the disease. This grading system was established by Glucksberg in 1974 (Ref. 5) and by an International Bone Marrow Transplant Registry (IBMTR) consensus in 1995 (Ref. 6). In brief, grade I denotes mild disease and a good clinical prognosis; grade II indicates moderately severe disease with further organ involvement (skin, gut and liver); and grade III and IV are the most severe life-threatening stages, with multiorgan involvement and increased mortality.

A three-phase model of aGVHD has been proposed (Refs 7, 8) and the genotype of both the recipient and donor may influence the disease severity (Fig. 1). The conditioning regimen and its damaging effect upon host tissues constitute the first phase, where cells – particularly antigen-presenting cells (APCs), such as dendritic cells (DCs) – become activated, resulting in the production of numerous cytokines including IL-1, TNF-α, IL-6 and interferon γ (IFN-γ). These cytokines induce the expression of host HLA class II antigens, and adhesion molecules (e.g. the intercellular cell adhesion molecule ICAM-1) important for recognition by donor T cells on target tissues. In addition, conditioning regimens, particularly myeloablation, cause endothelial and epithelial damage in the intestinal mucosa and other epithelial targets. This allows the translocation of bacterial components such as lipopolysaccharides across the lumenal epithelium and subsequent binding to pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain (NOD)-containing proteins (Refs 9, 10). This, in turn, elicits the additional release of IL-1 and TNF-α by host-derived macrophages (Ref. 11). The second phase centres on activation of the incoming donor T cells promoted by this inflammatory milieu, which requires the presence of host APCs (direct alloantigen presentation) (Ref. 12) and donor APCs (the 'indirect pathway', where processing and presentation of host antigen is by the donor

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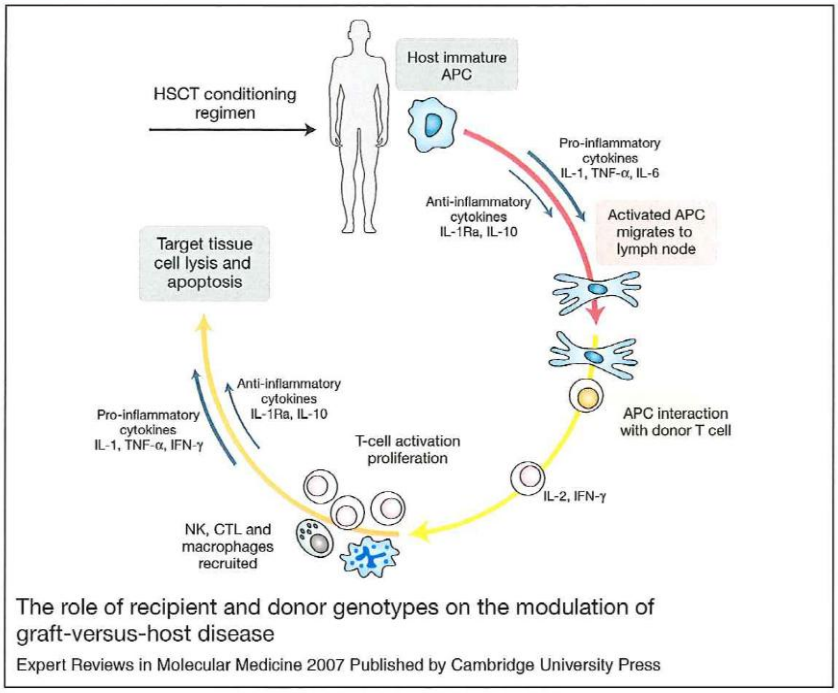

Figure 1. The role of recipient and donor genotypes on the modulation of graft-versus-host disease. The haematopoietic stem cell transplantation (HSCT) conditioning regimen activates host tissues, resulting in the production of inflammatory cytokines. This is influenced by the recipient genotype shown in red. Once the donor T cells are infused, they become activated in the inflammatory milieu and further cytokines are produced. Cytokine production by leukocytes can now be influenced by the donor genotype illustrated in yellow. The interaction of the donor and host genotype is illustrated in orange. If the risk genotypes (recipient/donor) are present (large grey arrow), the incidence or severity of graft-versus-host disease (GVHD) may be increased. If protective genotypes (recipient/donor) are present, the incidence or severity of GVHD may be reduced (small grey arrow). This figure is adapted, with permission, from Ref. 51 (© 2005 Taylor and Francis).

APC) (Ref. 13). A recent report showing that 100% APC (Langerhans cell) chimerism is achieved by day 100 in the skin of HSCT patients is consistent with the hypothesis of a transition from aGVHD to chronic GVHD (cGVHD) and related to indirect antigen presentation by donor APCs (Ref. 14). Activated donor T cells in the third phase of GVHD in turn release IL-2 and IFN-γ, promoting cytotoxic-T-cell and natural-killer-cell responses. Monocytes are stimulated in response to this and produce IL-1 and TNF-α. The final effector phase results in damage to host tissues (skin, gut and liver) and is mediated directly by cytokines (e.g. IL-1, TNF-α and IFN-γ), or together with cytotoxic T cells or natural killer cells, via pathways dependent on perforin and granzyme (Refs 15, 16).

Although the pathogenesis of aGVHD is relatively well understood, our understanding of the mechanisms responsible for cGVHD is relatively poor. This is due, at least in part, to the paucity of animal models for this condition. cGVHD is defined as occurring after 100 days

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post-transplant, usually after aGVHD, but it can also occur de novo. cGVHD is a multiorgan disease, defined as limited and extensive, and several grading systems exist (Refs 17, 18, 19) to assess its severity and extent. The occurrence of cGVHD seems to be dependent on the continued presence of host-reactive donor T cells (Ref. 20). However, it is unclear whether such lymphocytes are host-specific or simply host-reactive, as a result of donor and recipient sharing common epitopes.

Non-HLA polymorphisms in HSCT

Over the past decade, an increasing amount of data has demonstrated a role of non-HLA gene polymorphisms in HSCT outcome. For this review, we summarise results concerned with polymorphic genes for pro-inflammatory and anti-inflammatory cytokines and cytokine receptors. However, investigations involving genes of other immune response molecules have also been studied and are briefly reviewed. The vast majority of this research has been carried out on HLA-matched sibling transplants rather than on MUD HSCT cohorts. The data generated from non-HLA polymorphism research have been analysed with respect to transplant outcome, including aGVHD, cGVHD, transplant-related mortality, overall survival, relapse and infection (for recent review, see Ref. 21).

If polymorphic genes are located within the major histocompatibility complex (MHC) on chromosome 6 (e.g. *TNF* and the heat-shock protein *HSPA1*), it is likely that these genes will be identical between HLA-identical siblings. However, in both the HLA-matched and MUD transplant setting, genotypes of other genes including those of cytokines not situated on chromosome 6, such as *IL-1* and *IL-6* genes, will differ between patient and donor. In these cases, both patient and donor genotypes are examined for associations with HSCT outcome (Ref. 21).

HLA-matched sibling studies: cytokine and cytokine-receptor gene polymorphisms

TNFA

TNF-α is a multifunctional pro-inflammatory cytokine produced mainly by monocytes and macrophages, which has the ability to upregulate adhesion and MHC molecule expression and can directly induce cell death (effector phase). It also possesses anti-tumour activity and plays a major role in the GVHD immune response (Ref. 22). The gene encoding TNF-α (*TNFA*) is highly polymorphic and located within the class III region of the MHC complex on chromosome 6, close to the HLA region and lymphotoxin and *HSP* genes.

TNFA polymorphisms have been widely studied with respect to HSCT outcome. One of these polymorphisms is a SNP within the gene promoter region at position −308 (*TNFA* −308). This SNP has been linked with a diverse range of diseases and inconsistently associated with GVHD following HLA-matched sibling transplantation (Table 1). Several small studies have reported a positive association with aGVHD (Refs 23, 24); however, the majority of the larger studies found no such association (Refs 25, 26, 27, 28, 29, 30). *TNFA* −308, along with the *TNFB* +1069 polymorphism, has also been linked with toxic complications, with the highest incidence of such complications in *TNFA* and/or *TNFB* heterozygous recipients (Ref. 31).

Another SNP within *TNFA* is located at position +488. The A allele of this SNP has been associated with GVHD in two independent HLA-matched sibling cohorts. Patients with this allele developed significantly higher grades of aGVHD than +488A-negative patients. The A allele has also been associated with cGVHD and early death (Ref. 30).

A number of microsatellite polymorphisms occur within *TNFA*: *TNFa*, *TNFb*, *TNFc*, *TNFd* and *TNFe* (Refs 32, 33, 34). The *TNFd3* and *TNFa2* alleles have been associated with higher TNF-α production, whereas the *TNFa6* allele correlates with decreased production (Refs 4, 35, 36). In terms of HSCT, the *TNFd3* homozygous genotype is associated with an increased incidence of aGVHD and early death (Refs 25, 37), whereas the *TNFd4* allele correlates with the development of moderate to severe aGVHD (Ref. 27).

An important factor potentially influencing any association between *TNFA* polymorphisms and GVHD is the location of the gene within the MHC class III region. *TNFA* polymorphisms are in linkage disequilibrium with HLA class I and class II genotypes. As certain HLA genotypes are known to influence GVHD development, association with *TNFA* genotype may be secondary to or interact with the HLA associations (Refs 21, 38). Table 1 lists *TNFA* genotype associations with transplant outcome.

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Table 1. *TNFA* polymorphisms and haematopoietic stem cell transplantation outcome

Polymorphism	Genotype	Effect	Association	Study	Refs
TNFd	d3,d3	Increased TNF-α	Increased aGVHD; early mortality	HLA-matched sibling	25, 37
	d3	Increased TNF-α	None	HLA-matched sibling	26
	d4	Increased TNF-α	Moderate to severe aGVHD	HLA-matched sibling	27
	d3,d3/d4,d4	Increased TNF-α	Increased aGVHD	MUD	90, 91
	d3,d3/d4/d5	Increased TNF-α	Decreased survival	MUD	90
	d4/−1031C/TNF-a5		Increased TRM	MUD	89
−308G/A SNP	A	Increased TNF-α	Increased aGVHD	HLA-matched sibling MUD	23, 24
			Toxic complications	HLA-matched sibling MUD	31
			None	HLA-matched sibling	25, 26, 27, 28, 29
	−308/−238AG	Increased TNF-α	Delayed neutrophil engraftment	MUD	38
+448G/A SNP	A	NK	Severe aGVHD; cGVHD; early death	HLA-matched sibling	30
−1031T/C, −863C/A, −857C/T SNPs	−1031T, −863C, −857C	NK	Increased aGVHD; lower relapse	MUD	92

Abbreviations: aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; HLA, human leukocyte antigen; MUD, matched unrelated donor; NK, not known; SNP, single-nucleotide polymorphism; TNF, tumour necrosis factor; TRM, transplant-related mortality.

TNFRSF1B

The actions of TNF-α are mediated by two receptors: TNF superfamily receptor (TNFRSF) 1 and receptor 2. TNFRSF2 is most abundant on endothelial and haematopoietic cells and, upon TNF-α binding, it induces apoptosis of $CD8^+$ cells and stimulates haematopoietic cell proliferation (Ref. 39). The *TNFRSF1B* gene is located on chromosome 1 at position 1p36. A SNP (M/R) occurs within exon 6 of this gene at codon 196. The R allele of this SNP has been linked with the development of systemic lupus erythematosus (SLE), and the recipient RR genotype has been associated with an increased incidence of extensive cGVHD following HLA-matched sibling HSCT

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(Refs 40, 41). The R allele has also been found to associate with an exacerbated immune response and lower soluble TNFRSF2 levels compared with the M allele.

IL10

IL-10 is a potent inhibitor of T-cell proliferative responses and suppresses pro-inflammatory cytokine production. The gene of this cytokine maps to chromosome 1 (1q31-32) and is highly polymorphic, containing several microsatellites and five SNPs that have been resolved into conserved haplotypes representing high-level (GCC), intermediate-level (ATA) and low-level (ACC) producers of IL-10 (Ref. 42).

Several studies have examined the role of *IL10* polymorphisms in HSCT; however, at present, the data are conflicting. Initial studies demonstrated that the ACC haplotype and the longer *IL10* −1064 $(CA)_n$ microsatellite (>12) alleles associated with the development of severe (grade III–IV) aGVHD in HLA-matched sibling transplant recipients treated with cyclosporine alone or cyclosporine plus methotrexate (Refs 25, 37). The ATA/ATA of the recipient has been shown to decrease the risk of aGVHD in a large cohort of HLA-matched sibling transplants (Ref. 29). A study of peripheral blood stem cell (PBMC) transplants also demonstrated that the presence of some *IL10* haplotypes (ACC/ACC vs ATA/ATC vs ATA/ATA) increased the incidence of cGVHD, and individuals possessing the ATA haplotype required longer immunosuppression and were more susceptible to invasive pulmonary aspergillosis (Refs 43, 44). Several other studies have also reported associations between *IL10* polymorphisms and the risk of GVHD (Refs 21, 26, 28).

IFNG

IFN-γ is a pleiotropic cytokine with potent pro-inflammatory actions important in both the innate and adaptive immune response, and is essential for the induction and regulation of antimicrobial and anti-tumour immunity (Ref. 45). Within the first intron of the *IFNG* gene on chromosome 12q14-15 there is a $(CA)_n$ microsatellite polymorphism. This polymorphism has two common alleles – allele 2 and allele 3 – both of which exhibit differences in IFN-γ production in vitro. Allele 2 is associated with increased levels of IFN-γ, whereas allele 3 has been linked with decreased production. *IFNG* microsatellite alleles have also been linked with a variety of immunological diseases and, in addition, in HLA-matched sibling transplantation an association between recipient 3/3 homozygosity and an increased risk of aGVHD has been reported (Ref. 46). Other studies have also shown an association between the possession of the 3/3 genotype, or lack of the 2/2 genotype, and acute or chronic GVHD (Refs 47, 48). The mechanism behind these associations is currently unknown, but may be explained if IFN-γ has a negative feedback regulatory role, as seen in some murine models. Weekly injections of IFN-γ in a subacute murine model of GVHD prevent the condition and increase survival. By contrast, mice receiving stem cells from IFN-γ-knockout donors have been shown to develop accelerated lethal GVHD (Ref. 49). Presence of the *IFNG* 3/3 genotype has also been associated with increased risk of Epstein–Barr virus (EBV) reactions after sibling or MUD HSCT (Ref. 50).

IL1 family

There are ten members of the *IL1* family. IL-1α and IL-1β (genes *IL1A* and *IL1B*) are agonists, and the IL-1 receptor antagonist (IL-1Ra; gene *IL1RN*) is a specific receptor antagonist, which is relatively unique in cytokine biology. Binding of IL-1 to its receptor leads to induction of various genes including other cytokines (i.e. IL-6) and chemokines, nitric oxide synthase and type 2 cyclooxygenase, all of which are involved in the inflammatory response and stimulation of lymphocytes. The *IL1* family and its polymorphisms have therefore been associated with a wide range of inflammatory diseases.

Polymorphisms in *IL1A*, *IL1B* and *IL1RN* have been extensively investigated in HLA-matched sibling transplants, and their associations with HSCT outcome have recently been reviewed (Ref. 51). Allele 2 (donor-derived) of the *IL1RN* variable number tandem repeat (VNTR) downregulates IL-1 production and consequently correlates with less-severe aGVHD. The same allele in the recipient genotype is associated with aGVHD (Refs 28, 52). The presence of allele 2 (donor genotype) in either the VNTR or −889 polymorphisms of *IL1A* has also been associated with cGVHD (Ref. 53).

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IL6

IL-6 is a pro-inflammatory cytokine that plays a central role in immune, inflammatory and acute phase responses. It is produced by numerous cell types, induces the development of cytotoxic T cells in the presence of IL-2 and synergises with IL-13 to promote haematopoietic stem cell differentiation and maturation. Consequently, IL-6 plays a major role in the biology of GVHD and HSCT (Ref. 22).

The *IL6* gene is located on chromosome 7p21 and, like many other cytokine genes, has been found to be polymorphic – containing both microsatellites and SNPs (Refs 54, 55). A G/C SNP exists in the promoter region at position −174. In both normal and HSCT populations, the G allele of this SNP correlates with higher serum IL-6 levels (Ref. 56). Several studies have also found the G allele associated with both aGVHD and cGVHD following HLA-matched sibling transplantation (Refs 21, 26, 46, 57).

Transforming growth factor β (TGFB)

TGF-β is a pleiotropic cytokine with unique and potent immunoregulatory properties. Two SNPs exist within its gene, at positions −800 (G/A) and −509 (C/T), both of which associate with variations in plasma TGF-β concentration. The TT genotype of the −509 polymorphism is associated with greater TGF-β production (Ref. 58). In the HSCT setting, no association between these polymorphisms and transplant outcome has been reported (Refs 46, 59). However, a highly significant association was demonstrated between the high expression phenotype of the −509 C/T SNP and severe GVHD, when the *TGFB* genotype of patients with no or mild GVHD was compared with those who developed more severe grades of the condition (Ref. 60).

Polymorphisms also exist at codons 10 and 25 of *TGFB*, both of which result in amino acid substitutions (Leu/Pro and Arg/Pro respectively). A significant link between *TGFB* codon 10 polymorphism (donor-derived) and the development of aGVHD has been reported following paediatric HSCT (Ref. 61). The high-producer G/G genotype is also linked with the development of severe aGVHD following both sibling and MUD transplantation (Ref. 60). Several other polymorphisms within *TGFB* and *TGFBR* genes exist and have been reported to influence HSCT outcome in sibling transplant cohorts (Refs 59, 61).

Chemokines and chemokine receptors

Inflammation and tissue damage can be inflicted directly by cytokines or by cytotoxic T lymphocytes (CTLs), macrophages and polymorphonuclear granulocytes. The infiltration of such cells to sites of inflammation is dependent upon chemotaxis. Therefore, the relationship between chemokines and their receptors is also likely to be influenced by polymorphisms present within their genes. A recent renal transplant study identified polymorphisms that were associated with acute rejection in *CCR5* and *CCR2* genes (Ref. 62). A *CCR5* gene deletion mutation (Δ32) results in complete loss of chemokine receptor function and hence protection from aGVHD (Ref. 63). The loss of tissue-specific homing by T cells to the gut and liver – key GVHD target organs – has been illustrated in murine bone marrow transplant (BMT) studies on knockout mice (Refs 64, 65, 66). Genetic associations of other chemokines and their receptors will be analysed in the HSCT field given recent clinical (Refs 67, 68) and experimental (Refs 69, 70) data of chemokine and/or receptor expression in GVHD target organs. Patients can also be at risk of developing infections to which their immune system does not optimally respond. A *CCR5* gene polymorphism (−2554 G/T in the promoter) has been linked to susceptibility to cytomegalovirus (CMV) infection after transplantation (Ref. 71). The virus evading immune attack by T cells because homing to sites of inflammation is prevented.

Innate immunity

Other, non-cytokine genes associated with innate immunity have been assessed for their impact on HSCT outcome. There are a number of key molecules that allow mammals to sense pathogens, such as PRRs, which in turn activate the immune response via both the innate and adaptive pathways. These molecules obviously play a very important role in post-transplant complications, especially infectious episodes. Among these PRRs are the transmembrane TLRs, which have a potent ability to activate APCs, which subsequently provokes innate immunity (Refs 72, 73, 74). APCs also have ligands for different TLRs that are differentially expressed on the cell surface. TLR4 is a major

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receptor for lipopolysaccharides and has been implicated in the pathogenesis of GVHD. Polymorphisms in the *TLR4* gene have been associated with hyporesponsiveness to lipopolysaccharides. This leads to a reduced risk of aGVHD, but consequently results in an increased risk of Gram-negative bacteraemia. However, there was no hyporesponsiveness in those patients given prophylaxis for infection or GVHD (Ref. 75). Polymorphisms in the *TLR1* and *TLR6* genes have been investigated in patients with invasive aspergillosis following HSCT, with patient genotype being associated with an increased risk of infection (Ref. 76). These results suggest that the impact of patient genotype is influenced by the period of immunosuppression post transplant before donor immune reconstitution occurs.

Intracellular PRRs, such as NOD-like receptors, which are associated with inflammatory bowel disease, are important molecules for study in HSCT because the gastrointestinal tract is damaged by conditioning regimens and is a target organ for GVHD. There are three SNPs in *NOD2/CARD15* (nucleotide-binding oligomerisation domains containing 2/caspase recruitment domain family number 15) – SNP8, SNP12 and SNP13 – which are involved in defective NF-κB responses. Possession of two or more *NOD2/CARD15* variants is associated with more severe GVHD and increased transplantation-related mortality (TRM) in both HLA-matched siblings and matched unrelated transplants (Ref. 77). In a second cohort of HLA-matched siblings (Ref. 78), the genetic risk could be modulated through different types of gastrointestinal decontamination before transplantation. In T-cell-depleted transplants, possession of *NOD2/CARD15* SNPs is associated with lower disease-free survival rates (Ref. 79). These results illustrate that the altered immune response associated with *NOD* gene variants after HSCT is dependent on the type of immunomodulation (e.g. bacterial decontamination) and on the type of immunosuppression (e.g. T-cell depletion) administered.

The incidence of severe infections post transplant has been investigated by several groups. Severe bacterial infectious episodes are associated with myeloperoxidase gene (*MPO*) SNPs, with the AG or AA donor genotype rather than the wild-type CC genotype being associated with the most severe infections. Mannose-binding lectin (MBL) binds a range of pathogens and opsonises them for phagocytosis. Human MBL is encoded by *MBL2* on chromosome 10. SNPs in the promoter region affect serum MBL levels, and low MBL levels give an elevated risk of infection in immunocompromised individuals. In an Australian HSCT cohort, *MBL2* mutations in the recipient and/or donor genotype were associated with a risk of major infection following allogeneic HSCT (Ref. 80). However, this association was not observed in an independent study (Ref. 28).

FcγRIIa is expressed in Langerhan cells, dendritic cells, endothelial cells and leukaemic cells, and is genetically polymorphic. The *FcγRIIa*-R 131 genotype in the recipient has been associated with an increased incidence of severe infection, and time to neutrophil recovery is delayed when the donor genotype is *FcRIIIb* HNA-1a/HNA-1b (Ref. 28). In a more recent study (Ref. 81), 73 acute monocytic leukaemia (AML) patients were analysed for *FcγRIIa* polymorphisms as candidates for aGVHD. A mismatch between donor and patient *FcγRIIa*-R/R 131, *FcγRIIa*-H/H 131 and *FcγRIIa*-R/H 131 genotypes were associated with an increased probability of GVHD. The presence of FcγRIIa allotypes on DCs, Langerhan cells and endothelial cells suggests that they may be candidate minor histocompatibility antigens (mHags) through their recognition by donor T cells. This type of mismatching and potential relevance in GVHD of candidate mHags was also shown for the CD31 adhesion molecule (PECAM-1) (Ref. 82), where the Val/Asn/Gly haplotype in the donor significantly associated with incidence of GVHD and donor heterozygosity at codon 125 or 563 associated with worse overall survival. Several other HLA-matched sibling studies have examined associations between *PECAM1* polymorphisms and HSCT outcome with inconsistent results (Refs 82, 83, 84, 85).

Other polymorphisms associated with HSCT outcome

Steroid hormone receptors are implicated in the development of the immune system; consequently, these genes could have an impact on the reconstitution of the incoming donor graft. Steroid hormone receptor

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polymorphisms, such as the RFLPs within intron 8 of the vitamin D receptor (*VDR*) and intron 1 of the estrogen receptor (*ESR1*) genes, were found to correlate with aGVHD and lower overall survival (Refs 86, 87).

Other polymorphisms studied include the *FAS* −670 and *HSPA1L* 2763 SNPs. The *FAS* −670 G allele has been correlated with infection and cGVHD following HSCT (Ref. 30), whereas the *HSPA1L* SNP has been linked to the development of toxic complications in two independent transplant cohorts (Ref. 88).

MUD studies: cytokine and cytokine-receptor gene polymorphisms

TNFA

In MUD HSCT, the *TNFd* microsatellite polymorphism has been associated with several transplant outcomes, including GVHD, TRM and survival (Refs 89, 90). TNF-α serum levels and *TNFd* genotypes correlate with aGVHD, with patients homozygous for the *TNFd*3 or *TNFd*4 alleles having significantly higher TNF-α levels during conditioning and more frequently developing aGVHD (Ref. 91). More recently, *TNFd* has been associated with TRM rather than GVHD (Ref. 89). The presence of the *d*4 allele in conjunction with the *TNFA* 1031C allele in either recipients or donors significantly increases the incidence of TRM. A further allele associated with the *TNFd*4/1031C haplotype, *TNFa*5 has been linked with TRM. The *TNFd* microsatellite has also been associated with HSCT survival. A gradual decrease in survival rates has been observed for MUD transplant recipients possessing *TNFd*3/*d*3, *d*4 and *d*5 (Ref. 90).

TNFA −308 is another TNF-α polymorphism frequently examined in HSCT. In the MUD setting, the *TNFA**2 allele (donor-derived) of this SNP has been associated with severe aGVHD (III–IV) and strong linkage disequilibrium between the *TNFA* −308 and −238 SNPs and extended HLA haplotypes have been demonstrated. The *TNFA* −308/238 AG haplotype is also associated with significantly delayed neutrophil engraftment (Refs 23, 38).

TNFA −863 (C/A) and −857(C/T) polymorphisms in donors and/or recipients correlate with a higher incidence of GVHD and a lower rate of relapse. By restricting the analysis to HLA A-, HLA B- and DRB1-matched pairs, the effect of HLA linkage disequilibrium with *TNFA* was revealed. GVHD outcome was still linked with recipient genotype; however, the association with relapse was no longer observed (Ref. 92). Table 1 summarises *TNFA* associations in MUD transplant cohorts.

TNFRSF1B

Recipients of *TNFRSF1B* −196R-positive unrelated donors have a higher incidence of severe GVHD and a lower rate of relapse than patients receiving transplants from 196M homozygous donors (Ref. 92).

IL10

An association between allele length at the *IL10* −1064 $(CA)_n$ microsatellite and survival has been reported: longer recipient *IL10* alleles (>12) correlated with decreased survival (Ref. 90). In addition, in a mixed sibling and unrelated donor transplant cohort, the donors of cGVHD patients frequently possessed a greater number of CA repeats (allele 13 or above) (Ref. 23).

There appears to be a significant link between TRM and the *IL10* SNP GCC haplotype. Presence of the donor R2-GCC haplotype is associated with a higher incidence of TRM, whereas possession of the R3-GCC haplotype (donor-derived) decreases the risk of TRM (Ref. 89). These findings conflict with current HLA-matched sibling studies, as the majority of these have found associations with GVHD rather than TRM (Refs 29, 37, 92).

IFNG

The genotype of the *IFNG* intron 1 $(CA)_n$ microsatellite constitutes a risk for acute and chronic GVHD following HLA-matched sibling HSCT (Refs 46, 48). These associations have not yet been observed in the MUD setting. However, transplant recipients possessing the 3/3 genotype of this polymorphism have been shown to present with a significantly higher EBV copy number than those with other genotypes in a mixed cohort of sibling and MUD transplants (Ref. 50).

IL1 family

IL1 polymorphism research in the MUD setting is limited to one study performed on a small paediatric cohort. The presence of the *IL1A* −889 T allele in either the recipient or donor is associated with significantly improved survival

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and decreased TRM. No association with aGVHD was demonstrated; however, trends were observed between the incidence of cGVHD and the *IL1A* −889 A allele and allele 2 of the *IL1RN* VNTR (Ref. 93).

IL2

IL-2 is a pro-inflammatory cytokine that plays a key role in immune response production, and has been implicated in the initiation and maintenance of GVHD (Ref. 94). A T/G SNP exists within the *IL2* gene promoter region at position −330. The G allele of the polymorphism associates with early and sustained elevation of IL-2 production (high-producer genotype) and an increased risk for aGVHD after MUD HSCT (Ref. 95).

IL7R

IL-7 is a 25 kDa glycoprotein that plays an important role in the regulation of lymphopoiesis via IL-7 receptors (IL-7R). These receptors comprise a γc and an α chain. Sequencing of the α chain has revealed the existence of four SNPs (+510C/T, +1237A/G, +2087T/C and +3110A/G), all of which result in amino acid substitutions. The +1237 SNP within donors associates with survival and TRM following MUD transplantation, with the highest mortality being linked with the GG genotype. Considering the functions of IL-7, these findings seem to suggest a possible role for this SNP in T-cell and immune reconstitution following MUD HSCT (Ref. 96).

IL13

IL-13 is a Th2-type cytokine that suppresses the cytotoxic actions of macrophages, inhibits the production of pro-inflammatory cytokines and plays a major role in various inflammatory diseases (Ref. 97). In terms of HSCT, IL-13 research is currently extremely limited. However, its production has recently been associated with GVHD following MUD transplantation. Recipients who developed severe grades of aGVHD (III) had extremely high pre-transplant IL-13 production by donor T cells, whereas those developing little or no aGVHD had donors that produced no IL-13 pre HSCT (Ref. 98).

Several SNPs have been identified within *IL13* and linked with raised IgE levels and a number of immunological or inflammatory diseases (Refs 97, 99, 100, 101). To date, no studies associating these polymorphisms with HSCT outcome in any transplant setting have been published.

IL18

IL-18 is a pro-inflammatory cytokine that induces Th1-type cell differentiation and cytotoxic-T-cell function. The gene has three polymorphisms at positions −137 (G/C), −607 (C/A) and −656 (G/T) within its promoter region and three main haplotypes exist: GCG, CAT and GAT. The GCG haplotype in recipients is associated with a decreased risk of TRM at day 100 and 1 year post-transplant, and with improved overall survival. *IL18* haplotypes had no effect on aGVHD in this cohort (Ref. 102). However, associations between IL-18 and aGVHD have been demonstrated by well-established murine BMT models. Neutralisation of IL-18 by antibodies increases aGVHD-related mortality, whereas administration of the cytokine significantly improves survival and reduces the pathological features of aGVHD (Ref. 103).

Innate immunity and other non-HLA polymorphisms

Polymorphism studies involving other non-cytokine genes, particularly those associated with innate immune responses, have also been performed. In terms of MUD transplantation, only the NOD-like receptor polymorphisms have an impact on HSCT outcome. Possession of two or more *NOD2/CARD15* variants (linked with a defective antibacterial NF-κB response) increases the risk of severe aGVHD (grade III–IV) and TRM (Refs 77, 78).

Pharmacogenomics in HSCT

Studying genetic variability as a determinant of an individual's drug response has the potential to improve HSCT outcome. Consequently, several polymorphisms associated with drug metabolism have been studied in relation to HSCT (Ref. 21). To date, polymorphisms of genes involved in the metabolism of drugs used in conditioning regimens and prophylaxis strategies have been associated with GVHD, survival, toxicity and platelet recovery following HSCT (Refs 104, 105). A SNP (C677T) within the gene encoding methylenetetrahydrofolate reductase (*MTHFR*), an enzyme involved in methotrexate metabolism, has recently been associated with HSCT outcome. The 677 TT

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genotype (with lower enzyme activity) was linked with increased mucositis and delayed platelet engraftment in HSCT recipients receiving methotrexate (Ref. 104). These findings have been confirmed (Ref. 106); however, one study (Ref. 107) found no association with HSCT outcome. This SNP has also been investigated in a mixed cohort of sibling and unrelated transplants. An association between the CT and TT genotypes of HLA-matched siblings and a decreased incidence of GVHD was demonstrated (Ref. 108). By contrast, a link between the CT and TT genotypes and severe GVHD has also been observed (Ref. 105). More recently, associations between the C677T SNP and levels of liver enzymes, platelet recovery and TRM in HLA-matched sibling HSCT has been reported (Ref. 109).

SNPs associated with the P450 (*CYP*) cytochrome family and the multidrug-resistance 1 gene (*ABCB1*) are of interest, not only in HSCT but also in solid-organ transplantation. Recently, a P450 cytochrome SNP (*CYP3A5*) and four *ABCB1* SNPs [T–129C, C1236T, G2677(T,A) and C3435T] were investigated for any association with interindividual cyclosporine variation in renal transplant recipients. No association was observed with the *CYP* SNP; however, the recipient wild-type genotype in *ABCB1* C1236T was associated with lower values of dose-adjusted peak drug concentration and dose-adjusted area under the concentration-time curve, compared with carriers of the mutant allele (Ref. 110). In terms of HSCT, a P450 polymorphism (*CYP2B6*) has been investigated for any association with transplant outcome. Recipient alleles of the *CYP2B6* polymorphism were not only linked with lower P450 enzyme expression and higher accumulation of cytotoxic metabolites, but also with mucositis, haemorrhagic cystitis and veno-occlusive disease of the liver (Ref. 105). At present, no studies associating the *ABCB1* polymorphisms with HSCT outcome have been published.

Clinical implications and applications

SNPs are relatively abundant in the human genome, but only a proportion will give rise to functional alterations of the encoded proteins leading to biological variation and activity of cytokines, receptors, pharmacogenes or other molecules related to disease exacerbation.

Interaction between SNPs and the environmental factors that may play a role in HSCT outcome, such as exposure to high-dose chemotherapy and/or immunosuppressive drugs, may influence how certain cytokine gene SNPs interact or function. This has been described as the concept of environmentally determined genetic expression (EDGE). This type of approach may be particularly valuable in describing genetic association studies of complications after chemotherapy of HSCT, including not only GVHD, but also infection, mucositis and veno-occlusive disease (Ref. 111).

In HSCT, obvious problems of analysis arise owing to the heterogeneity of the patient cohort. This is reflected in the fact that although a number of polymorphisms are consistently associated with HSCT outcome (some of which are summarised in Table 2), the same SNP can be associated with aGVHD rather than cGVHD and/or survival or relapse depending on the transplant cohort – that is, HLA-matched sibling, MUD or mixed. For example, some HSCT transplant centres use peripheral blood stem cells rather than bone marrow, which may give rise to increased cGVHD in older patients. Other centres are increasingly using reduced-intensity conditioning regimes, giving rise to a smaller 'cytokine storm' and a later onset of acute or chronic GVHD. Some of the SNPs/alleles are relatively rare, which means that larger and more homogeneous data sets are required for a multifactorial analysis and prognostic index model generation. Any HSCT genotypic analysis needs to take into account the 'environment' – clinical risk factors, such as age, female-to-male transplants, CMV status of patient and donor, stage of disease at transplant and time from diagnosis to transplant – as well as the conditioning and therapeutic regimen, all of which play a major role. Several of these factors have been included in clinical risk models for determining the outcome of HSCT (Refs 26, 112, 113, 114, 115). In addition, the immune status of the recipient and donor, thymic function (Ref. 116) and immune reconstitution are critical factors in determining successful HSCT (Ref. 117).

Other problems arising in studying non-HLA genetics in diverse HSCT populations are the potential differences in allele frequencies between ethnic groups across, for example, Europe, Japan and the USA. One recent

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Table 2. Polymorphisms consistently associated with haematopoietic stem cell transplantation outcome

Gene	Polymorphism	Genotype	Effect	Association	Study	Refs
IFNG	$(CA)_n$ microsatellite	3,3+/2,2−	Lower IFN-γ	Increased aGVHD	HLA-matched sibling	46, 48
		3,3+	Lower IFN-γ	cGVHD	HLA-matched sibling	47
IL1RN	VNTR	A2+	Increased antagonist	cGVHD	HLA-matched sibling	28
					MUD	93
			Increased antagonist	Decreased aGVHD	HLA-matched sibling	52
IL6	−174G/C SNP	G	Increased IL-6	Increased aGVHD; cGVHD	HLA-matched sibling	26, 46, 57
IL10	$(CA)_n$ microsatellite	>12 Repeats		Severe aGVHD	HLA-matched sibling	25, 29
			Lower IL-10	cGVHD	HLA-matched sibling MUD	23
				Decreased survival	MUD	90
TNFRSF1B	−196M/R SNP	R	Lower soluble TNFRSF2	Increased severe GVHD; lower relapse	MUD	92
				cGVHD	HLA-matched sibling	40
NOD2/CARD15	SNP8, -12, -13	2+ variants	Altered NF-κB	Severe GVHD; increased TRM	HLA-matched sibling MUD	77, 78

Abbreviations: aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; HLA, human leukocyte antigen; IFNG, interferon γ; IL, interleukin; MUD, matched unrelated donor; NOD, nucleotide-binding oligomerisation domain; NF, nuclear factor; SNP, single nucleotide polymorphism; TNFRSF, tumour necrosis factor superfamily receptor; TRM, transplant-related mortality; VNTR, variable number tandem repeat.

study comparing the incidence of aGVHD in HLA-identical sibling transplants within the Japanese, white and African Americans, Scandinavians and the Irish (Ref. 118) showed significant differences between the American groups and the Irish compared with the Japanese or Scandinavians. Similar differences were described for TRM, and indicate that genetic differences other than HLA may affect transplant outcome. Several studies on allele

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frequencies in transplant populations worldwide and comparative studies are currently being undertaken (Refs 119, 120). Consequently, allele frequency data needs to be taken into account before analysis.

More complex statistical methods and bioinformatic tools still need to be developed and confirmed in second or third confirmatory prospective patient–donor cohorts before results will be sufficiently mature to be used in the clinic. One such algorithm for predicting mortality of patients with GVHD using only clinical parameters, such as bilirubin levels, treatment with steroids and performance score, has been developed (Ref. 121). Clinical models for overall survival for chronic myelogenous leukaemia (CML) also include clinical factors, which make up a risk score that can be applied to the clinic (Ref. 113). These types of clinical risk assessments may also benefit from the addition of non-HLA genotypes. An individualised scoring system for patients could then be used to assess therapies and clinical protocols. However, there are several problems in the reporting of genetic association with complex diseases (Ref. 122). Selection bias needs to be avoided and negative studies need to demonstrate that they were carried out on large enough cohorts with sufficient statistical power to detect any association. False-positive results owing to chance associations need also to be considered in the analysis. Negative associations have, for example, included the *TNFd* microsatellite, which has shown variable association with GVHD and outcome, with many studies demonstrating no association at all (summarised in Table 1) (Refs 21, 25, 26, 27, 28, 29, 89). Other negative associations include those for *IL1B* −511, *IL1RN* −9261, *IL6* −174, *IL10* −592A/C and −1082 A/G, *TNFA* −308, *IL4R* −1902 and −3223, *IL1RN* VNTR86 intron 2 and *IL2*, as well as negative associations with cord-blood transplants (Refs 29, 90). Complications arising post transplantation are related to the HSCT procedure itself; the functional genomic response of the patient or donor engrafting cells arise because of the allogeneic or infectious onslaught. However, genetic association with a complex disease requires identification of functional gene polymorphisms, which are potentially inherent in the development of the disorder. The mechanisms of gene association studies compared with methods used for analysis of the complications arising after HSCT are therefore very different. In the latter case, the heterogeneity of the HSCT cohort (type of transplant, diagnosis, and T-cell depletion strategies) needs to be taken into account to stratify and/or further analyse the data. This type of approach to data analysis is not necessarily applicable for patients with a complex disease, where subgroup analysis should be avoided (Ref. 123).

Despite these potential difficulties, research into non-HLA genetics and transplant outcome will give insight into the biology of HSCT, including the pathophysiology of GVHD and GVL. Genotyping cohorts of patients and donor pairs with, for example, *NOD2/CARD15* polymorphisms may aid in donor selection and/or altered HSCT protocols, whereas analysis of particular pharmacogenes may lead to altered prophylaxis regimens.

Research in progress

The aims of current research into the role of non-HLA genetics in predicting outcome are to develop an improved insight into the basic science of HSCT and to develop a risk or prognostic index for use in the clinical setting based on individual risk genotypes. These goals rely, to a large extent, on the standardised definition of clinical outcomes, especially when large and expensive studies are planned. A lack of such common language can profoundly affect the interpretation of biological studies, especially when observing outcomes such as GVHD or infection. A good example of a consensus on these topics is the recent US National Institutes of Health consensus on cGVHD (Ref. 124). Research initiatives require linking and coordination of HSCT research groupings with clinical information and patient and donor DNA. This approach is being followed by several groups across Europe (Ref. 125), which aim to improve the science of non-HLA genetics for translation to the clinic in the future.

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Further reading, resources and contacts

Schlomchik, W.D. (2007) Graft-versus-host disease. Nat Rev Immunol 7, 340-352
This review provides an excellent overview of the current literature of the mechanisms behind GVHD and the key molecules and cells that are involved, although it is heavily based upon murine model data.

Rossi, M. et al. (2005) Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J Immunol 175, 1373-1381
This brief review provides detail on dendritic cells, which instigate the adaptive immune response observed in GVHD.

Wysocki, C.A. et al. (2005) Leukocyte migration and graft-versus-host disease. Blood 105, 4191-4199
This review provides good background knowledge on the role of chemokines and their receptors in the homing of leukocytes to specific tissue sites – in particular those involved in GVHD.

Ruggeri, L. et al. (2006) Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. Immunol Rev 214, 202-218
This review gives and in-depth explanation of NK cell alloreactivity in the HSCT setting from animal models and clinical studies.

Features associated with this article

Figure
Figure 1. The role of recipient and donor genotypes on the modulation of graft-versus-host disease.

Tables
Table 1. *TNFA* polymorphisms and haematopoietic stem cell transplantation outcome.
Table 2. Polymorphisms consistently associated with haematopoietic stem cell transplantation outcome.

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