Cellular mechanisms of alloreactive immunity following stem cell transplantation

by

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A thesis submitted to

The University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

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March 2011

Abstract

Allogeneic stem cell transplantation is associated with a powerful T cell-mediated 'graft-versus-leukaemia' (GvL) effect and also 'graft-versus-host disease' (GvHD). Developing therapies to improve survival relies on greater understanding of these responses in order to enhance GvL and suppress GvHD. To gain an increased understanding of the mechanisms of GvHD I measured frequencies of Th1, Th17 and Treg subsets in the blood of 32 GvHD patients (during and outside of disease episodes) and in 21 patients who did not suffer GvHD. No associations between T cell subset frequencies or serum cytokine concentrations and incidence of GvHD were evident. My GvL work addressed whether T cell responses to cancer/testis antigens (CTAg) could be detected in a cohort of 41 patients who had undergone allogeneic stem cell transplantation for the management of acute myeloid leukaemia and multiple myeloma. CTAg-specific CD8+ T cell immune responses were observed within peripheral blood of five patients, with an average magnitude of 0.045% of the CD8+ T cell repertoire. T cell immunity was focussed against peptides derived from MAGE proteins and was increased within the bone marrow. These immune responses are likely to contribute to tumour eradication following transplantation and represent a potential novel mechanism for GvL.

For Tom and Doris

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List of Abbreviations

AF700	-	Alexa-fluor 700
AML	-	Acute myeloid leukaemia
APC	-	Allophycocyanin
BM	-	Bone marrow
BMMC	-	Bone marrow mononuclear cell
CFSE	-	Carboxyfluorescein succinimidyl ester
CR	-	Complete remission
CsA	-	Cyclosporine
CTL	-	Cytotoxic lymphocyte
DC	-	Dendritic cells
DFS	-	Disease free survival
DMSO	-	Dimethyl Sulphoxide
EDTA	-	Ethylenediaminetetracetic Acid
FCS	-	Foetal calf serum
FITC	-	Fluorescein isothiocyanate
FSC	-	Forward scatter
GvHD	-	Graft versus host disease
GvL	-	Graft versus leukaemia
HS	-	Human serum
HSC	-	Haematopoietic stem cell
ICS	-	Intracellular cytokine staining
IFN	-	Interferon
IL	-	Interleukin
LCL	-	Lymphoblastoid cell line
MHC	-	Major histocompatibility complex

MM	-	Multiple myeloma
MGUS	-	Monoclonal gammopathy of undetermined significance
MSC	-	Mesenchymal stem cell
MUD	-	Matched unrelated donor
PBMC	-	Peripheral blood mononuclear cell
PBS	-	Phosphate buffered saline
PC5	-	Pycoerythrin cyanin 5
PC7	-	Pycoerythrin cyanin 7
PE	-	Phycoerythrin
PMA	-	Phorbol 12-myristate 13-acetate
RIC	-	Reduced intensity conditioning
SSC	-	Side scatter
SCT	-	Stem cell transplantation
SEB	-	Staphylococcus enterotoxin B
TCL	-	T cell line
TBI	-	Total body irradiation
TGF	-	Tumour growth factor
Th	-	Helper T cell
TNF	-	Tumour necrosis factor

Chapter 1: Introduction

1.1 Bone Marrow and Haematopoiesis

1.1.1 Bone marrow structure and microenvironment

The medullary cavities of bones contain blood vessels and bone marrow, a rich microenvironment that fosters blood and stromal cell genesis. During foetal development, haematopoiesis takes place in the liver, spleen and in the medulla of a number of bones but throughout development is increasingly in flat bones so that by puberty blood production takes place predominantly in the sternum, vertebrae, iliac bones and ribs. In these bones the red marrow consists of long trabeculae within a sponge-like reticular framework. Spaces around this framework are filled with fat cells, stromal fibroblasts and haematopoietic precursors. Also found in the bone marrow are mesenchymal stem cells (MSC) which differentiate into fibroblasts, osteoblasts (bone formation), adipocytes, chondrocytes (cartilage generation) and myocytes (muscle). Activated, antigen experienced B cells differentiate into plasma cells which return to, and colonise the bone marrow.

Haematopoietic stem cells (HSC) express CD34 and stem cell antigen-1 (Sca1) which can be used to identify and sort HSCs. Within the bone marrow are osteoblastic and vascular niches (reviewed by Yin *et al*, 2006) formed by supporting cells. Bone marrow cells secrete cytokines which are a diverse group of proteins and glycoproteins (including interleukins and interferons) that promote a range of functions including growth, differentiation and proliferation. A key subgroup of cytokines are instrumental in cell trafficking and are known as chemokines (see section 1.3). Maintenance of these microenvironments is essential for healthy blood cell production. Amongst the array of cytokines present are G-CSF and CXCL12 which mobilise CXCR4 expressing HSC. These chemokines also play a role in HSC survival and proliferation (Cancelas et al., 2005, Lapidot et al., 2005).

1.1.2 Haematopoiesis

Mobilised HSCs divide, with one daughter cell remaining in the bone marrow, whilst the other becomes a multipotent progenitor (MPP) cell, then either a common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). The twin functions of self-renewal and differentiation are crucial to the haematopoietic process. Throughout the maturation process, the nascent haematopoietic cells migrate from the subendosteal region (at the inner bone surface) towards a central region and matured cells exit through a network of vascular sinuses (figure 1.1.1).

Common myeloid progenitors ultimately differentiate into granulocytes, macrophages, erythrocytes or megakaryocytes. Lymphoid progenitors form either NK cells, B cells or T cells (figure 1.1.2). Early B cells commit to the B cell lineage with rearrangement and expression of immunoglobulin genes, acquiring antigen specificity and expressing surface IgM and IgD within the bone marrow before leaving as immature B cells. Maturation occurs outside of the bone marrow, and mature B cells circulate through the lymphatic system until antigen encounter, undergoing somatic hypermutation of the immunoglobulin genes, altering antigen affinity. Most B cells are unsuccessful during differentiation and only a minority survive the developmental process. Activated B cells can either become short-lived plasma cells or enter follicles and form germinal centres (GC). Memory B cells are antigen experienced cells from the GC which can ultimately migrate to the bone marrow and form long-lived plasma cells (reviewed (Bonilla and Oettgen, 2010)).

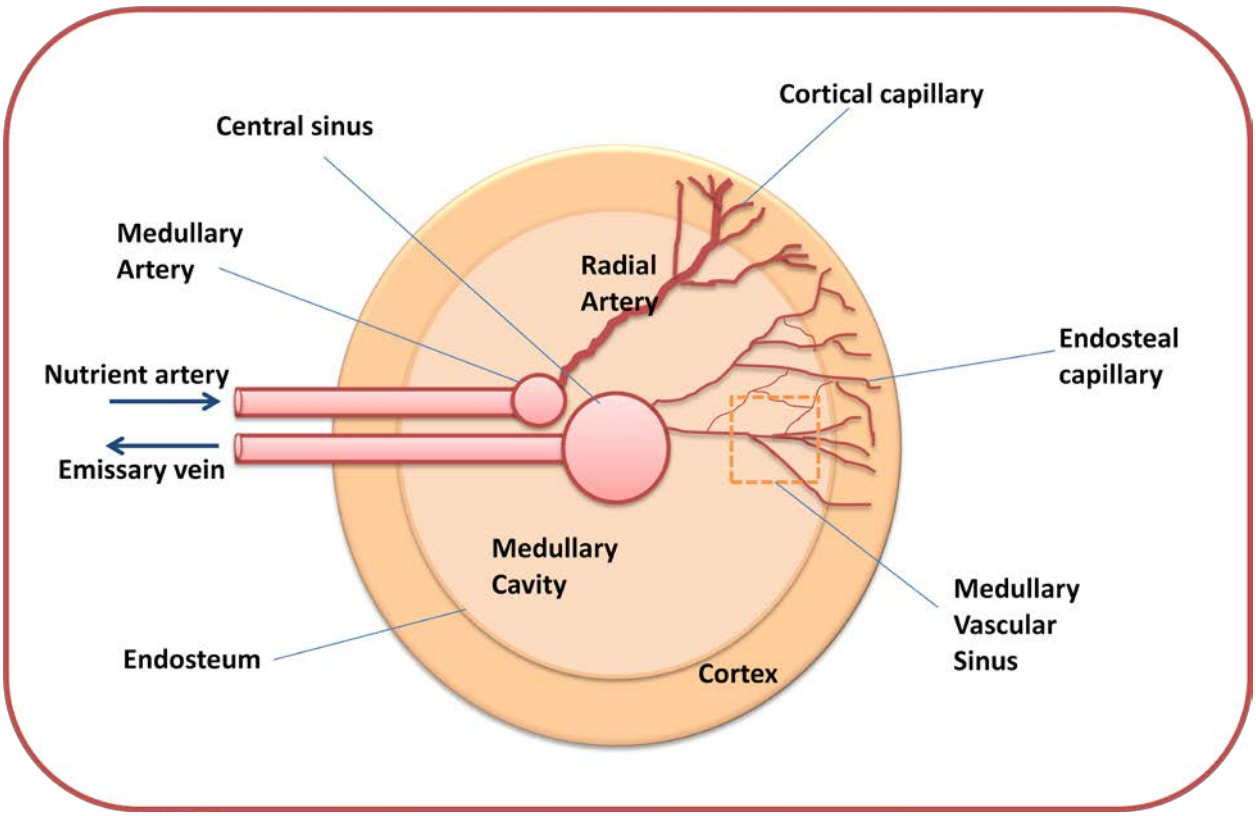


Figure 1.1.1 Structure and vascular network of bone marrow

The structure of bone marrow, with the vascular network bringing blood into the medullary cavity (nutrient artery) and exit channels (emissary vein, capillaries). Haematopoietic stem cells are found at the endosteum and migrate towards the central sinus as maturation occurs.

Adapted from (Nagasawa, 2006)

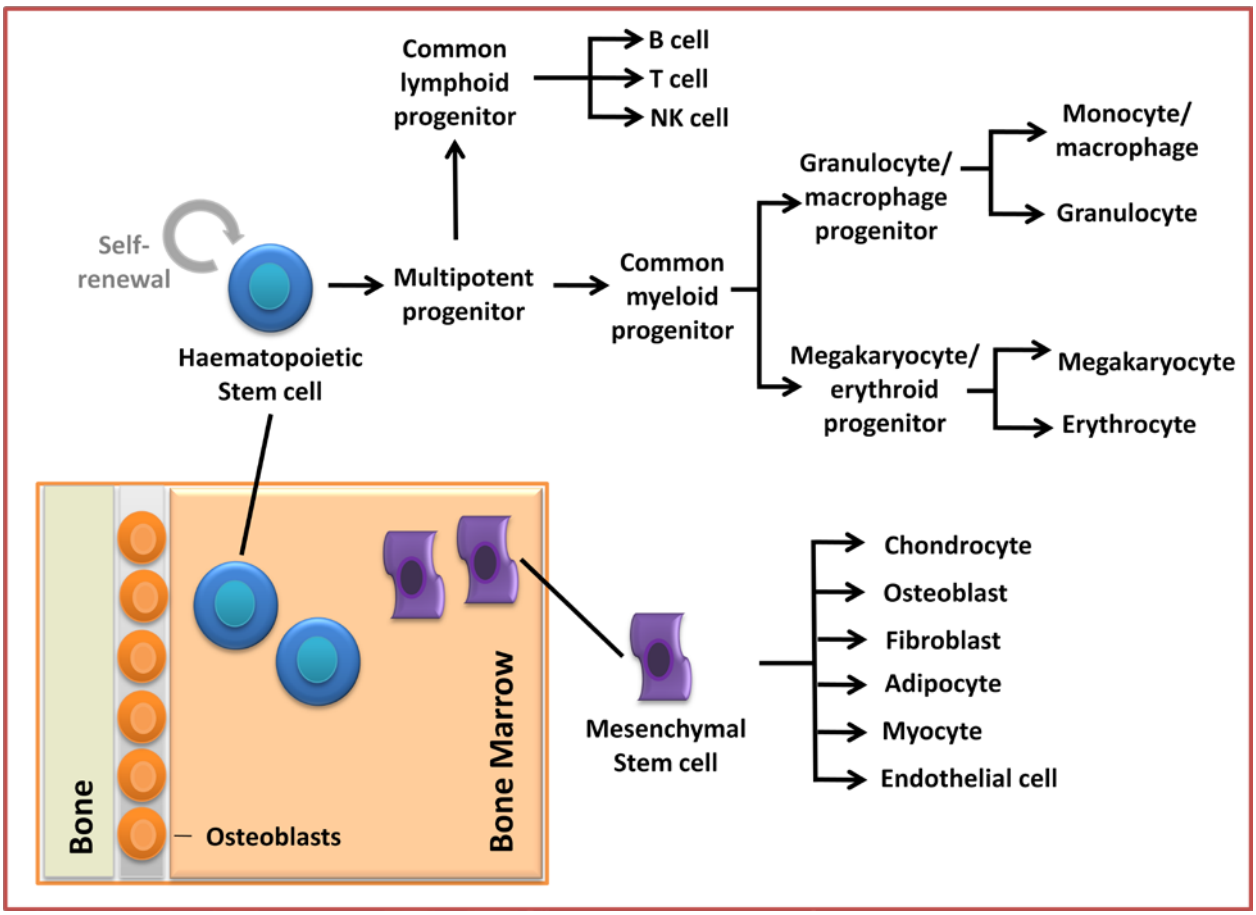


Figure 1.1.2 Differentiation paths of MSCs and HSCs

Haematopoietic stem cells differentiate into lymphoid or myeloid progenitor cells before leaving the bone marrow to mature into either lymphocytes (B cell, T cell & NK cell) or myeloid lineage cells (monocyte/macrophage, granulocyte, megakaryocyte, erythrocyte). Mesenchymal stem cells are also found within the bone marrow and differentiate into chondrocytes, osteoblasts, fibroblasts, adipocytes, myocytes or endothelial cells.

Adapted from (Yin and Li, 2006)

1.1.3 Thymic Selection

On arrival in the thymus, early T cell precursors do not express CD4 or CD8 which are cell surface co-receptors involved with antigen recognition, and are known as double negatives (DN) (figure 1.1.3). During these DN stages, the thymocytes can be subdivided on the basis of expression of CD44 and CD25, progressing from CD44+ CD25- to CD44+ CD25+ to CD44- CD25+ to CD44- CD25- which correspond to DN1 to DN4 respectively (reviewed by (Taub and Longo, 2005)). As they enter the DN2 stage, the cells express the interleukin-7 (IL-7) receptor CD127 and undergo IL-7 driven proliferation. As they pass through DN stages across the cortex, greater numbers of genes that define T cell identity are expressed, leading to greater lineage commitment. Up until the DN3 stage, it is still possible to differentiate along other pathways.

As they enter DN3a stage, T cell lineage is set and gene rearrangement proceeds for the variable regions of the T cell receptor (TCR) (Taghon et al., 2006). The TCR loci contains variable (V), diversity (D) and joining (J) segments, and during recombination, one of each are randomly spliced together. This allows an extremely diverse repertoire of TCRs, and only those that have successfully assembled a pre-TCR (TCRβ chain) can survive and pass through to DN3b stage. The TCRα chain is expressed to make a complete TCR, complexed at the cell surface with CD3. CD4 and CD8 are co-expressed to make double positive (DP) cells which then undergo selection based on their interactions with major histocompatibility complex (MHC) molecules. Positive selection allows for those DP clones that have low avidity binding to self-MHC, giving rise to a pool of T cells with the capacity to recognise foreign antigen presented by self-MHC. Those with a TCR that cannot recognise self-MHC are deleted. Negative selection eliminates DP clones that bind with a very high affinity for self-MHC/self-peptide in order to limit the expansion of pools of self-reactive T cells. This is known as

central tolerance. Single-positive T cells emerge from the selection process, and express CD4 following MHC class II interaction or CD8 resulting from selection by MHC class I. Matured, fully-differentiated but antigen naive T cells enter into circulation (figure 1.1.3).

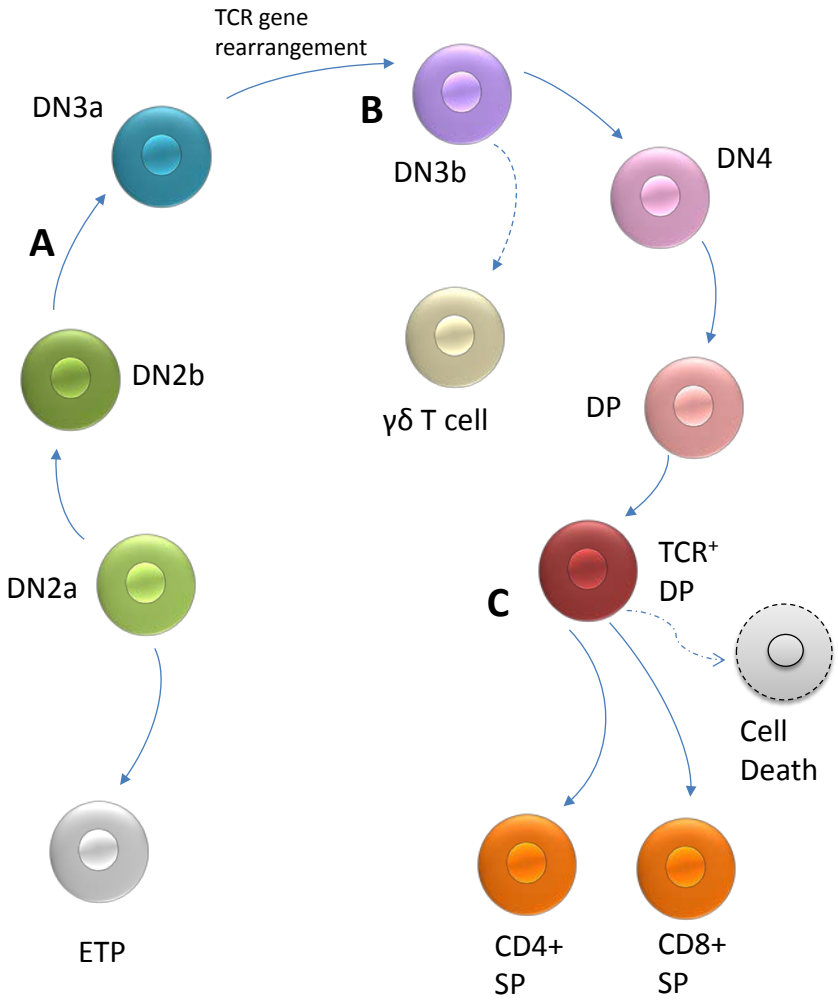


Figure 1.1.3 T Lymphocyte development in the thymus

Early T cell precursors (ETP) progress through the DN2 stage before complete commitment to T cell lineage (A). TCR gene rearrangement occurs between E3a and E3b at which point divergence along the αβ or γδ pathway occurs (B). Following DN4, the nascent lymphocytes become double positive (DP) for CD4 and CD8. Selection occurs after TCR formation to eliminate potential self reactive clones and those which cannot recognise self-MHC (C). Finally, T cells emerge as single positive CD4 or CD8 T cells.

1.2 Lymphocyte differentiation

The traditional paradigm of T cell lineage was that CD8+ T cells were cytotoxic, (thus also known as 'cytotoxic T cells' (CTLs)) and that CD4+ T cells were primarily for support, described as helper T cells (Th). Functional differences led to identification of two Th lineages named Th1 and Th2 based on cytokine secretion profiles (Mosmann et al., 1986). More recently, it has become clear that the CD4+ lymphocyte population has greater diversity and studies have identified an IL-17A secreting subset, the Th17 population (Harrington et al., 2005, Park et al., 2005) and crucially, a suppressive lineage known as regulatory T cells (Tregs) (figure 1.2.1). As described in section 1.1.3, MHC interactions in the thymus direct T cells along the CD4 or CD8 path.

On encounter with antigen, naive CD4+ T cells differentiate into subsets characterised by cytokine secretion profile and function which is used, along with lineage specific transcription factors to identify them. Cytokines present in the environment when the cells are activated direct the lineage pathway the cells will follow. For example, the presence of IFN-α, IFN-γ and IL-12 can drive a Th1 phenotype, but IL-1β and TGF-β lead to Th17 differentiation (figure 1.2.1). Differentiation is discussed further below, and antigen recognition is expanded upon in 1.4.

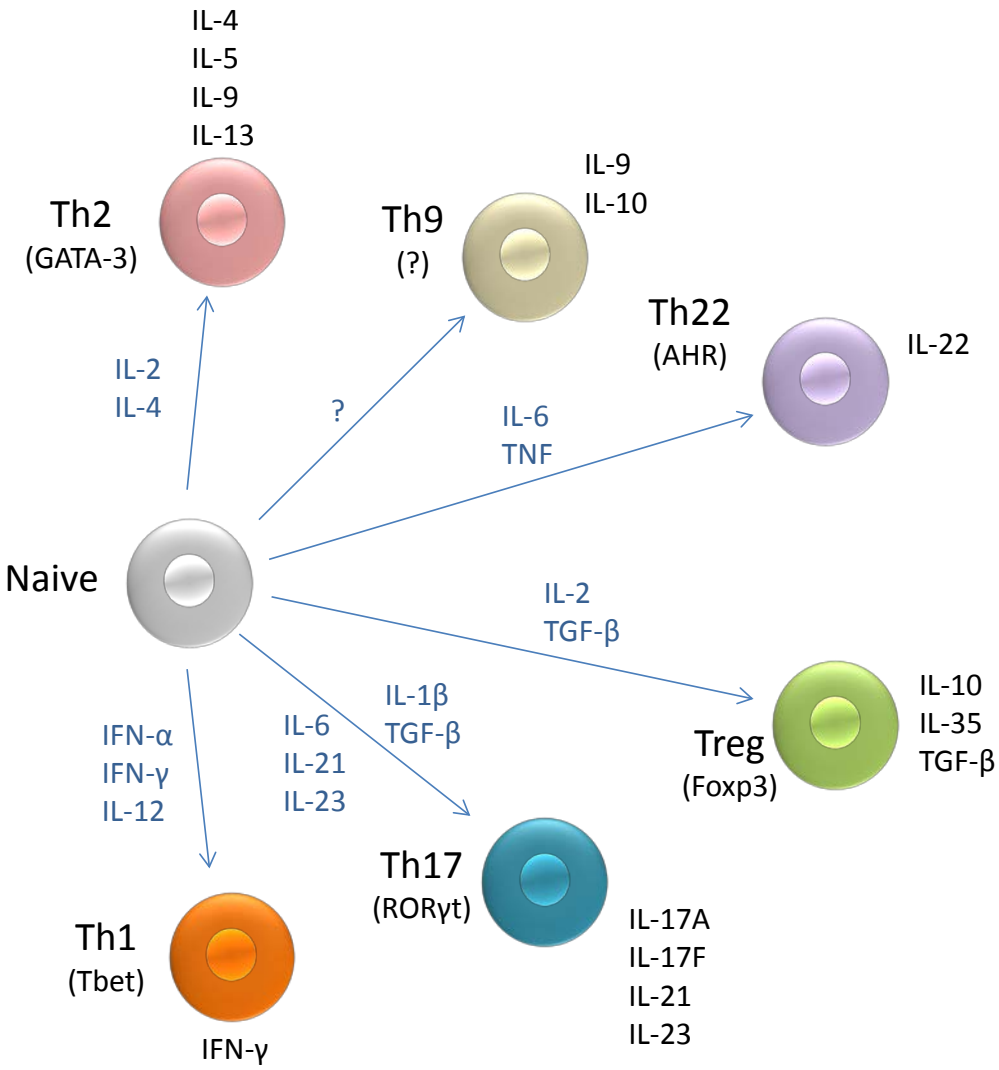


Figure 1.2.1 CD4+ T cell subsets and cytokines

Following antigen encounter, a naive T cell can differentiate along a number of paths dictated by the cytokines present in the environment, this gives rise to different subsets which can be defined by expression of transcription factors and cytokine secretion profiles. Here, the cytokines shown in blue represent those in the environment which drive the pathway towards Th2, Th9, Th22, Treg, Th17 or Th1 subsets. Their associated transcription factors are shown in brackets. The main cytokines secreted by each of the subsets are shown in black.

1.2.1 Effector subsets

Th1 cells produce IFN-γ and express the transcription factor Tbet. They are proinflammatory, influencing macrophage activation and the production of opsonising antibodies. Differentiation into this subset is thought to be induced by IL-12 and environmental interferons produced by dendritic cells (DC) (Parronchi et al., 1992). These combined signals induce transcription factor STAT1 which subsequently leads to Tbet induction. This leads to upregulation of the IL-12 receptor, enabling the cells to be IL-12 responsive, and generate high levels of IFN-γ (Szabo et al., 1997).

Th2 cells secrete IL-4, IL-5, IL-9 and IL-13 which are all involved in mediating responses to parasitic helminths. Binding of the IL-4 receptor induces STAT-6, leading to upregulation of GATA-3 which, in combination with IL-2-initiated STAT-5, allow Th2 differentiation (Pai et al., 2004, Zhu et al., 2001).

More recently, other lineages have been described, in particular Th17 which is characterised by production of IL-17A, IL-17F and IL-22. The transcription factor thought to be crucial for the functional characteristics of Th17 cells is RORγt. The exact nature of conditions required for the direction of naive T cells along this lineage is not entirely clear. Early murine studies showed that TGF-β and IL-6 were sufficient (Bettelli et al., 2006, Mangan et al., 2006). Subsequently, a critical role of IL-1β was reported along with suggestions that TGF-β was in fact unnecessary (Chung et al., 2009). This work indicated that cytokine requirements for initiation of the Th17 lineage differed between mouse and human. However, by stringent sorting of naive CD4+ T cells used in differentiation experiments and tightly controlling TGF-β concentrations, the necessity of this cytokine was confirmed (reviewed (Korn et al., 2009)). There were associations with an IL-12 family member known as IL-23. However, receptors for IL-23 are not upregulated until the Th17 lineage has begun to be established, so this

cytokine may have a greater role in maintaining the Th17 phenotype (McGeachy et al., 2009). Human Th17 cells have been shown to express CCR4, CCR6 and CD161 (Cosmi et al., 2008), and additionally secrete IL-26 and CCL20 (Wilson et al., 2007).

Other subsets have been described based on secretion of IL-9 (Dardalhon et al., 2008, Veldhoen et al., 2008) and IL-22 (Duhen et al., 2009, Trifari et al., 2009). Although these cytokines form part of the expression profiles of Th2 and Th17 respectively, these subsets were found to have a comparatively limited range of cytokines

Effector T cell subsets in autoimmune inflammation

A complication of stem cell transplantation is Graft-versus-Host Disease (GvHD) (see section 1.8.4), where the patient's tissues are damaged by T cells arising from the transplanted immune system, giving rise to symptoms that are not unlike some autoimmune conditions such as those affecting skin, or inflammatory bowel. A recent proteomic study demonstrated a number of proteins expressed in patients with autoimmune systemic sclerosis and also in sclerodermatous GvHD that were not found in healthy controls (Scambi et al., 2010). Therefore there may be similarities between the inflammatory processes affecting GvHD patients and people with autoimmune conditions. Autoimmune conditions are often manifest in skin and gut, as is GvHD.

Inflammatory bowel diseases (IBD) potentially arise from inappropriate responses to commensal gut flora leading to raised levels of proinflammatory cytokines such as IL-6, IL-12 and IL-23 (reviewed by (Kaser et al., 2010)). IL-6 has a role in promoting secretion of IFN-γ and TNF-α in Th1 cells (Yamamoto et al, 2000) and in directing T cells towards a Th17 phenotype rather than to become Tregs (Bettelli et al., 2006, Lee et al., 2009, Zhou et al., 2009). These roles would therefore lead to an increase of a proinflammatory infiltrate with

elevated Th17 levels. If a similar pathology occurs in gut GvHD, then raised serum IL-6 and increased frequencies of IL-17A secreting cells may be observed.

IL-12, along with TNF-α, drive T cells towards a Th1 phenotype and promote IFN-γ secretion. Murine experiments have associated increased levels of IL-12 with occurrences of experimental autoimmune encephalitis (EAE) and collagen-induced arthritis, and also linked the diseases to IL-23 (Cua et al., 2003, Murphy et al., 2003). These cytokines were shown to be pathogenic in uveitis. As IL-23 has been associated with driving, or at least maintaining the Th17 lineage, the action of both of these cytokines expands the Th1 and Th17 populations. IL-12 blocking antibodies abrogated colitis in mouse models, demonstrating the role of this cytokine in inflammatory bowel conditions (Neurath et al., 1995), and IL-23 was shown to drive innate and T cell-mediated intestinal inflammation (Hue et al., 2006). Indeed, the frequency of IL-17A secreting cells is substantially increased in Crohn's Disease mucosa (Annunziato et al., 2007).

IL-23 is overproduced by DCs in psoriasis lesions (Lee et al., 2004, Wilson et al., 2007) and one study found the frequency of IL-17A secreting cells was 6.2% in psoriatic plaques as compared to 0.5% in normal skin (Lowes et al., 2008). This raises the possibility that IL-17A secreting cells may also have a role in skin GvHD pathology.

These studies provided a rationale for investigating the frequencies of Th1 and Th17 subsets in GvHD patients.

1.2.2 Regulatory subsets

In order to prevent excessive or inappropriate immune mediated damage, the immune system has a regulatory component. T-lymphocyte mediated regulation is carried out by Tr1 and Treg cells. It has been suggested that the latter can either be of thymic origin, so called natural regulatory T cells (nTregs), or those induced in the circulatory system (iTregs) (Apostolou and von Boehmer, 2004, Cobbold et al., 2004, Curotto de Lafaille et al., 2001, Mucida et al., 2005). Despite having different origins, nTregs and iTregs are indistinguishable. Tregs are characterised by expression of the transcription factor Foxp3 following IL-2 and high levels of TGF-β stimulation of naive T cells. Mutations that lead to loss of functional Foxp3 result in fatal autoimmune conditions characterised by massive inflammation and a proinflammatory cytokine storm. Discovery of Foxp3 resulted from investigations into a rare and severe autoimmune condition known as Immune-Dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) (Chen et al., 2003, Fontenot et al., 2003, Hori et al., 2003).

Several mechanisms of suppressive action via cell to cell contact and through cytokine-dependent mechanisms, have been attributed to Tregs, which are either directed against effector T cells or antigen presenting cells (APC). Studies have shown that an important method of Treg-driven, *in vivo* suppression is via IL-10 secretion (Belkaid, 2007, McGeachy et al., 2009). This versatile cytokine causes inhibition of a number of cytokines and chemokines on a wide variety of cells, including APCs leading to inhibition of function and proliferation of effector T cells (reviewed (Moore et al., 2001)). Tregs can also inhibit DC function by inactivating extracellular ATP which has inflammatory effects on DCs, through the action of CD39 on Tregs, catalysing the ATP to AMP reaction (Borsellino et al., 2007). Surface LAG-3 on Tregs interacts with MHC on DCs, delaying maturation (Liang et al., 2008). Tregs

constitutively express CTLA-4 which binds to CD80 and CD86 on DCs, subsequently downregulating these molecules on the APC and prevents adequate co-stimulation through CD28 which is necessary for naive T cell activation (Wing et al., 2008).

Direct suppressive action against effector cells includes elimination by granzyme-mediated, perforin-dependent killing (Grossman et al., 2004) or by expression of galectin-1 which when bound, inhibits proinflammatory cytokine production and can induce apoptosis (Kubach et al., 2007). Tregs can suppress effector cells indirectly by secretion of immunosuppressive cytokines IL-10, TGF-β and IL-35, but can also upregulate IL-2Rα (CD25) and compete for environmental IL-2, thus reducing availability of this cytokine for effector cells (Shevach, 2009). Using Foxp3 to identify Tregs presents difficulties in functional studies due to the nature of intracellular staining protocols which affect cell function, so surface marker staining is essential for identification of viable Tregs, and CD25 was considered as a possible marker. However, CD25 alone is insufficient as only a small proportion of CD25+ T cells are regulatory. The highest 1-2% of CD25+ T cells are Tregs, but the exact boundary between $CD25^{hi}$ and $CD25^{mid}$ (which are not suppressive and do not express Foxp3+) is difficult to determine and leads to inaccuracies. Two studies identified that Tregs lack the IL-7Rα-chain (CD127). They noted that Foxp3+ CD4+ T cells with suppressive ability were highly enriched in the $CD25^{hi}$ $CD127^{lo}$ fraction (Liu et al., 2006, Seddiki et al., 2006).

Tr1 cells are Foxp3 –ve and are induced in the periphery by APCs in the presence of IL-10. They exert suppressive effects via the secretion of IL-10 and TGF-β (Groux et al., 1997, Levings et al., 2001).

1.2.3 Plasticity of T cell subsets

As the number of studies investigating the roles of T cell subsets increases, it has become apparent that cells do not always fit into rigidly defined subsets. For example, studies

identified CD4+, Tbet-expressing T cells that could produce both IL-17A and IFN-γ which were named Th17/Th1 or Th17-1. Stimulation of Th17 cells in the presence of IL-12 led to downregulation of Th17-associated transcription factors, upregulation of Tbet and led to IFN-γ secretion, whilst still maintaining the ability to secrete IL-17A (Annunziato et al., 2007). This suggests that perhaps T cells have the ability to amend their function in response to environmental factors, which would lead to a dynamic and responsive immune system. Plasticity of other T cell subsets has been reported, notably in the case of Tregs.

A number of studies have demonstrated a reorientation of Tregs into Th1, Th2, Th9 or Th17 effector subsets (Wan, 2010). Co-expression of Foxp3 and RORγt has been reported in both murine and human studies (Voo et al., 2009, Zhou et al., 2009). There have been reports of effector T cells expressing high levels of CD25, or Foxp3. It may well be that these cells are in transition from one phenotype to another. It is not clear how adaptable a T cell may be depending on its environment. This plasticity potentially has important implications for development of adoptive T cell therapies and vaccines. The consequences of adoptively transferring antigen-specific T cells to treat autoimmune conditions, or graft-versus-host disease in stem cell transplant patients, could be catastrophic if there is a switch from regulatory to effector function. Attempts to culture Tregs *in vitro* revealed that CD4+ Foxp3+ CD45RA populations would retain a regulatory phenotype, whereas those which were CD45RO+ switched to an effector phenotype (Hoffmann et al., 2006).

This blurring of the boundaries of defined subsets creates difficulties for studies of *in vivo* immune activity. The proportions of subsets could alter considerably at different times in different subjects and thus create a confusing picture of the dynamics of an immune response.

1.3 Chemokines and receptors in inflammation and regulation

Chemokines are a family of cytokines that play key roles in mediating immune reactions through roles in leukocyte chemotaxis and adhesion. They are described by two systems of nomenclature. The original system, based on function or expression, has evolved since the discovery of the first chemokines. More recently a unifying, global system was developed to simplify understanding of interactions, particularly due to reporting of the same proteins under different names. The 'traditional' nomenclature was often descriptive, for example, monokine induced by gamma interferon (MIG) or interferon-inducible protein 10 (IP-10), but the system was replaced by a new method based on structure and the order in which the chemokines were discovered (Murphy, 2002). Based on the position of cysteine residues, the names begin CC or CXC followed by L (for ligand) and the number related to historical discovery. For example, MIG was renamed CXCL9 and IP-10 became CXCL10. Both chemokines can bind to the same receptor, CXCR3, which is expressed on activated T cells. These chemokines are produced at sites of inflammation by monocytes and neutrophils, with CXCL10 additionally being secreted by endothelial cells, fibroblasts and keratinocytes, and serve to attract CXCR3 expressing T cells (Loetscher et al., 1996).

Chemokine receptor expression is thought to be related to lineage. Th1 cells have been shown to express CCR5, CXCR3 and CXCR6 (Loetscher et al., 1998, Sallusto et al., 1998, Siveke and Hamann, 1998) whilst Th2 express CCR4 (D'Ambrosio et al., 1998). There are receptors to facilitate migration to secondary lymphoid tissues, such as CCR6, CCR7, CXCR4 and CXCR5 (Hardtke et al., 2005, Lugering et al., 2005, Phillips and Ager, 2002, Williams, 2006).

Some early stage memory T cells were found to express both CCR4 and CXCR3 and thought to be at early stages of differentiation (Song et al., 2005), although it is possible that these

cells were of a lineage other than the classic Th1 or Th2 subsets, or in a transitional phase between subsets.

Lim et al investigated the chemokine receptor expressing profile of Th17 cells following various conflicting reports (Lim et al., 2008). They found that there was considerable overlap with Th1, Th2 and indeed Tregs. Their data revealed that the Th17 subset frequently expressed CCR2, CCR5, CCR7, CXCR3 and CXCR6 in common with Th1. They also share similarity of CCR4 and CCR7 with Th2, and like Foxp3 Tregs, express CCR4, CCR5, CCR6, CXCR3 and CXCR6. It was also intriguing that around 20% of IL-17A secreting cells in their study, also secreted IFN-γ. Such broad overlap in cytokine and chemokine expression with other subsets perhaps points to the flexibility of the Th17 phenotype, and that they are equally likely to migrate to sites recognised as destinations for Th1, Th2 and Tregs. It does not seem likely that there is a particular niche specifically targeted by Th17. This suggests that Th17, along with Th1 and Th2 are components of general inflammatory reactions.

Chemokine secretion and receptor expression are important in disease processes in order to recruit leukocytes to sites of infection, but aberrant expression or overexpression can lead to autoimmunity or allergy. CCR6 and its ligand, CCL20 are involved in recruitment of DCs and other immune cells to Peyer's patches, therefore having a role in mediating inflammation of the gut epithelium. High levels of CCL20 and upregulated CCR6 are associated with inflammatory bowel disease (IBD) (Cook et al., 2000, Kwon et al., 2002) (figure 1.3.1).

Upregulation of CCR4 and CCR10 has been detected in inflamed skin, along with elevated levels of ligands, CCL17/CCL22 (CCR4) and CCL27 (CCR10). This was in comparison to healthy skin, in which those receptors were virtually undetectable. This implies that these receptor/ligand pairs are instrumental in the migration of T cells in inflammatory skin conditions (Ebert et al., 2005).

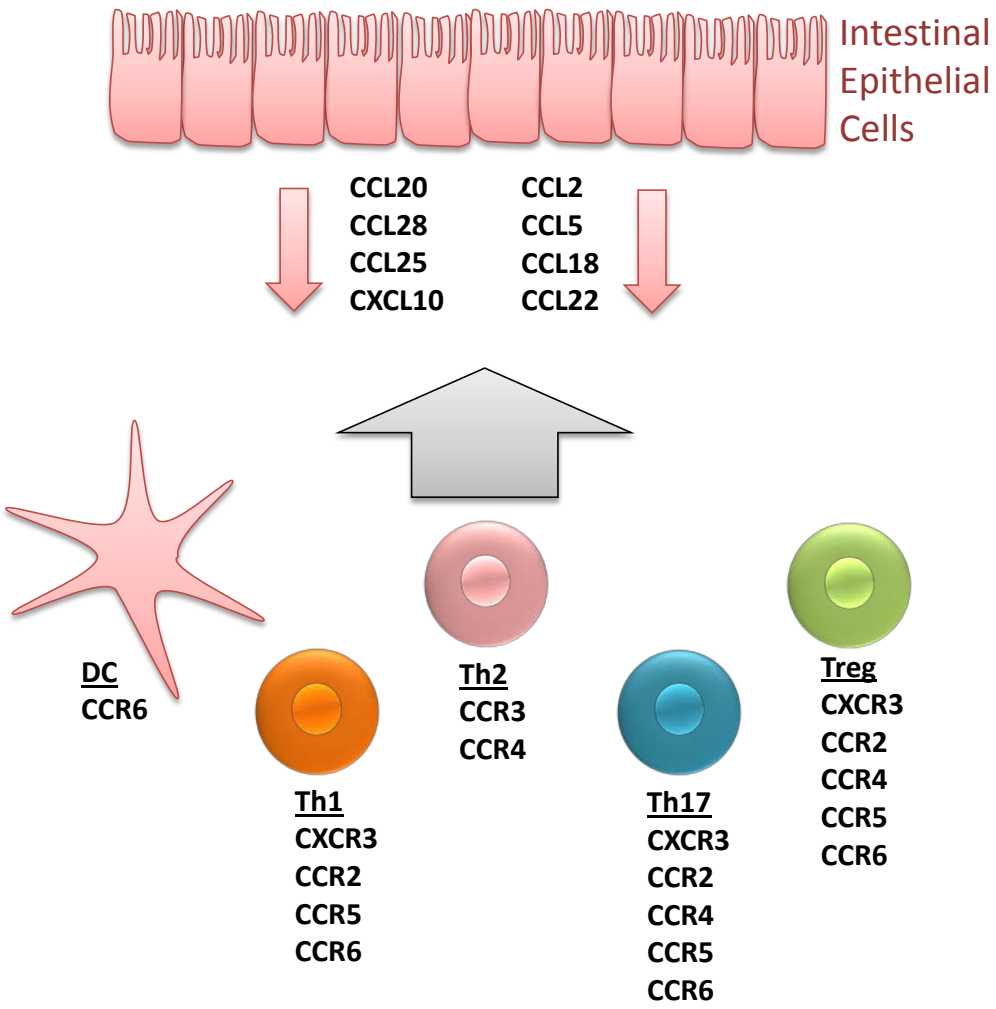


Figure 1.3.1 Selected chemokine receptors and ligands involved in recruitment of T cells and DCs to sites of intestinal inflammation

Chemokines are released during inflammation in the gut to recruit DCs and various T cell subsets. The diagram shows a selection of secreted chemokine ligands to the receptors expressed on DCs, Th1, Th2, Th17 and Tregs. The leukocytes migrate to the sites of inflammation. At these sites, increased levels of chemokines can be detected, along with high frequencies of T cell expressing the receptors (Adapted from (Nishimura et al., 2009)).

1.4 Antigen processing and T cell recognition

Antigen presenting cells (APC) express proteins on the cell surface that are able to present peptides to T cells and are encoded by highly polymorphic genes in a locus known as the major histocompatibility complex (MHC). MHC molecules that present to CD8+ T cells are class I type and are able to present peptides around 8 or 9 amino acids in length, whereas MHC class II molecules present longer peptides of around 15 amino acids to CD4+ T cells. It is thought that some MHC class II peptides could be considerably longer.

The discovery of the MHC was made by investigating rejection of transplanted skin grafts in mice and this concept of immune activity against cells of an allogeneic donor is known as alloreactivity. In humans this is responsible for transplanted organ rejection, or graft-versus-host disease (GvHD) and graft-versus-leukaemia (GvL) following allogeneic stem cell transplantation (SCT). The encoded MHC proteins are known as human leukocyte antigens (HLA) and each individual inherits three genes of both classes from each parent. Class I alleles are HLA-A, HLA-B and HLA-C (although a number of other alleles have been described), while class II are described as HLA-DP, HLA-DQ and HLA-DR. Across a population, there are a large number of heritable polymorphisms of each allele, which are numbered. For example, an individual may have a class I HLA type such as HLA-A2, HLA-A3, HLA-B4, HLA-B7, HLA-Cw3, HLA-Cw7, if different polymorphisms are inherited from each parent. It is not uncommon to find individuals who are homozygous for one or more allele, particularly for polymorphisms that are particularly prevalent in a population. Similar inheritance patterns occur for class II genes, so any individual may have between 6 and 12 MHC genes (Klein and Sato, 2000a, b).

1.4.1 Antigen processing

Although any cell expressing MHC molecules can engage T cells in an antigen dependent manner, certain cells can process antigen with much greater efficiency. The members of this group are known as professional antigen presenting cells (APCs) and include B cells, macrophages and particularly dendritic cells (DCs) (Mellman et al., 1998, Robadey et al., 1996, Schneider and Sercarz, 1997).

In general, antigens presented by MHC class I are from endogenous proteins, whereas exogenous proteins taken up by endocytosis are presented by MHC class II, although proteins from either source can be presented by both MHC molecules. The concept of exogenous antigen presentation to CD8+ T cells via the MHC class I pathway is known as 'cross-presentation' (Bevan, 1976). This concept is important for tumour immunology.

Endogenous proteins come from a variety of sources such as viral proteins and defective ribosomal products (DRiPs), which are ubiquitinated in the cytosol and directed to the proteosome to begin fragmentation (Shastri et al., 2002, Yewdell et al., 2001). Post-translationally modified proteins, such as phosphorylated peptides can be presented by MHC class I molecules (Zarling et al., 2000). Non-contiguous sequences have been found to be spliced together as MHC class I presented peptides which considerably increases the available pool of antigens which can be recognised by CD8+ T cells (Hanada et al., 2004, Vigneron et al., 2004).

The process of exogenous antigen 'cross-presentation' may be the principle, or only way of enabling CD8+ responses to tumour antigens. These ingested antigens can be transported into the cytosol for proteosomal degradation and MHC class I molecule loading in a TAP-dependent manner (Harding and Song, 1994, Kovacsovics et al., 1995, Norbury et al., 1995, Rodriguez et al., 1999), but can also be loaded in the endocytic pathway independently of

TAP (Bachmann et al., 1995, Kleijmeer et al., 2001, Pfeifer et al., 1993). After entering the proteosome, a series of degradation steps occur in order to trim the antigen to a suitable length for loading onto MHC class I molecules (reviewed by (Shastri et al., 2002). The peptides encounter the nascent MHC class I molecules which are chaperoned and stabilised by calnexin and calreticulin, and associated with transporter associated perotein (TAP) in the endoplasmic reticulum (ER) (Ortmann et al., 1994). After peptide binding, the MHC-bound peptide complex is transported to the cell surface for presentation to CD8+ T cells.

MHC class II presented antigens are principally derived from exogenous proteins, although it seems endogenously derived peptides are not excluded (Lechler et al., 1996). Following endocytosis, the antigens pass along the endocytic pathway which consists of early endosomes (EE), late endosomes (LE) and lysosymes which subject the proteins to progressively more acidic pH to facilitate denaturation (Lennon-Dumenil et al., 2002). The denatured proteins are exposed to a variety of enzymes, cleaving them for MHC class II loading.

The MHC class II molecules themselves are formed in the ER and the peptide binding region is occupied by a chaperone protein known as the invariant chain, which both stabilises the complex and directs it to the endocytic pathway (Anderson and Miller, 1992, Ghosh et al., 1995). The cleaved peptide is then 'exchanged' for the invariant chain in a reaction catalysed by a protein known as HLA-DM (Sherman et al., 1995). The peptide-MHC class II complex can then be presented on the surface of the cell for recognition by CD4+ T cells.

1.4.2 Antigen recognition

The T cell receptor (TCR) binds to the peptide/MHC complex on the DC, along with either CD4 or CD8 co-receptor. The CD4 and CD8 molecules determine which type of MHC molecule the T cell receptor can interact with. CD8 binds MHC I molecules while CD4 binds

MHCII, in both cases increasing the avidity of the TCR-MHC interaction. Thus, CD8-expressing T cells recognise peptides complexed with MHC I molecules while CD4-expressing T cells recognise peptides complexed with MHC II. CD80 and CD86, also expressed on the DC provide a co-stimulatory signal, and recruit either CD28 or CTLA-4 to this 'immunological synapse'. CD28 is more readily available, but CTLA-4 has higher affinity. The co-stimulatory signals provided by these molecules determine the outcome of this T cell/APC encounter. CD28 signalling drives T cell proliferation and differentiation, whereas CTLA-4 signalling inhibits T cell activation. The affinity of the TCR signal is proportional to the concentration of CTLA-4 recruited, which suggests that this is mechanism of tolerance to prevent very high affinity TCR clone reactions (Pentcheva-Hoang et al., 2004).

These activated T cells can respond to antigen by lysing infected cells and/or secreting cytokines (as described in 1.2). There are two principle, proposed mechanisms of killing infected cells. In the perforin/granzyme mediated mechanism, the T cell secretes perforin which binds to the target cell membrane and is polymerised to form an aqueous channel. Granzymes are then secreted and enter the infected cell through this channel and initiates the caspase-dependent apoptotic pathway. Alternatively, T cells can express CD95L (Fas ligand) to utilise the Fas/Fas ligand pathway. This is particularly important for CD4+ T cells. CD95L has a relatively long half-life on the surface which, even after TCR signalling has ceased, can lead to cell killing of neighbouring CD95 (Fas) expressing cells – a concept known as bystander killing (reviewed by (Russell and Ley, 2002).

1.5 Tumour immunology

1.5.1 Immunosurveillance

The concept of adaptive immune responses that clear malignantly transformed cells which have escaped DNA repair mechanisms and tumour suppressor pathways was proposed by Thomas and Burnet (Burnet, 1967, Thomas, 1959). This process was described by Burnet as 'immunosurveillance'.

However the theory is controversial, with the scientific community split as to whether immunosurveillance does in fact happen. The concept that the immune system can identify and destroy pre-malignant or malignant growths before they become clinically apparent is difficult to prove. Therefore, most evidence comes from mouse models. It was observed that lymphocyte deficient mouse strains, such as RAG2 knockout strains (which results in lack of B, T and NKT cells), have a higher incidence of induced and spontaneous tumour formation as compared to wild type. Similar findings in mice which lacked the ability to form a compete TCR (TCR $\beta^{-/-}$ or TCR $\delta^{-/-}$) indicate a substantial role for T cells in cancer prevention, which argues for an important role for the adaptive immune system in immunosurveillance (reviewed (Dunn et al., 2004)).

Detractors of this theory point out that tumours still develop despite the presence of a functioning immune system. Spontaneous tumours do occur in otherwise healthy individuals, although the incidence increases with age. This may be linked to general 'immunosenescence', a progressive deterioration in functional cells, possibly due to chronic demands on the immune system, such as cytomegalovirus (CMV) infection. However, counter arguments to the immunosurveillance theory point to childhood cancer, or the fact that elderly persons still have sufficient immune capabilities to combat infections as evidence against immune control of tumours.

If immune surveillance does clear some or most potential tumours, the immune system is also exerting selective pressures on nascent tumour cells. If a cancerous cell is a product of disordered gene expression, then some of the aberrantly expressed genes may equip the tumour cells with immune escape mechanisms. Therefore, those cells which can become tolerated by the immune system may thrive. This is known as 'immunoediting' and consists of three stages. Firstly, there is the classical 'immunosurveillance' mechanism, whereby potentially malignant cells are destroyed, which is described as 'elimination'. With the development of certain, limited escape mechanisms, the immune system may have reduced efficiency at transformed cell clearance. However, some control is maintained and this stage is described as 'equilibrium'. Finally, further selective pressure allows the neoplastic cells to evolve to evade the patient's immune system, which is termed 'escape' (figure 1.5.1.1) (reviewed (Dunn et al., 2004)).

1.5.2 Tumour antigens

There are a number of ways that the immune system can be alerted to the presence of a tumour cell. Tumour antigens presented by MHC class I and II molecules can be recognised by CD8+ and CD4+ T cells respectively and elicit adaptive immune reactions. The first tumour antigen was identified in 1991 (van der Bruggen et al., 1991) and several more have since been identified.

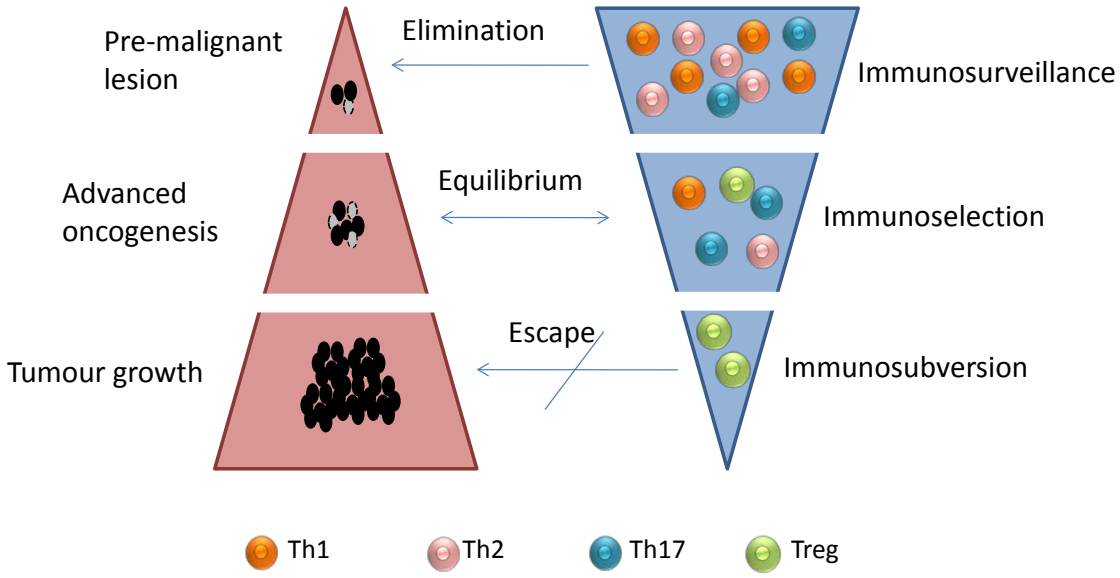


Figure 1.5.1.1 Process of immunoediting of tumours

The three stages of elimination, equilibrium and escape are shown between the increasing presence of tumour cells (red triangle) and the decreasing tumour-suppressive influence of the immune system (blue triangle). Black ovals indicate transformed cells with those being killed in grey. T cell subsets are indicated by the key. A variety of other cells such as B cells, NK cells, neutrophils and macrophages are also thought to be involved with the immunoediting process. Relative proportions of cell numbers are not intended to be representative, although increasing frequencies of Tregs are likely to play a role in tolerance.

(Adapted from (Zitvogel et al., 2006)).

Tumour antigens can be divided into five groups:

1. Mutation: these are generated by point mutations in ubiquitously expressed genes. They are generally tumour specific, but not found in many different patients, thus limiting their immunotherapeutic potential. Examples include MART-2 (Kawakami et al., 2001) and MUM-1 (Coulie et al., 2001).
2. Shared Tumour-specific: Antigens expressed only by tumours and germline (or other immune-privileged sites). For example Cancer-Testis Antigens (CTAgs) are expressed by a wide variety of tumours, but not in normal, healthy tissue. This restricted tissue expression offers a potential for immunotherapy. The MAGE family of CTAgs are examples of Shared Tumour-specific antigens (van der Bruggen et al., 1991). CTAgs are discussed in more detail later.
3. Differentiation: These are also expressed in the tissue of origin of the malignancy which could result in potentially severe autoimmune complications if used as immunotherapy targets. For example, carcinoembryonic antigen (CEA) is expressed by many gut carcinomas, but also in normal gut epithelia. If the normal tissue is likely to be surgically removed, as in the case of prostate specific antigen (PSA), then autoimmune reactions are of limited importance.
4. Overexpression: Although expressed in healthy tissue, these antigens are greatly upregulated in tumour tissue. Low expression in non-tumour sites means that there may not be a great risk of autoimmune damage to these tissues, but the threshold is not easy to determine. An example is alpha-foetoprotein.
5. Viral: A number of tumours are strongly associated with viruses and viral proteins may be expressed by these tumours, but also potentially non-transformed virally

infected tissues. Human papilloma virus (HPV) and Epstein-Barr Virus (EBV) are linked to tumours and/or lymphomas.

(Boon and van der Bruggen, 1996, Houghton et al., 2001, Old, 2003, Parmiani et al., 2007, Rosenberg, 1999, Van den Eynde and van der Bruggen, 1997, Van Der Bruggen et al., 2002)

Comprehensive lists of described peptides from tumour antigens can be found at www.cancerimmunity.org (with the exception of viral antigen peptides).

Immune system cells can also be alerted to tumour by expression of NKG2D ligands such as MICA and MICB or the minor histocompatibility antigen H60. Intrinsic processes in the tumour can lead to uric acid production and expression of toll-like receptor ligands, such as heat shock proteins. These both serve to generate a proinflammatory reaction through the innate system.

1.5.3 Cancer Testis Antigens

The family of tumour antigens termed Cancer Testis Antigens (CTAgs) are a group of shared tumour specific antigens characterised by expression on germline cells and tumour cells only. The first CTAg was identified by Pierre van der Bruggen in 1991 (van der Bruggen et al., 1991), using a melanoma cell line MZ2-MEL that could be killed by autologous CTLs. This antigen was named MAGE-1, which was subsequently renamed MAGE-A1. A similar strategy was adopted by colleague Thierry Boon which resulted in the identification of MAGE-A3, followed by BAGE and GAGE gene families (Boel et al., 1995, Gaugler et al., 1996, Van den Eynde et al., 1995). By studying the mRNA expression of these genes in various tissues, it became apparent that expression was restricted to testes, an immunologically protected site, and tumour cells. This seemed to make these antigens ideal targets for immunotherapy because as long as an effective immune response can be mounted against them, there is no

apparent risk of healthy tissue damage. Since then, around 100 CTAg gene families have been identified. Details can be found at www.cta.lncc.br. The development of a serological technique for cloning tumour antigens that elicit a high titre IgG response has helped research in this field considerably and is known as SEREX (serological analysis of recombinant cDNA expression libraries) (Sahin et al., 1998). Some antigens are expressed in the testis but also in other immune-protected tissues such as the placenta or retina, and have been shown to be expressed by tumours. One such antigen was identified in renal cell carcinoma and was termed RAGE-1 (Gaugler et al., 1996). Although not strictly defined as a CTAg, it has been included in this study and grouped as a CTAg throughout this report.

There is incomplete data regarding protein expression for many CTAgs, but those that have been studied have found heterogeneous expression within tumours as some cells may express multiple antigens, whilst others have no CTAg expression. In some tumours, only a small proportion (fewer than 1%) of cells in the tumour mass express CTAgs, but strong expression is seen in those cells that are positive for this gene expression (Gedye et al., 2009). There has been speculation that these expression patterns could indicate that the CTAg expressing cells could be cancer stem cells (Old, 2007). It is also unclear whether many CTAg genes are regulated post-transcription, which means that despite gene expression, there may not be any detectable protein. This has important implications for their potential immunogenicity which would affect their possible role in immunosurveillance or as an immunotherapeutic target (Caballero and Chen, 2009).

The function of these genes, when either normally or aberrantly expressed, is not well understood. Both MAGE and GAGE families have been shown to confer some resistance to apoptosis (Cilensek et al., 2002, Yang et al., 2007) and there have been various suggestions of other anti-apoptotic, cell survival and proliferative functions. With these functions, along

with sites of normal expression in the testis and placenta, it is possible to speculate that CTAgs are involved in gametogenesis and placentation. It may be that during tumourigenesis, these functions are initiated without appropriate regulation and expression of differentiation genes (Old, 2007).

The limited tissue expression of CTAgs should prevent immune tolerance developing in healthy individuals, but it is not clear to what extent these proteins are immunogenic when expressed by tumour cells. It is certainly true that humoral and T cell responses can be detected *ex vivo* in human cancer patients, but it is not necessarily apparent as to whether the responses are controlling the disease to any degree, or if they are merely following the disease progress but unable to contain it. It may be that early on there is some immune control through CTAg responses but as the tumour develops evasion mechanisms, all or part of the response is hampered by development of tolerance. For example, humoral responses have been demonstrated in multiple myeloma (Atanackovic et al., 2007), and T cell responses have also been detected in multiple myeloma patients (Goodyear et al., 2005, Goodyear et al., 2008). In the latter study, CD4+ effector memory phenotype CTAg-specific T cells were mainly seen in patients diagnosed with monoclonal gammopathy of uncertain significance (MGUS), which can be a pre-myeloma condition. These T cells were cloned and could lyse tumour target cells. However, CTAg responses in patients with advanced multiple myeloma were predominantly CD8+ with an effector memory RA phenotype, with limited ability to destroy tumour cells during *in vitro* assays. This suggests that as the disease progresses, the immune response is altered, potentially becoming impaired.

However, it may be that if immunity to CTAgs can be boosted, particularly whilst there is still an effective immune response, or if tolerance has been broken by stem cell transplantation, then CTAgs could be useful immunotherapy targets. There have been numerous vaccine

trials, particularly in melanoma patients using MAGE-A3 or NY-ESO-1. Around 20% of patients vaccinated with either MAGE-A3 peptide recombinant virus, or peptide pulsed DCs showed tumour regressions. Although this is not extremely high, it is approximately 20 times higher than reported for spontaneous melanoma regression (Lonchay et al., 2004). There have been phase II and III trials of recombinant MAGE-A3 protein vaccines in melanoma and non-small cell carcinoma patients which have shown increases in overall survival (reviewed (Caballero and Chen, 2009)).

It is possible that a vaccine strategy, perhaps alongside a tolerance breaking intervention, could prove beneficial. In a study using NY-ESO-1 recombinant protein vaccine along with CTLA-4 blockade, 8 of 15 patients had a clinical response of which 5 had NY-ESO-1 antibodies as opposed to the 7 non-responders who had no detectable NY-ESO-1 anibodies (Yuan et al., 2008). Following stem cell transplantation, previously tolerised T cells would have been eradicated, and it is unlikely that donors would have CTAg-specific Tregs, so initiating a CTAg-specific immune response could lead to improved survival.

1.5.4 Tumour evasion and escape

Genomic instability in tumours can produce a wide variety of mutations, and it follows that those that confer a favourable advantage to the tumour by preventing destruction by the immune system will be passed on through further divisions. The interplay of these evolutionary processes with the selective pressure brought to bear by the immune system can result in the tumour being edited sufficiently to evade and escape the immune defences.

Just as the immune system has a variety of methods to detect and kill malignant cells, the tumour can develop various mechanisms of evasion. Defects in antigen processing pathways and downregulation of MHC molecules can protect the tumour (Algarra et al., 2000, Marincola et al., 2000). It was found that tumours of melanoma patients in vaccine trials

with gp100 and MART-1 tumour antigen peptides, lost expression of the target antigens or underwent a complete loss of the MHC molecules on which the antigens were presented. This suggests that the tumours evolved to resist the therapeutically manipulated immune attack (Jager et al., 1997, Khong et al., 2004).

Defects in the IFN-γ receptor signalling pathway confer resistance to IFN-γ in human lung adenocarcinoma cells (Kaplan et al., 1998). Rather than develop resistance to a certain cytokine, some tumours secrete immunosuppressive cytokines such as IL-10 or TGF-β (Khong and Restifo, 2002) and one study found that secretion of soluble MICA/B led to downregulation of NKG2D on T cells and NK cells (Groh et al., 2002).

Tumour cells can also hijack the regulatory side of the immune system to aid escape. Accumulations of Tregs have been seen in tumour sites which may have been induced by TGF-β secretion by neoplastic cells. Evidence has shown that some tumours can act on certain DC subsets to induce a regulatory phenotype in CD4+ $CD25^{hi}$ T cells (Ghiringhelli et al., 2005).

Despite elaborate immune defence mechanisms, tumour cells can still evolve sufficiently to evade detection, or subvert tolerogenic mechanisms to survive and proliferate (as described above).

1.6 Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is a clonal proliferative disorder caused by genetic mutations within haematopoietic progenitor cells, and leads to a failure of differentiation and the accumulation of leukaemic blast cells in the bone marrow, peripheral blood and organs. Typically, the leukaemic blasts in AML are myeloid or monocytic progenitors, but erythroid and megakaryocytic forms of AML also occur. The result of this is severely attenuated blood production, impaired immunity and sometimes organ failure which, if untreated, is likely to lead to death within a few months.

AML is the most common adult acute leukaemia with nearly 2000 cases diagnosed per year. Patients have a median age at diagnosis of 70. AML can occur *de novo* or be preceded by myelodyspolasia or myeloproliferative disorders and there may be evidence of these pre-leukaemic conditions at diagnosis. A double-hit model was proposed that suggested mutations leading to constituitive expression of RAS (cell surface receptor) or FLT-3 and C-KIT (tyrosine kinases), in combination with overexpression of HOX genes, would increase proliferation and block differentiation respectively. Either of these mutations singly would lead to a myeloproliferative disorder, but together would cause AML (Frohling et al., 2002). However, it is possible that several mutations are necessary to initiate AML.

1.6.1 Diagnosis of AML

In the UK, AML is categorised under the World Health Organisation (WHO) system which has four major groups, and incorporates cytogenetic data and levels of accumulated blast cells in the bone marrow. AML is suspected if there are 20% blast cells in the marrow, and these should be shown to be myeloid in origin. At least 20% should stain positive for common myeloid differentiation markers such as CD33 or CD13. Other critical myeloid markers include cytoplasmic myeloperoxidase. Acute promyelocytic leukaemia is a distinct form of

leukaemia which is treated with all-trans retinoic acid (ATRA) and athracycline chemotherapy, or arsenic, with a relatively good prognosis. Forms of AML with good prognosis such as core-binding factor AML with t(8;21) and inv16 usually respond well to chemotherapy alone. Genetic abnormalities with poor prognosis in AML include monosomy 5, monosomy 7 and complex cytogenetics, and these patients are unlikely to have a good prognosis even with transplantation. The presence of FLt-3 abnormalities also carries a poor prognosis.

1.6.2 Immune evasion by AML

The bone marrow microenvironment is very permissive to cancer cell homing, survival and proliferation due to having high concentrations of growth factors required for haematopoiesis. Solid tumour metastases often home to, and thrive in the bone marrow, and this niche has been implicated in resistance to chemotherapy in leukaemic patients. The CXCR4/CXCL12 axis has been show to promote tumour blast survival by preventing apoptosis. Chronic myeloid leukaemia patients have been treated very successfully with imatinib, but complete eradication of disease in the bone marrow is not achieved due to CXCR4/CXCL12 interactions (Vianello et al., 2010). There are numerous other interactions with the microenvironment such as blast cell integrins interacting with stromal ligands such as VCAM-1 to facilitate survival. Overexpression of blast cell receptors allows diseased tissue to out-compete non-diseased marrow, further disrupting normal haematopoietic function and reducing immune capacity. Direct cellular interactions and secretion of soluble factors serve to impair the immune response to the tumour (Buggins et al., 2001). For example, blast cell production of indoleamine 2,3-dioxygenase can promote Foxp3 expression in T cells and potentially convert local effector cells to an immunosuppressive phenotype (Curti et al., 2007).

1.6.3 Treatment of AML

Treatment of AML consists of several courses of chemotherapy which aim to induce remission within the first 1 or 2 courses, followed by consolidation chemotherapy. There are increasing attempts to have tailored treatments, often with novel experimental agents, according to the genetic subgroup and risk category of AML in the current AML17 trial in the UK. Standard induction chemotherapy consists of a combination of an anthracycline, such as danurubicin or idarubicin with cytosine arabinocide (Ara C). A monoclonal antibody such as gentuzumab ozogomycin, which targets CD33, can be used, but its optimal usage in AML is still being explored. Patients are typically in hospital with myelosuppression for several weeks following each course of chemotherapy before neutrophil and platelet recovery occur.

However, the majority of patients relapse, and salvage chemotherapy only leads to second remission in 50% of cases, often only for a few months (Craddock et al., 2005). The prognosis of AML depends critically on the age and performance status of the patient, and on the genetic subtype of AML.

Elderly patients often have AML with genetic abnormalities that confer unfavourable prognosis, and long-term survivors are rare.

Where a HLA-matched donor is available, allogeneic stem cell transplantation may be the most favourable option. This is discussed further in section 1.8.

1.7 Multiple Myeloma

Multiple myeloma (MM) is a plasma cell tumour with approximately 3000 new cases per year in the UK and the median age at diagnosis is 65. It is characterised by accumulations of malignant monoclonal plasma cells in the bone marrow leading to anaemia, renal impairment, bone destruction, hypercalcaemia and severe immune dysfunction, with associated susceptibility to infection. A premalignant condition called monoclonal gammopathy of uncertain significance (MGUS) can exist in asymptomatic individuals and in a minority of cases transforms to active myeloma requiring treatment. For the majority of patients, treatment leads to a plateau phase of variable duration. Relapse inevitably occurs requiring further treatment, but the disease usually becomes resistant.

A characteristic feature of the disease is unrestrained production by the malignant plasma cells of monoclonal immunoglobulin, a paraprotein which is secreted into the blood. This abnormal protein can be measured and identified by electrophoresis. Around 30-50% of patients have excessive concentrations of immunoglobulin light chain which can pass through the kidney and be secreted in urine. A Bence-Jones protein test of the patient's urine sample can be carried out to identify these light chains to aid diagnosis and disease monitoring. More recently, new tests have been devised to measure the free light chain in serum, which may ultimately replace the Bence-Jones urine test (Pratt, 2008).

1.7.1 Immune evasion by myeloma cells

Myeloma cells in the bone marrow, like the malignant AML cells, have a complex interaction with the microenvironment which is crucial for maintenance and progression of the disease, and contributes to immune disruption. Myeloma cells produce a variety of immunomodulatory cytokines including IL-10 and TGF-β, which act on local T cells and DCs, driving the local immune microenvironment towards an immunosuppressive state (Pratt et

al., 2007). The tumour cells do express MHC molecules and co-stimulatory markers CD80 and CD86 although expression is generally low and may be restricted to a limited number of malignant plasma cells (Yi et al., 1997).

1.7.2 Treatment of myeloma

Myeloma patients are given bisphosphonate which inhibits excessive osteoclast bone reabsorption and limit bone damage. In the last decade, three novel agents, thalidomide, bortezomib and lenalidomide have greatly improved the treatment options for multiple myeloma, translating into significant improvements in survival. Before these agents, melphalan and prednisolone were given in combination to older patients, whilst younger patients received other combinations such as vincristine and doxorubicin/Adriamycin™ (VAD), with or without cyclophosphomide (C-VAD), along with steroids methylprednisolone or dexamethasone.

The challenge is now to find optimal use of these three novel agents, both together and also with the more established drugs. It is likely that many other novel agents will be developed, such as more immunomodulatory drugs, proteasome inhibitors, HDAC inhibitors and heat shock protein inhibitors. Currently the gold standard initial treatment in the UK is cyclophosphamide, thalidomide and dexamethasone (CTD), which is being compared to cyclophosphamide, lenalidomide and dexamethasone (CRD) in the UK Myeloma XI trial.

For those patients who are young and fit enough, stem cell transplantation is still considered to be the best option. Autologous stem cell transplantation is commonly used for myeloma patients whereby stem cells are collected after an initial few months of treatment to reduce the tumour load and returned to the patient following conditioning therapy. If a HLA-matched donor is available, and the patient is determined to be likely to withstand

treatment-related toxicity, then allogeneic stem cell transplantation can be considered. This study is focusses on allogeneic transplantation which is discussed further in section 1.8.

1.8 Allogeneic stem cell transplantation

The principle of stem cell transplantation (SCT) is to ablate the patient's immune system with chemotherapeutic agents and/or irradiation and then harvest haematopoietic stem cells from a HLA-matched donor for infusion into the patient. The stem cells will then home to the bone marrow and begin to repopulate the depleted immune system through homeostatic mechanisms. Stem cells can be harvested from the patient, frozen and reintroduced following conditioning (autologous (autograft)) or from a healthy, HLA matched donor (allogeneic (allograft)).

1.8.1 Stem cell donors

Donors are selected on the basis of how closely they are matched to the patient's HLA typing. HLA-mismatches give to a greater risk of graft-versus-host disease, leading to potentially fatal tissue damage to the patient. The first choice would be a fully matched sibling, but if this is not available, then searching bone marrow registries such as the Anthony Nolan Trust, may yield a matched unrelated donor (MUD). In some cases, a partially matched parent or child can be used, which is known as a haploidentical transplant and is more commonly used for paediatric patients. However no patients in this study had haploidentical donors. Another potential source of stem cells is cord blood, which can be used where a HLA-identical donor is not available. There is a considerably lower risk of GvHD which allows less stringent HLA-matching (Rocha et al., 2001, Wagner et al., 1996). but there is an increased risk of early mortality due to infection resulting from delayed

reconstitution (Rubinstein et al., 1998). However, there were no cord blood transplants in this study.

Traditionally, stem cells were extracted directly from the bone marrow cavity, although this practice has largely been replaced by administration of G-CSF to the donor which has the effect of mobilising the stem cells into the peripheral blood allowing them to be harvested. The choice of procedure is usually made by the donor. After collection, the stem cells can be cryopreserved and stored until infusion into the patient.

1.8.2 Transplant conditioning

In the vast majority of cases of patients with acute leukaemia, conventional chemotherapy has been used to achieve remission prior to commencing transplant conditioning. The patient is admitted to a bone marrow transplant centre and remains an in-patient throughout the procedure, and for several weeks into the post-transplantation period.

Myeloablative conditioning consists of cyclophosphomide along with either total body irradiation (TBI) (CyTBI) or administration of busulphan (Bu/Cy). These transplants are the most effective anti-leukaemic therapies currently in use, with long-term disease free survival (DFS) in the region of 60-70% for patients transplanted in first complete remission (CR) for acute myeloid leukaemia, with relapse rates up to 30% lower than for patients treated with conventional chemotherapy (Craddock, 2008). However, the intense nature of the conditioning regimens means that it is not a viable treatment option for older or more infirm patients.

In order to expand the range of eligible patients for allogeneic-SCT, reduced intensity conditioning (RIC) regimens were developed (Childs et al., 1999, Corradini et al., 2002, Giralt et al., 1997, Kottaridis et al., 2000, McSweeney et al., 2001, Slavin et al., 1998). RIC

transplants use less chemotherapy than fully myeloablative transplants and have more immunosupporession. RIC conditioning leads to a mixed chimeric state in the bone marrow, often needing donor lymphocyte infusions (DLI) to achieve full donor chimerism. The strategy relies on an immunological graft-versus-leukaemia/tumour effect rather than intensive tumour reducing chemotherapy. RIC transplants have a lower mortality (around 15-20%) and long term DFS can be achieved in patients for 20-30% of patients in their fifth and sixth decades. It is unclear which subgroups of AML patients are most likely to benefit from RIC transplantation and it is even less clear in myeloma patients where GvHD linked to graft-versus-myeloma presents a major hurdle to allogeneic transplantation in myeloma. Typically, TBI is not used, although sometimes reduced dose irradiation is utilised. A typical combination of chemotherapy agents is fludarabine, melphalan and campath.

Patients are kept in a protected environment, with a low-microbial diet due to their very high-risk of infection as a result of immune system depletion. Prophylactic antimicrobial drugs such as ciprofloxacin, fluconazole, septrin and acyclovir are administered, and cyclosporine is given to reduce the risk of early onset GvHD. This is usually maintained at a constant dose for some time into the post-transplant period, before being reduced over time until it can be completely withdrawn. The length of time before tapering is based on a clinical assessment of risk, as is how sharply the dose is reduced. If, during the tapering period, the patient begins to show signs of GvHD, then the physician may increase the dose again. This staged withdrawal is carefully managed to minimise GvHD risk whilst allowing sufficient immunity to infections without compromising GvL.

The composition of the conditioning regimens has implications, not just for depleting the immune system, but also for the dynamics of reconstitution. Campath is a monoclonal antibody directed against CD52 on the surface of many myeloid, lymphoid and some CD34+

cells of the bone marrow, leading to antibody mediated cell death. It can be delivered *in vivo* as part of the patient's conditioning or administered to the stem cells *in vitro* for a T-deplete graft prior to infusion. The antibody persists in the patient at cytotoxic levels for considerably longer after *in vivo* delivery which delays T cell reconstitution (Morris et al., 2003). Reduced dosage of campath during conditioning improves reconstitution but carries an increased risk of GvHD (Chakraverty et al., 2010). Conversely it is well-known that there is a higher risk of relapse with greater T cell depletion.

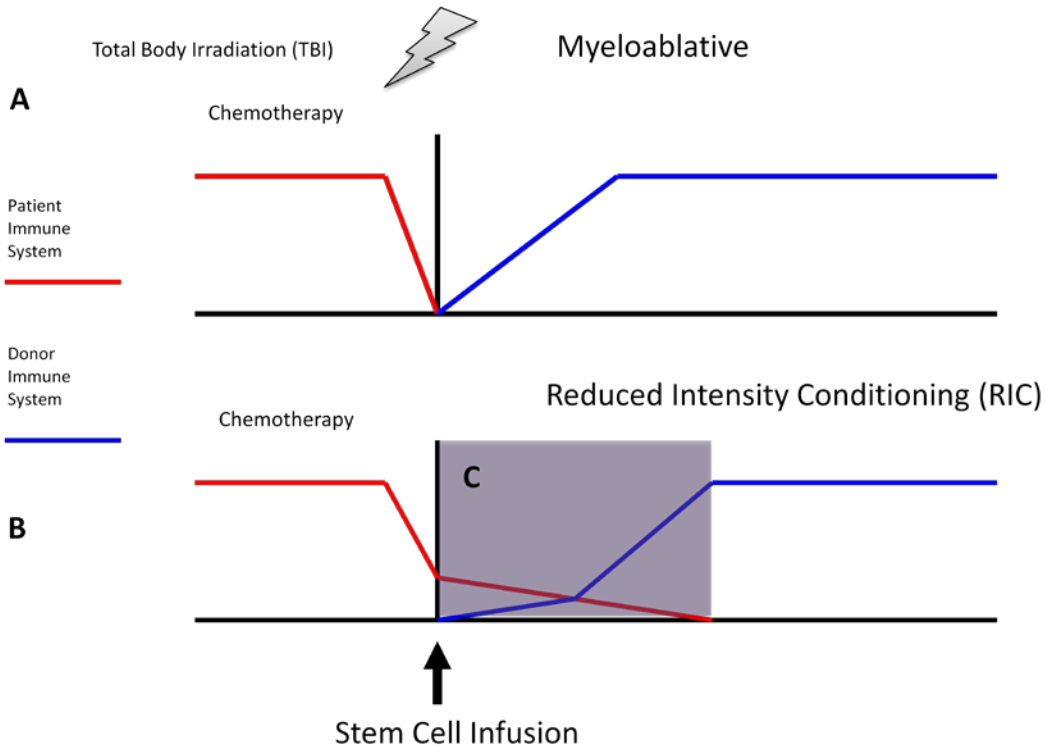


Figure 1.8.2.1 Types of allogeneic transplantation

Myeloablative conditioning (A) destroys the patient immune system and tumour cells with high dose chemotherapy and total body irradiation. Following infusion of stem cells harvested from the HLA-matched donor, homeostatic mechanisms cause an accelerated reconstitution to restore the immune system to normal levels. For patients who would not withstand the harsh conditioning regimens, a reduced intensity conditioning (RIC) protocol can be followed (B) whereby the immune system is depleted with less toxic chemotherapy, usually without irradiation. Following infusion of the donor stem cells, there is a period of mixed chimerism (shaded area, C) before the newly transplanted system 'out-competes' the damaged patient cells.

1.8.3 Graft-versus-Leukaemia

The presence of a functioning adaptive immune system is crucial to the success of the transplantation procedure. As described in the previous section, cyclosporine is given prophylactically to prevent GvHD, and by reducing the dose within the first 20 days following transplantation, relapse rates can be reduced by up to 30% (Bacigalupo et al., 1991, Bacigalupo et al., 2000). This suggests a link between T cell activity and relapse prevention. Occurrence of GvHD is associated with a decreased risk of relapse (Horowitz et al, 1990) and as GvHD is thought to be T cell mediated, it would follow that GvL has a T cell component. Further evidence came from experiments demonstrating that further input of T cells following transplantation enhances the GvL effect and prevents relapse (Kolb et al, 1990). However, despite the obvious benefit of a GvL effect, GvHD is a serious cause or morbidity and mortality. Therefore, in order to optimise stem cell transplantation as a therapy, strategies to enhance GvL whilst preventing GvHD must be developed.

If targets of GvL can be elucidated, then there is the possiblilty of immunotherapeutic targeting to enhance the anti-tumour effect. Despite careful matching of HLA types between donor and patient, disparities between polymorphic genes in the donor and recipient can lead to immunogenic minor histocompatibility antigens (mHAgs) (Goulmy et al., 1976). If expression of these antigens is restricted to the haematopoietic system, then they can be targeted to induce anti-leukaemia immunity. For example, HA-1 can elicit an immune reaction from the donor immune system with a single amino acid difference (den Haan et al., 1998). This minor difference affects the stability of MHC presentation and immunogenicity (Nicholls et al., 2009, Spierings et al., 2009). The difference of one amino acid substituted for another can alter the efficiency of TAP-transportation and lead to increased immunogenicity, as is the case with HA-8 (Brickner et al, 2001). HA-1 and HA-2

specific T cells were isolated from three relapsed allograft patients following DLI. There followed a complete remission of the disease and chimerism of the patients was restored to 100% (Marijt et al., 2003). This demonstrates the potential of tumour-specific immunity. Other examples of haematopoietic system restricted mHAgs are HA-2, HB-1 and LRH-1 (reviewed (Goulmy, 2006)).

The tumour cells can also become visible to the donor immune system through the aberrant or overexpression of tumour associated antigens (TAA). These may have been expressed prior to transplantation but not induced a strong anti-tumour response due to tolerance developing. However, the transplanted donor immune system will not be tolerised to these antigens. The number of tumour cells will have been diminished by the conditioning treatment. Therefore, the opportunity arises to eradicate residual tumour cells which may occur naturally, but the response could be strengthened by targeted immunotherapy.

A number of studies have shown that Wilm's tumour antigen (WT-1) is overexpressed in acute leukaemias and has the potential to elicit robust T cell responses in patients (Oka et al., 2000, Rezvani et al., 2005). Vaccine trials using WT-1 in combination with PR-1 to treat myeloid leukaemia patients showed reduced tumour load suggesting a vaccine induced anti-tumour effect (Rezvani et al., 2008). CD8 memory T cell responses have been shown to PRAME-1 in acute lymphoblastic leukaemia (ALL), acute and chronic myeloid leukaemia (CML) patients (Rezvani et al., 2009). MUC-1, another TAA, has been shown to be expressed in both AML and MM (Brossart et al., 2001), with a positive-correlation between expression and reduced risk of relapse (Kapp et al., 2009). Peptides from these antigens could be utilised as part of a vaccine strategy.

Although these overexpressed antigens have been shown to be well tolerated, with little toxicity in the vaccine trials, the nature of their tissue expression means the possibility of

inducing GvHD cannot be ignored. Therefore, the possibility of using CTAgs for immunotherapy post transplantation should be considered as there is no expression in healthy tissues, making it unlikely that GvHD reactions would be initiated. Several CTAgs have been shown to be expressed in MM such as MAGE, LAGE and NY-ESO (Pellat-Deceunynck et al., 2000, van Baren et al., 1999, van Rhee et al., 2005). T cell responses to CTAg proteins are found in MM patients (Goodyear et al., 2005, Goodyear et al., 2008) indicating the immunogenic potential in the post-transplantation setting. A number of CTAgs including RAGE-1, MAGE-C1, MGEA-6, GAGE-3, and some MAGE-A family members were shown to have RNA expression in AML (Guinn et al., 2005). If CTAg genes are being expressed in AML patients, then it is possible that they could elicit T cell immunity which could constitute a component of the GvL effect.

1.8.4 Graft-versus-host disease

Graft-versus-host disease (GvHD) is an important clinical complication following allo-SCT and is a significant cause of morbidity and mortality. As mentioned above, the condition is closely linked to the protective GvL effect, with a large T cell contribution. Tissue targets are not well understood, although certain mHAgs have been related to GvHD. For example HY antigen, which is expressed in male tissue, can be the target of a T cell response if the donor was female, as functional CD8+ HY-specific T cells have been detected in women who have had a male pregnancy (James et al., 2003, Piper et al., 2007b, Verdijk et al., 2004). However, there are likely to be a very wide range of potential tissue target antigens, so there is a need to understand the mechanisms of pathogenesis in order to modulate the harmful effects of GvHD without compromising GvL. Current treatments can be broadly immunosuppressive, or pan-T cell inhibiting which carry a risk of relapse due to suppressing anti-tumour immunity.

GvHD is classified into acute and chronic disease, with the acute form occurring within the first 100 days following transplantation. Acute GvHD tends to be directed against skin, liver and gut tissues, whereas chronic GvHD has broader effects, resembling autoimmune conditions such as systemic sclerosis. The pathophysiology of acute GvHD has been described as a three-step process (figure 1.8.4.1) beginning with damage to host tissue which may lead to proinflammatory responses from these cells, or lipopolysaccharides (LPS) being released into the bloodstream, and result in the release of inflammatory cytokines such as IL-1, TNF-α and IFN-γ. MHC molecules and co-stimulatory molecules are upregulated on activated APCs. During the next stage, the donor immune cells respond to the APCs by proliferating and secreting further inflammatory cytokines and chemokines, including IFN-γ and IL-2. In the third stage, or effector phase, there is tissue damage caused by activated T cells and NK cells, which also secrete further cytokines. Chemoattractant chemokines such as CCL2, CCL3, CXCL2, CXCL9-11 CCL17 and CCL27 recruit further effector cells to the site (reviewed (Ferrara et al., 2009)).

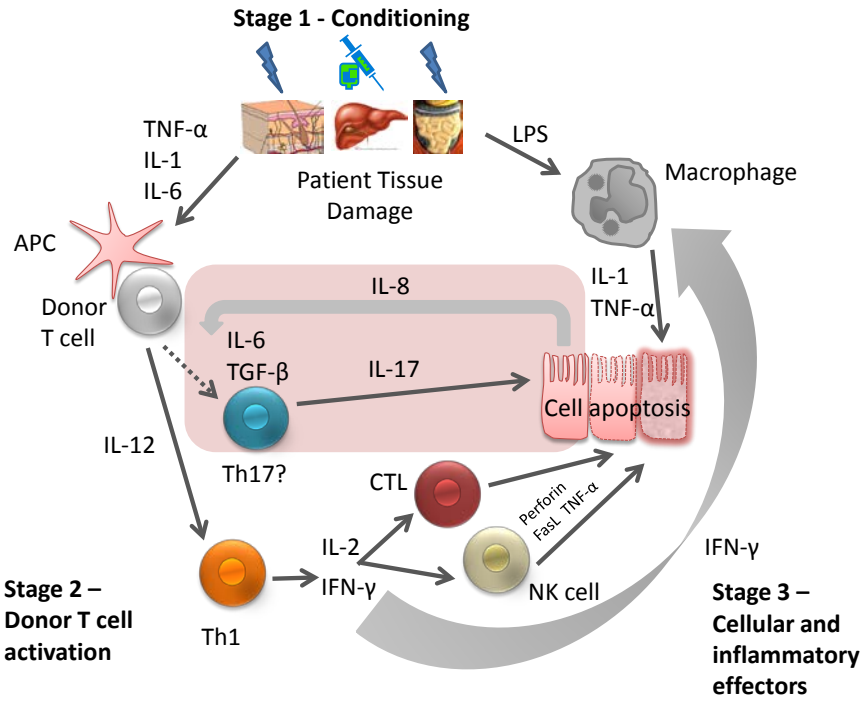


Figure 1.8.4.1 Three stage pathophysiology of GvHD

Tissue trauma during conditioning leads to the release of bacterial lipopolysaccharide (LPS) into the circulation, triggering macrophages to secrete IL-1 and TNF-α which upregulate MHC on host tissues, facilitating mHAg recognition. Stage 2 is characterised by activation of donor T cells which differentiate into Th1 cells in the presence of IL-12. The boxed area shows the proposed role of Th17 cells in the pathophysiology of GvHD, beginning with the differentiation in the presence of IL-6 and TGF-β. IL2 and IFN-γ secreted by Th1 cells leads to further T cell expansion and activation of NK cells, both of which damage host tissues through perforin and granzyme and Fas ligand in stage three. The action of IL-17A on host tissues induces IL-8 production which has chemoattractant effects, leading to the recruitment of further T cells.

(Adapted from (Hill and Ferrara, 2000))

Skin involvement is the most common manifestation of acute GvHD, and patients develop a pruritic, macropapular rash. Gut GvHD leads to diarrhoea, possibly with bleeding as a result of mucosal ulceration. Severity of the disease is classified by a staging system from grade I to grade IV. Higher grade disease is associated with a poor prognosis with long term survival for grade III being around 25%, down to 5% for grade IV (reviewed (Ferrara et al., 2009)).

The role of T cells in GvHD has been the target of extensive investigation. As indicated previously, depletion of T cells has a preventative effect in GvHD. However, disease is reduced in patients who have either a CD4+ or CD8+ T cell depleted graft, suggesting that there is involvement of both subsets, although the fact that there is some disease indicates that either subset may independently cause some level of GvHD (Champlin et al., 1990, Gallardo et al., 1997). Polarisation of T cell subsets is of great interest, and early experiments suggested that acute GvHD reactions were predominantly of a Th1 nature, and chronic GvHD was mediated by Th2 cells (Allen et al., 1993, De Wit et al., 1993). However, this model is too simple, particularly in the light of discoveries that the T cell repertoire is more dynamic that the Th1/Th2 paradigm. In studies using mice deficient in the Th2 associated cytokine IL4, decreased GvHD was observed, whereas in mice lacking the Th1 associated cytokine IFN-γ a more severe pathology was induced (Murphy et al., 1998, Yang et al., 1998). It is most likely that there is a complex interaction between innate immune cells, natural killer (NK) cells Th1, Th2, Th17 and Treg subsets.

There is conflicting data concerning the role of Tregs in GvHD. It would seem logical that the occurrence of GvHD would be associated with a decrease in Tregs. This has been shown in one study of acute GvHD patients which showed a decreased frequency of Tregs as disease severity increased (Magenau et al., 2010). However two studies of Tregs in chronic GvHD found opposing trends. One study showed that Tregs were increased in GvHD, although the

lymph node homing marker CD62L was decreased on these cells (Clark et al., 2004). The other study stated that Tregs were increased (Zorn et al., 2005). It is difficult to compare these studies directly as Clark *et al* looked at absolute numbers of cells that were CD4+ $CD25^{hi}$ whereas Zorn *et al* examined frequencies of Tregs, and used Foxp3 expression to characterise the cells. However, such inconsistencies are unsurprising in the context of recent findings concerning the phenotypic flexibility of T cell subsets, and the complex nature of the regulatory immune system. It is interesting to note that the CD4+ CD25hi CD62L- population described by Clark *et al* were also shown to be CD45RO+ as opposed to CD45RA expressing. Hoffmann et al published that homogeneous T cell lines with a regulatory phenotype could only be generated from a CD4+ CD25hi CD62L+ CD45RA+ population. This may indicate that some functional plasticity is involved (Hoffmann et al., 2006). As the inflammatory disease progresses, cells which had previously had an effector phenotype, may have become regulatory.

With this in mind, it is perhaps unsurprising that the limited literature on the role of Th17 cells in GvHD is not consistent. Mouse studies have shown that in one case the absence of IL-17A augments Th1 differentiation leading to increased GvHD (Yi et al., 2008), but in another IL-17A does contribute directly to GvHD, albeit without influencing overall survival (Kappel et al., 2009). A third study found that lethal GvHD was mediated by Th17 cells with severe pulmonary and cutaneous manifestations (Carlson et al., 2009). Other evidence points to GvHD potentially being mediated by different T cell subsets synergistically, independently or altered differentiation. In mouse models, allogeneic Th17 cells were shown to cause GvHD independently, although not in a syngeneic setting, which suggested an antigen dependent mechanism. In the same system, Th1 cells could independently cause GvHD. However, polarised Th17 cells did not maintain their phenotype and began to

produce large amounts of IFN-γ. Furthermore, blocking differentiation into a Th17 phenotype did not affect GvHD development (Iclozan et al., 2010). This indicates that Th17 cells can contribute to, but are not necessary for GvHD.

Understanding of T cell homing mechanisms in GvHD may yield ways to disrupt the disease. Studies have shown that a number of chemokines including CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL9, CXCL10, CXCL11, CCL17 and CCL27 are overexpressed in affected tissues, and a higher frequency of CXCR3 and CCR5 expressing T cells causing in GvHD sites (Duffner et al., 2003, Wysocki et al., 2005b). CCR5 expressing Tregs have also shown to modulate GvHD in disease sites (Wysocki et al., 2005a). Acute skin GvHD patients have elevated CXCL10 and recruit CXCR3 expressing T cells to disease sites (Piper et al., 2007a). Further characterisation of T cell trafficking chemokines and receptors in both acute and chronic GvHD may well provide insights into mechanisms that could be targeted for therapeutic intervention.

1.8.5 Separating GvL and GvHD

There are a number of potential strategies for enhancing the GvL effect whilst simultaneously diminishing GvHD. Better reconstitution can be achieved in patients who have received a T-cell depleted graft by subsequent donor lymphocyte infusion (DLI). This is carried out after the first few weeks in order to avoid the highly inflammatory environment in this period, when there is increased activation of DCs and MHC class II is upregulated on epithelial cells (Li et al., 2006). Mouse models showed that delayed DLI in mHAg mismatched animals enhanced GvL without increasing GvHD (Klingebiel et al., 2008), although GvHD is increased if the DLI is given to lymphopenic recipients as compared to non-lymphopenic mice (Miller et al., 2007). Unfortunately in human systems, this approach has had inconsistent results with regards to separation of GvL and GvHD, particularly in acute leukaemia and myeloma (Kolb et al., 2004). DLI is often successfully used to treat relapse in

allograft patients with less rapidly growing tumours such as chronic myeloid leukaemia (Dazzi et al., 2000), but this approach has been less successful in AML, partly because the blast-reduction chemotherapy required can promote a proinflammatory environment, leading to increased risk of GvHD post-DLI (Edinger, 2008).

Selection and expansion of regulatory T cells for reinfusion has been shown to suppress GvHD (Taylor et al., 2002) but not at the expense of the GvL effect (in murine models) (Edinger et al., 2003). Caution should be exercised with this approach due to the potential plasticity of the reinfused cells, as a conversion to pro-inflammatory effector cells may exacerbate GvHD. It has been reported that murine transplant recipients treated by extracorporeal photopheresis, where leukocytes are treated *ex vivo* with 8-methyloxypsoralen and exposed to UVA radiation, have increased frequencies of Tregs and reduced GvHD incidence (Gatza et al., 2008).

The immune environment could be manipulated by therapeutically targeting APC populations. Selective depletion of CD11b+ donor DCs or addition of CD11b- donor DCs can enhance GvL without a corresponding increase of GvHD in murine studies (Li and Waller, 2004). Certain subsets of DCs which express indoleamine 2,3-dioxygenase (IDO), which is an immunomodulatory enzyme, are thought to be tolerogenic by suppressing effector responses and expanding Tregs (Chen et al., 2008). It has been proposed that expansion of 'regulatory DCs' may prevent GvHD without diminishing GvL (Li et al., 2009). However, this has only been shown in preclinical models and is yet to be demonstrated in human transplant recipients.

A potential strategy to limit GvHD is to use cytokine blockades such as anti-TNF therapy in order to modulate the cytokine-mediated proinflammatory environment. Elevated plasma levels of this cytokine have been associated with increased severity of GvHD and higher

mortality (Choi et al., 2008). Soluble TNF-α suppression in a murine allograft model resulted in decreased GvHD without compromising GvL (Borsotti et al., 2007), although consistent administration of a TNF-α antibody, infliximab, over three weeks resulted in an increased frequency of severe infectious complications (Hamadani et al., 2008). Another study showed that this strategy was effective for reducing GvHD without inhibiting GvL, but also reported infectious complications (Couriel et al., 2004). It may be that a certain dose could be beneficial as a component of a combined strategy. However, these studies underline the fact that caution must be exercised when using immunosuppressive strategies to combat GvHD, so that necessary immune function is not excessively compromised.

By further understanding the mechanisms of both GvL and GvHD, it may be possible to achieve the goal of augmenting anti-tumour immunology whilst simultaneously diminishing GvHD pathogenesis in order to improve the overall survival and quality of life for allo-SCT patients, and potentially make this a viable treatment option for a greater number of people.

The aims of this project were two-fold. One was to investigate the presence of CTAg immunity following allo-SCT in order to determine the potential of these genes to be exploited for immunotherapy. The other was to attempt to elucidate the roles of T cell subsets in the pathogenesis of GvHD, and whether there were any patterns that could be utilised or interrupted to treat or prevent GvHD.

Chapter 2: Materials and Methods

2.1 Patients

2.1.1 GvL Study Patients

41 patients with a primary diagnosis of AML or MM who were undergoing an allogeneic stem cell transplant were studied. Written informed consent was obtained prior to joining the study and appropriate ethical approval was obtained (South Birmingham Regional Ethics Committee). 35 patients had a diagnosis of AML with the remaining 6 undergoing treatment for MM. 20 patients received a myeloablative conditioning regimen consisting of cyclophosphamide (60mg/kg x 2 days) and 14.4 Gy total body irradiation (TBI), and 21 received a reduced intensity conditioning (RIC) regimen incorporating fludarabine (25mg/m^2 x 5 days), campath (140mg/m^2) and melphalan (10mg x 5 days) (table 4.1.1).

2.1.2 GvHD Study Patients

Patients who were undergoing allogeneic stem cell transplantation for treatment of various haematological malignancies at University Hospital Birmingham NHS Trust and Heartlands and Solihull NHS Trust were recruited into the study. Written informed consent was obtained prior to joining the study and appropriate ethical approval was obtained (South Birmingham Regional Ethics Committee). Peripheral blood samples from 55 patients were studied. The patients were divided into three cohorts which consisted of patients who had had acute GvHD (n=18), chronic GvHD (n=15) or no GvHD (n=22). Samples were analysed during GvHD and before or after GvHD episodes. The PBMC samples were split and then either stained with antibodies to Foxp3 in order to quantify Tregs, or a combination of IFN-γ and IL-17A antibodies to measure frequencies of Th1 and Th17 cells respectively. This co-staining allowed identification of the Th17-1 subset which secretes both IFN-γ and IL-17A. We also used antibodies to investigate the expression of chemokines CCR6, CXCR3 and CCR4 in relation to Th1 and Th17 subsets.

2.1.3 Isolation of PBMCs from patient whole blood samples

Heparinised peripheral blood samples of 42ml were taken pre-transplantation and then at several time points in the post transplant period. A further sample was taken into a tube without anticoagulant. This was left to clot at room temperature or at 37^{o}C, before being centrifuged at 2200rpm for 5 minutes and a 2ml serum sample removed for storage at -80^{o}C. Bone marrow samples were obtained at the time of routine clinical monitoring and mononuclear cells (MC) were isolated from all samples by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The whole blood was diluted in an equal volume of RPMI 1640 (Invitrogen, Paisley, UK) prior to being layered onto lymphoprep and centrifuged at 1994 rpm for 30 minutes, brake off. A 2ml sample of the plasma was removed and frozen at -20^{o}C. The lymphocyte layer was removed by transfer pipette to a sterile tube containing 20ml RPMI 1640 (Invitrogen). Further washes were carried out by centrifuging at 1500rpm for ten minutes, then 1200rpm and finally 1000rpm with the supernatant being discarded each time and the pellet resuspended in 20ml RPMI. During and after the final wash, the resuspension media was RPMI supplemented with 10% Human Serum (HS) (TCS Biosciences, Buckinghamshire, UK) or Foetal Calf Serum (FCS) (PAA, Somerset, UK), penicillin/streptomycin (Invitrogen) and L-Glutamine (Invitrogen).

All IFN-γ capture assays were carried out using freshly isolated peripheral blood (PBMC) or bone marrow (BMMC) samples. Foxp3 staining and intracellular cytokine staining experiments were carried out on cryopreserved PBMC samples.

2.1.4 Cell freezing and thawing

Cells were centrifuged for 5 minutes at 1200 rpm and the supernatant was removed. The pellet was resuspended in the residual supernatant by gently flicking the tube and was then placed on ice for 10 minutes. 1 ml freezing media, consisting of FCS (PAA) containing 10%

dimethyl sulphoxide (DMSO) (Sigma) was added dropwise for each vial to be frozen and then transferred to chilled cryovials. The vials were placed in a freezing container which allowed the cells to be surrounded by, but kept separate from isopropanol, for controlled temperature reduction of 1^{o}C per minute. The container was placed in a -80^{o}C freezer and after 24 hours the cells were removed to -180^{o}C storage in a liquid nitrogen freezer.

To thaw the cryopreserved cells, the vials were removed from liquid nitrogen storage and placed in a waterbath at 37^{o}C and monitored closely until the cryovial contents were in liquid phase. The cells were immediately transferred to 10 ml culture medium consisting of RPMI (Sigma) with 10% FCS (PAA), supplemented with penicillin/streptomycin/L-glutamine, and washed by centrifuging at 1200 rpm before resuspension in an appropriate volume of culture medium.

2.1.5 Generation of B95.8 lymphoblastoid cell lines (LCL)

PBMCs were isolated from whole blood as described in 2.1.3. Cells were resuspended in B95.8 tissue culture supernatant (prepared in house) at 1x10^{7} cells per ml and incubated for one hour at 37^{o}C/5% CO_2, with gentle agitation every 15 minutes.

The cells were then washed twice by adding 10ml LCL culture medium (RPMI 1640, 10% FCS, pen/strep, L-glutamine) and centrifuging for 5 minutes at 1200rpm. After the final wash, the cells were resuspended in 2ml LCL medium supplemented with 2µg/ml cyclosporine (CSA) (Sandoz, Surrey, UK) and incubated in 2 wells of a 48 well culture plate, at 37^{o}C/5% CO_2. After 7 days, half of the media was replaced with CSA supplemented LCL medium. Cultures were monitored for the presence of clumps of cells and expanded into 24 well plates and then T25 tissue culture flasks, where appropriate. The media continued to be replaced with CSA supplemented medium when yellow for 14-21 days, after which time CSA was no longer

added. Cells continued to be grown in T25 or T75 flasks and medium was changed twice per week, and the cultures were split weekly to prevent overgrowth.

2.2 Detection of CTAg-specific T cells by IFN-γ Cytokine Secretion Assay

A panel of 20 peptides (Alta Biosciences, Birmingham, UK) from ten CTAg gene families was chosen on the basis of having been previously identified as T cell epitopes (as detailed on the website of the Academy of Cancer Immunity) and also shown to have RNA expression in AML and/or MM (Guinn et al., 2005, van Baren et al., 1999). The peptides, their HLA restriction and gene derivation are detailed in table 4.1.2 (Chapter 4). Peptide-specific T cells were identified from PBMCs using interferon-gamma cytokine secretion detection and enrichment assay kit (CSA) (Miltenyi Biotec®, Bergisch Gladbach, Germany)) according to manufacturer's instructions. Freshly isolated PBMC were seeded into wells of tissue culture plates (Iwaki) in RPMI 1640 media (Invitrogen) supplemented with 10% human serum (H+D Supplies) and L-glutamine (Invitrogen) at a cell density of $1x10^7$/ml and left overnight without stimulation at 37^oC in 5% CO_2. Peptides were then added either individually or in pools of not more than 6 at a final concentration of 10μg/ml. An equivalent volume of DMSO (Sigma, UK) was added to a negative control well which was then used to determine gate positioning for FACS analysis, as shown in figure 1. Staphylococcal enterotoxin B (SEB) (Sigma, UK) was used as a positive control at a concentration of 1μg/ml. Following a three hour stimulation period, the cells were labelled for five minutes with IFN-γ catch reagent and incubated with continuous rotation for 45 minutes at 37^oC. The cells were then labelled with IFN-γ detection reagent conjugated to PE fluorochrome, followed by anti-PE magnetic beads. Magnetic selection was carried out either manually using MS columns (Miltenyi Biotech, Bergisch Gladbach, Germany) for double positive selection, or using the equivalent "posseld" selection programme on an autoMACs (Miltenyi Biotech, Bergisch Gladbach, Germany). Pre- and

post-selection samples were labelled with CD4-FITC, CD8-PC5 monoclonal antibodies (Beckman Coulter, High Wycombe, UK) and propidium iodide (1 µg/ml, Sigma, UK) to exclude dead cells. Flow cytometric analysis was carried out using either Beckman Coulter XL-2 flow cytometer (Beckman Coulter, High Wycombe, UK) with WinMDI software for analysis (Scripps Institute, La Jolla, USA) or a BD LSRII with FACS DIVA analysis software (BD Biosciences). The percentage of antigen-specific T cells in the CD4+ or CD8+ pool, was calculated using the number of cytokine secreting cells in either the positively selected fraction or pre-selected sample gated on CD4+ or CD8+ cells within the PBMCs. Any IFN-γ secreting cells detected in the unstimulated negative control were considered to be background and subtracted from the frequency of cells producing IFN-γ following peptide stimulation.

2.3 Foxp3 staining

PBMCs were washed three times with cold phosphate-buffered saline (PBS) and stained with surface antibodies and a dead cell exclusion dye. The antibodies were CD127 FITC (eBioscience, Hatfield, UK), CD25 PC5 (Beckman Coulter, High Wycombe), CD4 PC7 (eBioscience), CD8 Pacific Blue (eBioscience), CD45RA AF700 (Biolegend, San Diego, USA) and CD3 APC-Cy7 (Biolegend). A LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen, Paisley, UK) was used to exclude dead cells.

The PBMCs were then fixed and permeabilised using the Foxp3 staining buffer kit (eBioscience) according to manufacturer's instructions. The sample was split and stained either with Foxp3 PE antibody (eBioscience) or Rat IgG1 PE Isotype control antibody. Samples were analysed on a BD LSRII (BD Biosciences, Oxford, UK).

2.4 Intracellular cytokine staining for Th1/Th17

The portion of PBMC sample not used for Foxp3 quantification was stained with surface antibodies to CXCR3 (CD183) PE (BD Biosciences), CCR4 PC7 (BD Pharmingen, Oxford, UK), CD8 AmCyan (BD Biosciences), CCR6 APC (R&D Systems, Abingdon, UK), CD3 APC-Cy7 (Biolegend). The sample was resuspended in RPMI (Invitrogen or Sigma-Aldrich, Gillingham, UK) containing 10% foetal calf serum (PAA) and then split. One part was stimulated for 6 hours at 37^{o}C 5% CO_2 with 25ng/ml PMA and 400ng/ml ionomycin (Sigma-Aldrich) and the other left unstimulated, both having monensin added at 1.25µg/ml within 1 hour of incubation. After 6 hours the tubes were transferred to a refrigerator and left overnight at 4^{o}C. The cells were washed three times with, and resuspended in, cold PBS before staining with LIVE/DEAD fixable dead cell staining kit (Invitrogen). The samples were then fixed for 30 minutes using 4% paraformaldehyde (Sigma-Aldrich) before washing with MACS buffer (PBS, 0.5% Bovine Serum Albumin (BSA) (Invitrogen), 2mM EDTA (Sigma)) and permeabilised for 5 minutes at room temperature, in the dark, with 0.5% saponin (Sigma-Aldrich). Some cells were removed for isotype control staining with anti-mouse IgG1 Pacific Blue or AF700 and then the samples were stained with IL-17A Pacific Blue (Biolegend) and IFN-γ AF700 (Biolegend) antibodies. Samples were washed with MACS buffer and then analysed on an LSRII (BD Biosciences) flow cytometer. Our CD4+ population was identified by gating on CD3+ CD8- cells within the lymphocyte gate, due to potential down-regulation of CD4 on the surface following stimulation. Statistical tests were carried out with Prism 5 (Graphpad Software, San Diego, USA), using a paired t-test (for two time point or group comparisons) or a Mann-Whitney test (more than two time point comparisons), and Spearman's non-parametric test was used to determine correlations.

2.5 Luminex analysis of serum cytokines

Concentrations of cytokines and chemokines present in the serum of patients were measured by using a 25-plex luminex kit (Panomics, Milan, Italy) according to manufacturer's instructions. Serum samples were stored at -80^{o}C and were thawed at the time of carrying out the assay. The panel of cytokines and chemokines measured is shown in table 5.4.1 (Chapter 5).

A 96 well filter plate was prepared by adding 150µl reading buffer and incubating for 5 minutes at room temperature. The buffer was removed through the filter by using a vacuum filter pump designed for extraction of liquids through the plate filter. 50µl of antibody beads were added to each well and excess buffer was removed by filtration. The beads were then washed with 150µl wash buffer, removed by filtration and blotting the base of the plate. 25µl assay buffer was added per well, followed by 25µl of samples and standards to appropriate wells. The plate was sealed and wrapped in foil, then incubated for one hour at room temperature on a plate shaker (500rpm).

Buffer was removed by vacuum filtration and the plate was washed three times with wash buffer. 25µl mixed detection antibodies were added to each well and the plate was sealed to incubate for 30 minutes at room temperature on a plate shaker (500rpm). Buffer was then removed by vacuum filtration and the plate was washed three times with washing buffer.

Streptavidin-PE was added at 50µl per well and the plate was sealed for a further incubation on a plate shaker (500rpm) for 30 minutes at room temperature. Solution was removed by vacuum filtration and the plate was washed three times. 120µl/well reading buffer was added and the plate resealed and incubated for 5 minutes on the plate shaker. The plate

was then read on a luminex reader. Statistical tests were carried out with Prism 5 (Graphpad Software, San Diego, USA), using a paired t-test (for two time point or group comparisons)

2.6 Interferon-γ ELISA

This assay was used to test specificity of T cell clones by establishing recognition of peptide loaded target cells. In most cases, peptide-loaded LCLs were used as target cells. LCLs were resuspended in a minimal volume of RPMI 1640 with peptide added at to a concentration of 10µg/ml and incubated for one hour at 37^{o}C. Negative control LCLs had an equivalent volume of DMSO added instead of peptide. The targets were then washed and resuspended at 3x10^{5}cells/ml in T cell line medium (TCL) (RPMI 1640, 10% HS, pen/strep, L-glutamine). 100µl of LCL suspension was added to wells of a 96 well U-bottomed tissue culture plate (Iwaki, Japan). T cell clones were suspended at 1x10^{4} cells/ml in TCL medium, and 100µl added to the targets. Control wells of T cells only, targets only and LCLs that had either not been loaded with peptide or loaded with an irrelevant peptide, were also set up. The plates centrifuged at 1000rpm for 5 minutes, then incubated for 12-18 hours at 37^{o}C/5% CO_2, and centrifuged again. 150µl supernatant was removed and the concentrations of IFN-γ were established.

A Maxisorp™ plate (Thermo Scientific Nunc, Leicestershire, UK) was coated with 0.75µg/ml anti-human IFN-γ antibody (Endogen) in 50µl coating buffer (0.1M Na2HP04 (pH9)), sealed and kept at 4^{o}C overnight. The antibody was then removed and the plate blotted onto tissue. 200µl blocking buffer (PBS, 1% BSA, 0.05% Tween 20 (Sigma-Aldrich)) was added per well and the plate left for 2 hours at room temperature. Following the incubation, the plate was washed three times with washing buffer (PBS, 0.05% Tween 20) and 50µl test supernatants, along with standards (2000pg to 15pg IFN-γ in doubling dilutions) were added.

The plate was then washed 4 times with 200µl/well washing buffer prior to 50µl/well biotinylated anti-human IFN-γ antibody (Endogen) (0.75µg/ml in blocking buffer) being added for one hour at room temperature. The plate was then washed 4 times and 50µl/well Extravidin-Peroxidase (Sigma-Aldrich) (diluted 1/1000 in blocking buffer) was added for 30 minutes at room temperature. After 8 more washes, 100µl/well TMB (Tebu Bio, Cambridgeshire, UK) was added and the plate was left for up to 20 minutes at room temperature in the dark. The reaction was stopped by the addition of 100µl 0.5M sulphuric acid and the plate was read at 450nm on an absorbance plate reader.

2.7 Chromium-release Cytotoxicity Assay

This assay was utilised to assess the cytotoxic killing ability of the T cell clones by measuring the percentage of target cells lysed following co-culture. Peptide-loaded LCLs were used as target cells and were prepared in the manner described in section 2.6. After peptide loading, the LCLs were incubated with 100µCi $Na_2{}^{51}CrO_4$ for one hour at 37^{o}C/5% CO2 and agitated every 15 minutes before being washed with T cell line (TCL) medium (RPMI 1640, 10% HS, pen/strep, L-glutamine).

Targets and clones were resuspended in TCL medium to give effector to target ratios 30:1, 10:1 or 5:1 in a total volume of 200µl per well during co-culture. Target only wells were set up for spontaneous lysis (200 µl TCL medium) and maximum lysis (100µl TCL medium and 100µl 1% SDS) measurements. The plates were centrifuged at 1000rpm for 5 minutes and then incubated for 4-6 hours for CD8+ T cell clones or 12-16 hours for CD4+ T cell clones. Following the incubation period, 100µl supernatant was harvested and measured using a Topcounter gamma-radiation counter (Hewlett-Packard), which calculated the percentage of target lysis according to the following formula:

$$\% \text{ specific lysis} = \frac{\text{sample release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

Positive values were considered to be those above a 20% threshold.

2.8 T cell cloning

T cell responses were cloned using this method for further characterisation and functional assays. The T cell culture (TCC) medium used throughout was RPMI 1640, 5% HS, 5% FCS, pen/strep, L-glutamine.

Feeder cells consisting of allogeneic LCLs and overnight PHA-activated buffy coats from three donors were irradiated (40Gy). The feeder cells were washed 4 times in TCC medium and resuspended at $1x10^5$ LCL and $1x10^6$ per ml. The mixture was supplemented with 20U/ml IL-2 (Chiron), IL-4 (Peprotech) (10ng/ml), IL-7 (Peprotech) (10ng/ml), IL-15 (Peprotech) (8ng/ml), IL-21 (Peprotech) (8ng/ml) and PHA (Sigma) (10µ/ml). Final concentrations were half of these amounts. 100µl of this mixture was added to wells of 96 well U bottomed tissue culture plates (Iwaki).

T cells for cloning were washed and aliquoted into different volumes of TCC medium to give concentrations of 0.3 cells, 1 cell, 3 cells and 10 cells per 100µl, and added to wells of feeder cells to give a total volume of 200µl per well. The plates were then wrapped in foil and incubated at $37^oC/5\%CO_2$. On day 7, 100µl of media was replaced with TCC containing IL-2 (20U/ml) to give a final concentration of 10U/ml.

From day 14, the plates were monitored for clonal outgrowths. If growth was observed, the contents of the well were transferred to a well in a 48 well tissue culture plate containing 1 ml of feeder cell/cytokine mix as described earlier. Half of the media in the wells was

replaced twice weekly with TCC medium supplemented with either IL-2 alone, or with IL-2, IL-7, IL-15 and IL-21 at the concentrations described earlier. Every two weeks, irradiated feeder cells were also added to the wells at the same cell concentrations as the original cloning mix. Clones were tested for function and specificity at least 7 days after the most recent addition of feeder cells.

2.9 T cell clone rapid expansion protocol

This method was used to expand the numbers of T cell clones in a short time in order to provide the Regional Genetics Laboratory with enough cells to extract sufficient DNA for their standard chimerism analysis.

The media used throughout was RPMI 1640 (Invitrogen) with 5% HS (TCS Biosciences), 5% FCS (PAA), pen/strep (Invitrogen), 2mM L-Glutamine (Invitrogen), 25µM β-Mercaptoethanol (Invitrogen), 12.5mM HEPES (Invitrogen). To a T25 tissue culture flask (Iwaki) containing 25ml of this media, $1x10^5$ clone was added, along with $5x10^6$ 40Gy irradiated LCLs, $2.5x10^7$ 40Gy-irradiated allogeneic buffy (from three donors) and 30ng/ml OKT3 monoclonal antibody. The flask was incubated at $37^oC/5\%$ CO_2.

On the second day, IL-2 (Chiron) was added to a final concentration of 50U/ml. The mixture was centrifuged at 1200rpm for 5 minutes on day 4, and then the pellet resuspended in 25 ml culture medium with 50U/ml IL-2. On day 8, half of the media was replaced with fresh culture medium containing 100U/ml IL-2 (to give a final concentration of 50U/ml). This step was repeated on day 11 and on day 14 the cells were harvested and sent for DNA analysis.

2.10 CFSE Proliferation Assay

Cells were washed three times in 10ml phosphate buffered saline (PBS) by centrifuging at 1200rpm for five minutes and resuspending in 10ml PBS for each wash. After the third wash the cells were counted and resuspended at a concentration of $2x10^7$ cells/ml. Meanwhile, CFSE (Invitrogen) was diluted from the stock solution of 10mM (in DMSO) to 2µM, before being added an equal volume to the cell suspension, giving a final CFSE concentration of 1µM. The cells were vortexed gently and incubated in a 37°C water bath for 10 minutes, with occasional agitation. After the incubation period, culture medium (RPMI 1640, 10% Human Serum, penicillin/streptomycin, L-Glutamine), pre-warmed to 37°C was added at an equal volume to the cell/CFSE suspension in order to stop the reaction. A further three washes were carried out as described before in 10ml PBS and the cells were resuspended at an appropriate concentration for further experiments.

2.11 Immunohistochemistry

Slides with paraffin-embedded bone marrow trephine samples were obtained from the Pathology Department of the Queen Elizabeth Hospital, Birmingham. Slides were incubated in a dry oven at 60°C for 20 minutes before being transferred to preheated W/Cap (pH8) in a coplin jar being heated to 98°C in a waterbath. After 30 minutes the jar was removed from the waterbath and left to cool for 20 minutes at room temperature, then washed under a running cold tap for 5 minutes. The jar was filled with 0.3% H_2O_2 in methanol for 10 minutes and washed again under a cold tap for 5 minutes.

The slides were then laid out on a staining rack and 10µg/ml MAGE antibody (Invitrogen) or IgG2a isotype antibodies were added and left for one hour at room temperature. The slides were washed by stirring in TBS buffer for 30 minutes before being returned to the staining rack.

Two drops from a dropper bottle of envision solution (Dako: Chemate/Envision kit) were added to each slide and incubated under cover for 30 minutes at room temperature. The slides were then washed again for thirty minutes by stirring in TBS buffer and returned to the staining rack. DAB (Dako) was added for 5 minutes and washed off with water, followed by addition of Mayer's haematoxylin solution which was washed off after 30 seconds. The slides were washed under a cold tap for 2 minutes, then under a hot tap for one minute before a further wash for 1 minute in cold water.

A liquid mounting solution was then added to the area of the slide where the sample was located and a glass coverslip was placed carefully on the slide. The sample was then examined under a light microscope and the level of staining assessed.

2.12 CD137 Assay

Cryopreserved PBMCs were thawed, washed in RPMI 1640 (Invitrogen) and resuspended at $1x10^7$ cells/ml in T cell culture medium (RPMI 1640, 10% human serum, 0.5% penicillin/streptomycin, 0.5% L-glutamine). Cells were incubated with 10 µg/ml CTAg peptide(s) for 24 hours (37^oC, 5% CO_2). Equivalent volumes of DMSO (Sigma) were added to negative control wells and 1 µg/ml SEB was used for positive controls. Each well was harvested, washed in MACS buffer (PBS, 0.5% Bovine Serum Albumin (BSA) (Invitrogen), 2mM EDTA (Sigma)) then stained with anti-CD137-PE (BD Biosciences), along with CD3-APC-Cy7(BD Biosciences) and CD8-AmCyan (BD Biosciences) to identify T cells. CD14-ECD (Beckman Coulter), CD19-ECD (Beckman Coulter), CD56-PC7 (BD Biosciences) and 1µg/ml propidium iodide (Sigma) were also added to exclude other cell populations and dead cells. Responding cells could either be assessed directly by flow cytometry or enriched using PE-microbeads (Miltenyi Biotech).

2.13 Peptide-stimulated T cell line

Freshly isolated PBMC were resuspended in 1 ml serum-free RPMI 1640 media (Invitrogen) and 30µM peptide was added. The cells were incubated for 1 hour and then T cell line (TCL) media (RPMI 1640 with 10% Human Serum (TCS Biosciences)), supplemented with 25 ng/ml IL-7 (Peprotech) and 2 ng/ml (Peprotech), was added at a volume to achieve a cell concentration of $3x10^6$ cells/ml. Each 1 ml of cell suspension was transferred to one well of a 24 well cell culture plate. On day three, a further 1 ml of TCL media was added containing IL-2 (Chiron) to a concentration of 20 U/ml. Every three days, 1 ml of media was removed to be replaced with another 1 ml of TCL media with 40 U/ml IL-2 (for a final concentration of 20 U/ml). Cells were harvested after 10-14 days.

Chapter 3: Identifying Alloreactive T cells by CFSE Proliferation Assay

3.1 Introduction

An important contribution to the success of allogeneic stem cell transplantation is the development of GvL. The association between the occurrence of GvHD and a decrease in the likelihood of relapse (Horowitz et al., 1990) suggests that some form of GvL is also developing. However, GvHD continues to be a major cause of morbidity and mortality. Clinical strategies to prevent or treat GvHD are often immunosuppressive and have the disadvantage of potentially undermining GvL. Both GvL and GvHD are T cell mediated and as targets of GvHD are extremely varied and not well characterised, broadly immunosuppressive treatments are used. Many patients receive prophylactic cyclosporine following transplantation, and a clinical decision is made to reduce the dose over time until it can safely be withdrawn. During this tapering period, if the patient shows signs of developing GvHD-like symptoms, the dose is again increased. Alternatively, patients may be treated with courses of steroids such as prednisolone.

It would be advantageous to be able to distinguish between pathogenic T cells that cause GvHD and those providing beneficial GvL. Both of these processes are thought to be caused by alloreactive T cells which develop from the donor immune system that has been transplanted into the patient. The identity of tumour targets that elicit a GvL reaction are poorly characterised currently. However, if alloreactive T cells that cause GvL can be identified in patients, it may be possible to establish their specificity or isolate them for immunotherapy. T cells that have the capacity to respond to the patient's tumour could be cloned and expanded, perhaps while the patient is receiving immunosuppressive GvHD treatment, and then reintroduced to the patient, leading to a more robust GvL response.

Alloreactive cells could be identified by culturing T cells isolated from the patient following transplantation, with irradiated samples taken before transplant conditioning. Those cells

that proliferate are potentially alloreactive. An identical parallel experiment that uses donor cells instead of the pre-transplant sample should not induce proliferation if the patient's immune system is fully donor - this can be established by chimerism analysis. Therefore, the level of background, non-alloreactive proliferation can be established. Labelling the T cells from the post-transplant sample with a dye such as CFSE allows the proliferating cells to be identified through progressive loss of the dye during cell division (Lyons, 2000, Lyons and Parish, 1994). The CFSE-dim population can then be sorted to allow cloning of potentially alloreactive cells. Previous studies have used this fluorescence based method to identify rare antigen-specific CD4 cells (Mannering et al., 2003), and alloreactive T cells in murine studies (Pachnio et al., 2006, Suchin et al., 2001). This chapter seeks to establish whether this approach could be used in a human setting.

3.2 Use of CFSE to identify alloreactive cells

5, 6-carboxylfluorescein diacetate succinimidyl ester (CFSE) is initially non-fluorescent and is converted to the fluorescent anionic form by intracellular esterases. When CFSE labelled cells undergo division, the dye is distributed equally between the daughter cells (Weston and Parish, 1990). Therefore, cells that have proliferated have a lower concentration of CFSE and can be differentiated from the non-dividing population by FACS, due to having less-bright staining. An example of CFSE staining is shown in figure 3.2.1. A detailed method for CFSE staining is given in Chapter 2 (2.10).

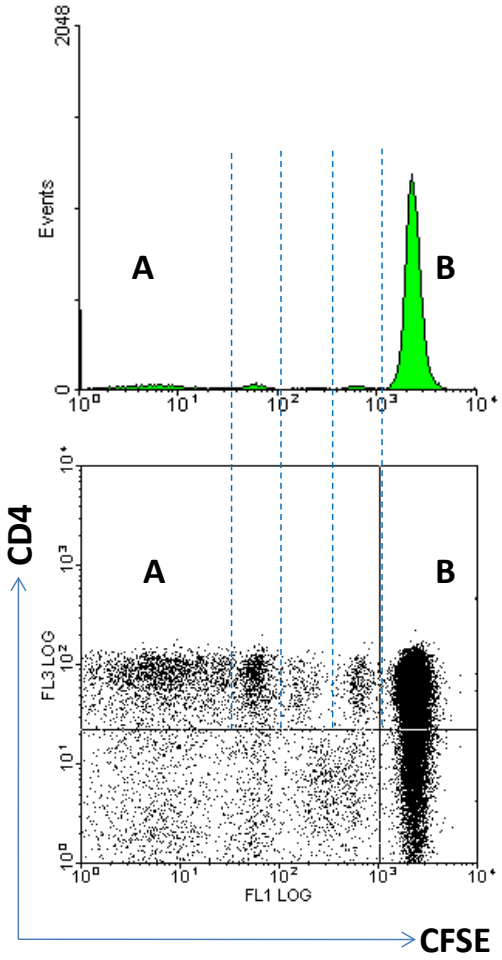


Figure 3.2.1 Example of FACS staining of CFSE labelled CD4+ T cells

Dividing populations of T cells can be seen, segregated by dotted lines as a histogram and by dot plot, where progressive loss of CFSE denotes increased numbers of divisions. Cells which have undergone the most numbers of divisions are shown in area A and undivided cells are in B. CD4+ T cells are above the quadrant in the dot plot. Those below may be CD8+.

The method used for stimulating alloreactive T cell proliferation is to co-culture CFSE-labelled PBMCs with irradiated pre-transplant samples or donor samples. This would be ideally done with tumour samples from the patient, but due to lack of availability of these, a pre-transplant sample of peripheral blood was used with the expectation that there would be some tumour present. There was not a practical and reliable method to test each sample for the quantity of tumour. However, there should have been a degree of tolerance to non-tumour specific antigens and any reactivity to these would be indicated by proliferation in the patient's post- transplant sample when co-cultured with the donor sample. T cells specific for mHAgs may also be detected.

3.3 Optimisation of CFSE proliferation assay as a method for detecting alloreactive cells

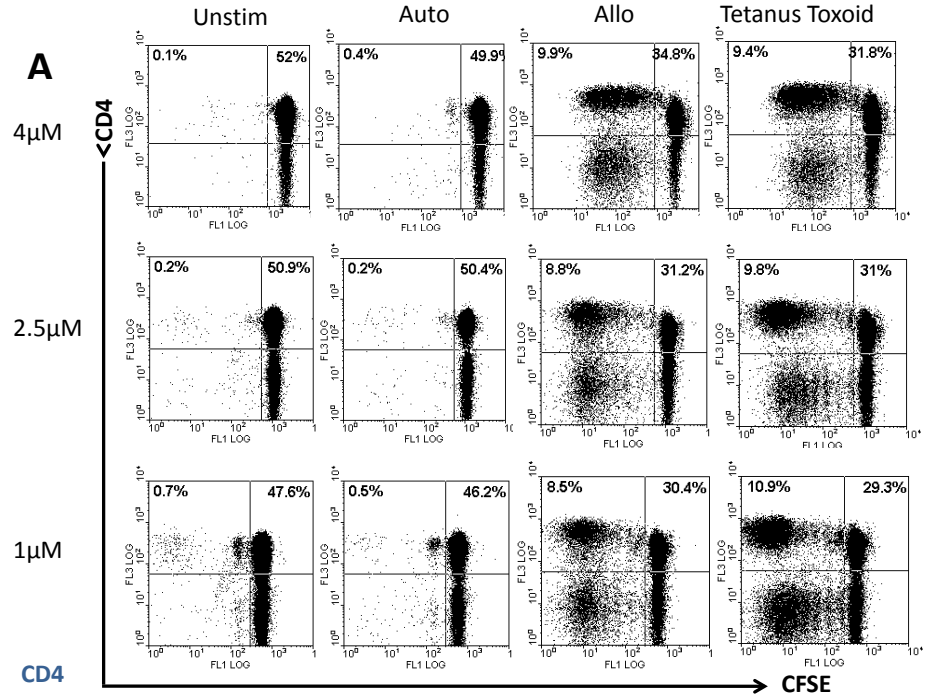
One of the challenges of this study was the limited availability of clinical material, particularly the pre-transplant samples. Therefore it was necessary to ensure the method was optimised and tested on healthy donor samples before applying the technique to clinical material.

3.3.1 Titration of CFSE

An important consideration when choosing an assay to identify, sort and ultimately clone Ag-specific T cells is whether the cells will be viable at the end of the experiment. A concern for this assay was the potential toxicity of the CFSE dye. Higher concentrations can cause cell death, inhibit cellular proliferation and alter the expression of surface markers (Last'ovicka et al., 2009). Therefore it was necessary to establish the lowest concentration that would allow dividing cells to be differentiated from the non-proliferating population. PBMCs from a healthy donor were labelled with reducing concentrations of CFSE (4µM, 2.5µM and 1µM) and stimulated either by co-culture with irradiated autologous or

allogeneic cells, or with Tetanus Toxoid. After seven days in culture at 37^{o}C 5% CO_2, the cells were stained with CD4 ECD or CD8 PC5 and analysed by flow cytometry (Coulter XL). Data is shown in figure 3.3.1.1 as dot plots, with percentages of proliferating and non-proliferating CD4+ or CD8+ T cells shown.

Following stimulation with Tetanus Toxoid, both CD4+ and CD8+ T cells exhibit greater proliferation at 1µM than 2.5 or 4µM CFSE labelling. Where 4µM CFSE was used, 9.4% proliferation of CD4+ T cells was observed, but 10.9% after labelling with 1µM (figure 3.3.1.1A). 10.19% of CD8+ T cells proliferated after labelling with 1µM CFSE, but only 4.2% proliferated in response to the same stimulus when labelled with 4µM CFSE (figure 3.3.1.1B). This may be due to toxicity at the higher concentrations or perhaps the mechanism for recognition of the Toxoid is impaired. After allogeneic stimulation there is a slight reduction in the percentage of proliferating cells between the maximum and minimum concentration of CFSE used for labelling. This reduction was only 1.4%, from 9.9% at 4µM to 8.5% at 1µM only for CD4+ T cells (figure 3.3.1.1A) and 0.5% from 8.6% to 8.1% for CD8+ T cells (figure 3.3.1.1B). When 2.5µM CFSE was used for labelling, the frequency of dividing CD8+ T cells was considerably lower than where 1 or 4µM was used. The reason for this is unclear, but may simply be a technical problem during that assay. Overall, the conclusion drawn was that the lowest concentration would be most suitable for the assay when considering the potential toxicity of CFSE.


A
Unstim
Auto
Allo
Tetanus Toxoid
4µM
2.5µM
1µM
CD4
CFSE
0.1%
52%
0.4%
49.9%
9.9%
34.8%
9.4%
31.8%
0.2%
50.9%
0.2%
50.4%
8.8%
31.2%
9.8%
31%
0.7%
47.6%
0.5%
46.2%
8.5%
30.4%
10.9%
29.3%

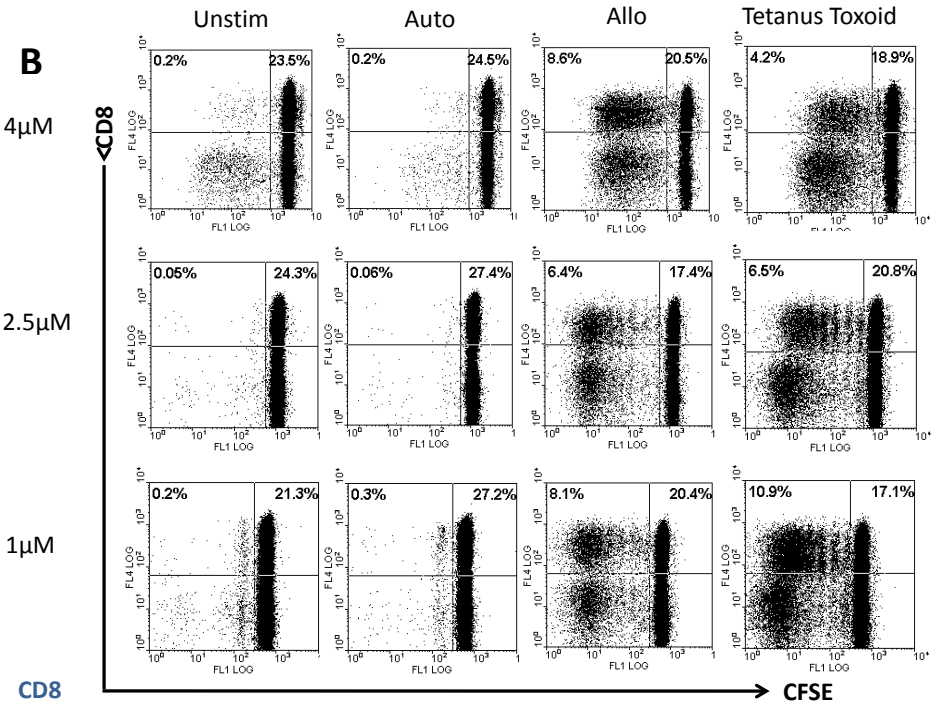

B
Unstim
Auto
Allo
Tetanus Toxoid
4µM
2.5µM
1µM
CD8
CFSE
0.2%
23.5%
0.2%
24.5%
8.6%
20.5%
4.2%
18.9%
0.05%
24.3%
0.06%
27.4%
6.4%
17.4%
6.5%
20.8%
0.2%
21.3%
0.3%
27.2%
8.1%
20.4%
10.9%
17.1%

Figure 3.3.1.1 Titration of CFSE

Dot plots showing proliferation of CD4+ (A) and CD8+ (B) (y-axis) T cells after seven days in culture, following stimulation with irradiated autologous PBMCs, allogeneic PBMCs or tetanus toxoid (TT). Proliferating T cells were measured by labelling with CFSE (x-axis) titrated from 4mM to 1mM (top to bottom, decreasing concentration). Percentages shown are dividing (upper left quadrant) or non-dividing (upper right quadrant) CD4+ or CD8+ T cells as proportions of the total lymphocyte gate. Proliferating T cells were isolated from peripheral blood of healthy donors, as were irradiated target cells used for stimulation. Autologous stimulation was by cells from the same donor, and the allogeneic stimuli cells were from a different, HLA-mismatched donor.

3.3.2 Proliferation over time

A time course experiment was set up using healthy donor cells to determine the optimum co-culture time that allowed sufficient proliferation to occur. PBMCs were isolated from the blood of four donors and an aliquot of each was irradiated to use as autologous stimuli. The remaining PBMCs were labelled with CFSE and stained with antibodies against CD4 and CD8.

Cells from each donor were plated into 24 well plates and co-cultured with irradiated autologous or allogeneic cells, or left unstimulated. Samples were analysed on a Coulter XL flow cytometer at days 7, 10 and 14. The percentages of dividing cells are shown in figure 3.3.2.1 for each condition, as means for all donors. Proliferation occurred in response to allogeneic stimulus between days 7 and 10, and stabilised up to day 14 in the CD4+ population. There was a decrease in the number of CD8+ T cells that had proliferated following day 10 which may be due to cell death. Therefore the optimum time to harvest co-culture assays to determine proliferation was around day 10 (figure 3.3.2.1).

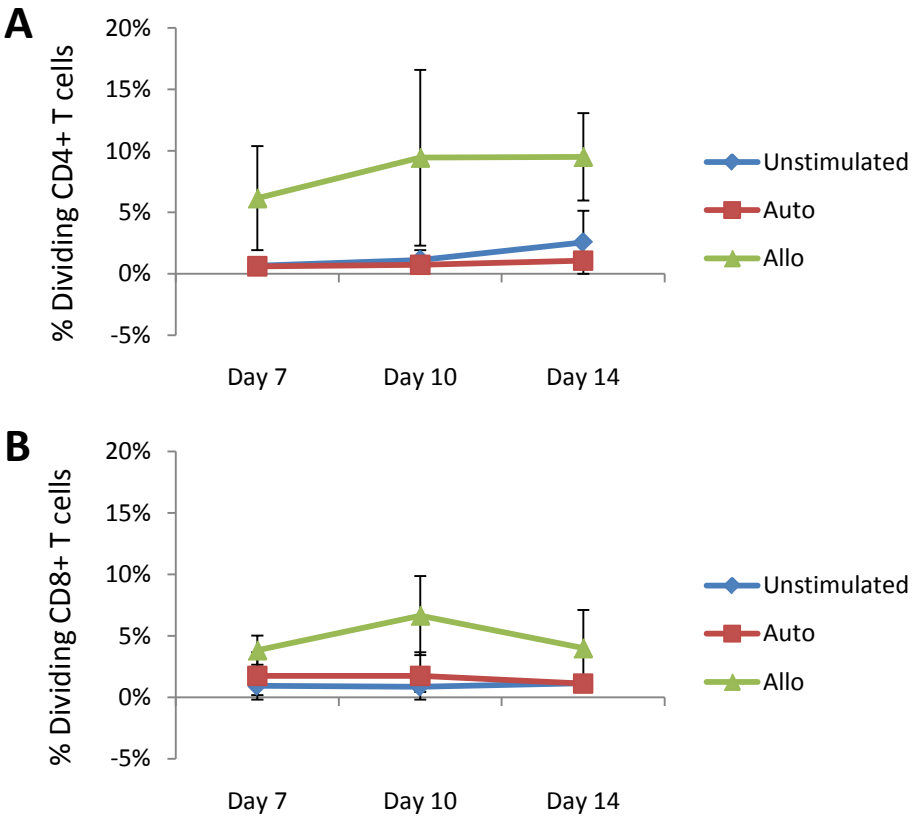


Figure 3.3.2.1 Proliferation over time

Mean percentages of proliferating $CFSE_{low}$ CD4 (A) or CD8 (B) cells from four healthy donors are shown with standard deviation (y-axis), when cultured for up to 14 days with irradiated allogeneic PBMC from a HLA-mismatched healthy donor (green line), autologous PBMC (red line) or no stimulation (blue line). Cells were harvested at days 7, 10 and 14, stained with CD4 and CD8 antibodies, then the percentages of proliferating cells were established by flow cytometry.

3.3.3 Supplementation of culture medium with cytokines

In order to assist the growth of proliferating cells and reduce the risk of cell death, the culture medium could be supplemented with cytokines. However, this would carry a risk of driving non-specific proliferation. Experiments were carried out similar to those in described in 3.3.2 but with IL-2 supplemented at different concentrations. Figure 3.3.3.1A shows that in healthy donors there was little difference in proliferation when 20U/ml IL-2 was added, in comparison to those wells without IL-2 addition. Where IL-2 was supplemented at 500U/ml there was a slight decrease in proliferation following allogeneic stimulation. It is not clear why this would be, but high levels of IL-2 have been associated with maintenance of a suppressive environment (Antony et al., 2006, Fontenot et al., 2005). The data shown is a representative example of several similar experiments.

Figure 3.3.3.1B shows proliferation when CFSE-labelled patient PBMCs were co-cultured with irradiated autologous (donor) or allogeneic (pre-transplant) cells in the presence of varying concentrations of IL-2. Whilst the presence of high concentrations of IL-2 seems to favour allogeneic CD8 proliferation, there is a slight increase in autologous cell division. However, there is a marked increase in autologous CD4 proliferation, to similar levels as with allogeneic targets. This result does suggest that addition of IL-2 can promote non-specific proliferation.

The proliferation inducing effects of supplementing the standard culture medium with IL-7 and IL-15 are demonstrated in figure 3.3.3.2. Healthy donor CFSE-labelled PBMCs were cultured alone, or co-cultured with irradiated allogeneic cells. These experiments were carried out using either standard T cell culture medium (RPMI with 10% Human Serum, 1% penicillin/streptomycin, 1% L-glutamine) or the same media supplemented with IL-7 (25ng/ml) and IL-15 (2ng/ml). Whilst considerable increases in allogeneic proliferation were

observed in the presence of additional IL-7 and IL-15, it should be noted that in the absence of allogeneic stimuli, increases were still nearly five-fold (CD4) and ten-fold (CD8), suggesting that higher concentrations of these cytokines lead to considerable non-specific proliferation.

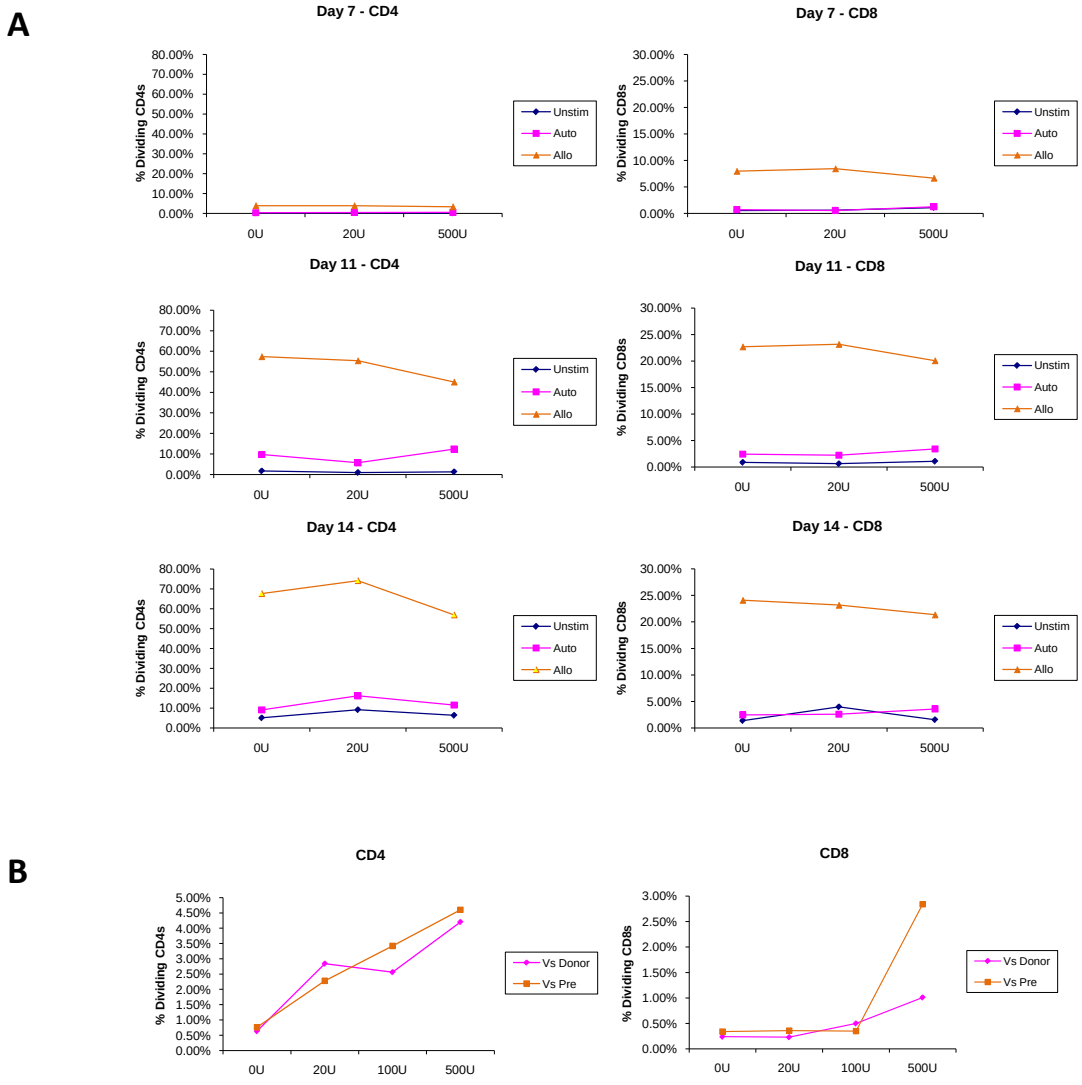


Figure 3.3.3.1 Effect of adding IL-2 to cultures

CFSE labelled PBMCs from a healthy donor (A) were cultured in media only (blue line), or co-cultured with irradiated autologous (pink line) or allogeneic (orange line) PBMC in the presence of varying concentrations of IL-2 (x-axis). Similarly, PBMCs isolated from a stem cell transplant patient (B) and CFSE labelled were co-cultured with irradiated autologous PBMC (from the HLA-matched donor, pink line) or allogeneic PBMC (isolated from the patient prior to transplant conditioning, orange line). The frequency of proliferating CD4+ T cells (left column) or CD8+ T cells (right column) was determined by the percentages of $CFSE_{low}$ cells (y-axis).

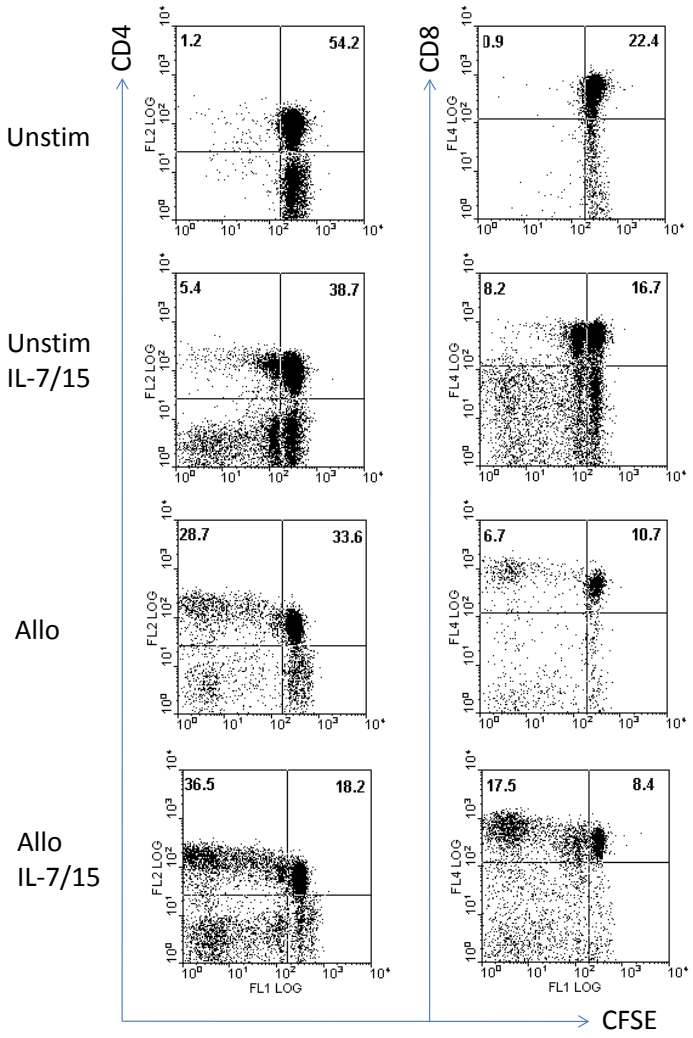


Figure 3.3.3.2 Proliferative effects of supplementing media with IL-7 and IL-15

CFSE labelled PBMC from a healthy donor were cultured in media only, or co-cultured with irradiated allogeneic PBMC with or without IL-7 and IL-15 (y-axis). The frequency of proliferating CD4+ T cells (left column) or CD8+ T cells (right column) was determined by the percentages of $CFSE_{low}$ cells (upper left quadrants) compared to percentages of undivided, $CFSE^{hi}$ T cells (upper right quadrants), shown as percentages of the entire lymphocyte population.

3.4 Detection and cloning of alloreactive T cells

Having optimised the culture conditions for the assay, it was necessary to test the principle further in healthy donor samples and then investigate whether this method shown successfully in a mouse model could be applied to human allograft patients.

3.4.1 Alloreactive T cell detection in healthy donor and transplant patients

PBMCs were isolated from the blood of healthy donors and split into fractions that were either irradiated or labelled with CFSE, a sample of which were analysed for staining by flow cytometry on day 1. Cells from the irradiated fraction were either used in autologous co-culture or as allogeneic stimulation for labelled T cells from other donors. Co-cultures were set up as described earlier. Cells harvested after 10 days and stained for CD4 and CD8 for analysis on a Coulter XL flow cytometer. Example dot plots are shown in figure 3.4.1.1A. The proportions of proliferating cells were recorded for both autologous and allogeneic cultures for each donor and are shown in figure 3.4.1.2.A. In almost all cases, there was greater proliferation in the allogeneic cultures, although to differing degrees. The differences may be attributable to levels of HLA mismatch between donors. Complete HLA-typing data was not available for all donors.

Peripheral blood samples were donated by allograft patients when attending the bone marrow transplant patient clinics. PBMCs were isolated from these fresh samples and stained with CFSE. Samples of the CFSE-labelled cells were assessed for CFSE-staining levels by flow cytometry on day 1.

Cryopreserved PBMCs isolated from blood samples that had previously been taken from either the HLA-matched donor (autologous), or the patient prior to beginning transplant conditioning (allogeneic), were thawed and irradiated. Tumour samples collected at the time of diagnosis were used for allogeneic stimulation instead of the pre-transplant PBMCs,

where available. Co-culture assays were set-up as before and cells harvested after 10 days before being stained with anti-CD4 ECD and anti-CD8 PC5 for analysis using a Coulter XL flow cytometer. Sample dot plots are shown in figure 3.4.1.1B. A summary of autologous and allogeneic proliferation of cells from all patients is shown in figure 3.4.1.2B. If the autologous proliferation is seen as 'background', then there was little evidence of specific proliferation in the allogeneic wells. That the high levels and incidence of allogeneic response seen in the healthy donors was not seen in the patients in not surprising due to the patient and donor pairs being HLA-matched. Other reasons for the lack of responses could be that the pre-transplant PBMC samples are an inadequate source of allo-antigen, or that the post-transplant PBMCs from the patients were in poor condition due to *in-vivo* immunosuppression.

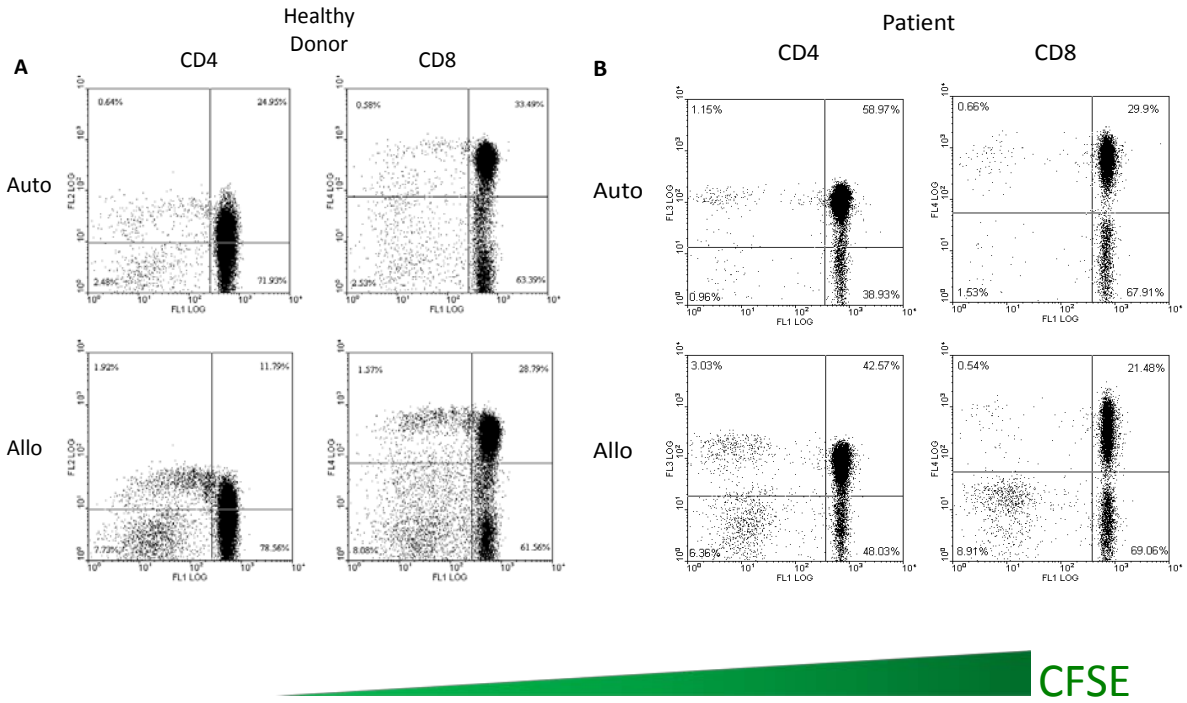


Figure 3.4.1.1 Measurement of proliferation by CFSE-labelling

Example dot plots are shown from CFSE proliferation assays to determine the frequencies of proliferating CD4 or CD8 (y axis) T cells following autologous or allogeneic stimulation with irradiated PBMC. Healthy donor T cells (A) were stimulated with PBMC from the same donor (auto) or from a different healthy donor (allo). Stem cell transplant patient (B) T cells were stimulated with PBMC from the HLA-matched stem cell donor (auto) or PBMC isolated from the patient before transplant conditioning. The frequencies of proliferating T cells were determined by $CFSE_{low}$ cells (x-axis) and are shown in the upper left quadrants as percentages of Total lymphocytes, and the percentages of non-dividing T cells in the upper right quadrants.

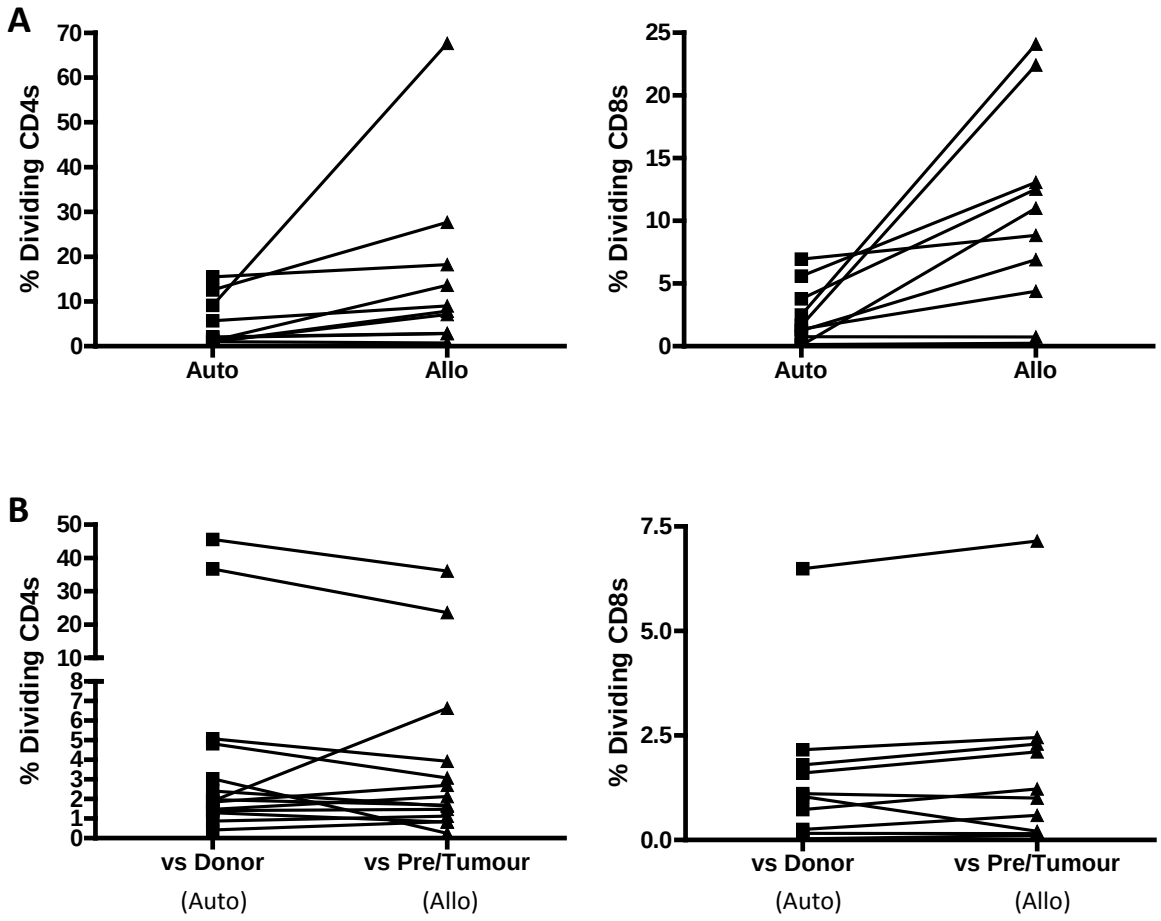


Figure 3.4.1.2 Comparison of proliferation induced by co-culture with autologous or allogeneic cells

The percentages of dividing CD4+ (left) and CD8+ (right) T cells measured by CFSE loss following co-culture with allogeneic or autologous cells for all healthy donors (A) and allograft patients (B) are shown. Freshly isolated PBMCs from patients were co-cultured with irradiated PBMC from their HLA-matched donor (autologous) or PBMC isolated from the patient prior to transplant conditioning, or tumour (allogeneic) (x-axis).

3.4.2 Generation of alloreactive T cell clones

Despite a lack of clear allo-specific proliferation, there were instances of proliferation above background in the allogeneic cultures and attempts were made to isolate alloreactive T cells by FACS and generate clones by limiting dilution. The frequencies of dividing cells are shown in table 3.4.2.1. In some cases, such as patient TS142PM, the autologous response was higher than from allogeneic stimulation. However, some samples had robust responses to allogeneic stimuli above background, for example KaM153PM at 17 weeks post-transplantation had 1.32% alloreactive T cells (table & figure 3.4.2.1). T cell clones were grown from two patients, KaM153PM and KS114PM.

The sorted cells were cloned by limiting dilution as per the method outlined in Chapter 2 (section 2.8). Unfortunately the clones generated from KaM153PM were lost due to a contaminated feeder cell product. Clones from KS114PM were tested for specificity against a tumour sample taken from the patient at diagnosis, and also against PBMCs from the donor, in order to demonstrate whether these clones were alloreactive and tumour-specific. Results of IFN-γ ELISA recognition assays and ^{51}Cr-release killing assays are presented in figure 3.4.2.2. Donor LCLs were used as autologous targets as there was no other donor material available, and it is acknowledged that there was the risk of response to EBV proteins. However, in the ^{51}Cr release assay there was no lysis of donor LCLs, suggesting that this was unlikely to be a problem. Some lysis of tumour was evident for all clones, but only four breached the threshold of 20% to be considered positive results. IFN-γ ELISA data was available for three of these and for two clones, concentrations were more than double for reactions to tumour cells than to donor LCLs (which were similar low concentrations to the negative control). However, the concentrations against the tumour were between 400 and 600 pg/ml suggesting only weak responses. Three clones were strongly positive for IFN-γ

production against tumour over background levels. Unfortunately killing assay data was only available for one of these and lysis was considerably below the threshold. From the data available, clone 1E12 had the best correlation between IFN-γ production and killing ability. However, some CD4+ T cell subsets do have robust cytokine responses, but are not necessarily cytotoxic.

Patient	Time point (weeks post-transplant)	% CD3 (above background)	% CD4 (above background)	% CD8 (above background)
KS114PM	114 (21 post-DLI)		0.39	0.17
KS114PM	120 (27 post-DLI)		4.48*	0.49
RJ140PM	45	0.25		
TB142PM	43	0		
KaM153PM	17	1.32		
KaM153PM	26		0.21	0.03

Table 3.4.2.1 Alloreactive T cell frequencies from cell sorting experiments

CFSE-labelled patient PBMC samples were co-cultured with irradiated autologous PBMC (from HLA-matched stem cell donor) or allogeneic PBMC (isolated from patient blood prior to transplant conditioning). After 11 days, the cells were harvested and stained either for CD4/CD8 or for CD3 alone and the $CFSE_{low}$ CD3+/CD4+CD8+ T cells were FACS separated for T cell cloning by limiting dilution. The percentages of proliferating cells are shown in the table for each sorting experiment. The frequencies shown were calculated by subtracting any proliferation following autologous stimulation from the percentage of dividing cells after allogeneic stimulation. The time post-transplant is shown in weeks along with the portion of that time following donor lymphocyte infusion (DLI) (if applicable). The figure for proliferating CD4+ T cells at 120 weeks post-transplant from patient KS114PM (*) is elevated due to gating complications. However, a clear population was observed.

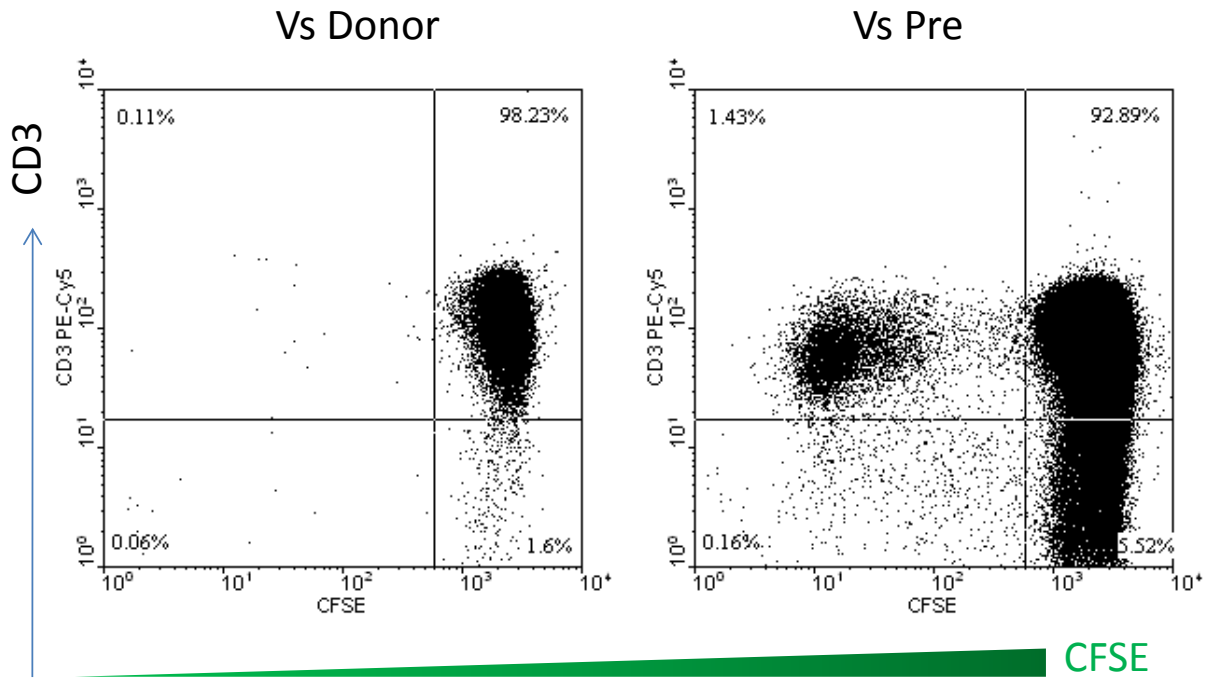


Figure 3.4.2.1 Sample dot plots from cell sorter showing proliferation in response to pre-transplant patient PBMC

A CFSE-labelled PBMC sample from patient KaM153PM was co-cultured with irradiated autologous PBMC (from the patient's HLA-matched stem cell donor, left) or allogeneic PBMC (isolated from patient blood prior to transplant conditioning, right). After 11 days, the cells were harvested and stained for CD3 and the $CFSE_{low}$ CD3+ T cells were FACS separated for T cell cloning by limiting dilution. As 0.11% T cells proliferated during autologous cell co-culture, as compared to 1.43% during allogeneic cell stimulation, then 1.32% of dividing T cells were considered to be responding to allogeneic stimulation, and therefore were allogeneic.

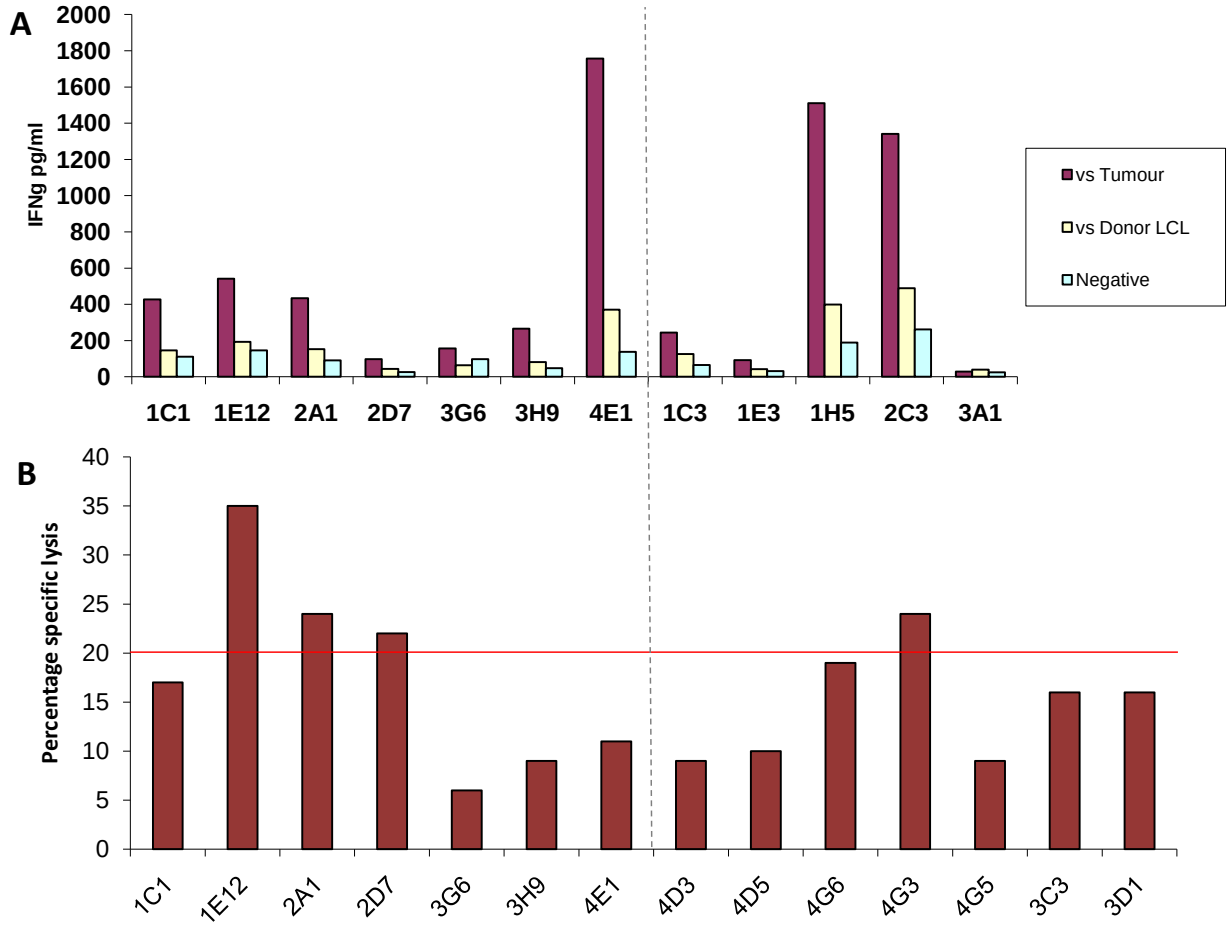


Figure 3.4.2.2 Investigation into functions of T cells cloned from KS114PM

T cell clones from patient KS114PM were tested by IFN-γ ELISA (A) for reactivity against, and ability to kill (B) patient tumour cells and LCLs derived from the HLA-matched transplant donor. Killing ability was assessed by specific lysis of target cells in a ^{51}Cr release assay. There was no lysis of donor LCLs, and killing above a threshold of 20% was considered positive (red line).

Both ELISA and ^{51}Cr data was available for clones to the left of the dotted line and are shown in the same columns. For clones to the right of the dotted line, results are available for only one assay.

3.5 Discussion

The data from this study confirmed that CFSE could be used to detect proliferating T cells in co-culture assays and higher levels of cell division clearly occurred in an allogeneic culture when using mismatched healthy donor cells. The attempts to translate the findings of previous mouse studies that alloreactive T cells could be detected by this method following stem cell transplantation into a human clinical scenario proved somewhat less conclusive. It is to be expected that the effect would be far more subtle in the transplant setting as having a high degree of histocompatibility between donor and patient is designed to prevent large donor responses to patient tissues, which would lead to GvHD. Therefore, the idea of this study was to try to detect those cells that would respond to tumour. As such, tumour reactive T cells are likely to be at low frequency it may well be that this method is not sufficiently sensitive, and that even low levels of background proliferation may prevent detection of tumour specific T cells. Very few clones isolated from a patient in this study exhibited killing ability and/or cytokine release in response to tumour. Only two of around 20 clones tested, produced IFN-γ to more than double background levels and also killed above threshold. One of these clones was the most effective at lysing tumour cells.

There are a number of potential reasons for the apparent inconsistency between cytokine production and killing ability. Clones often lose killing function whilst retaining the ability to produce cytokines. This is particularly true of CD8+ cells. Cell sorting was carried out by selecting CD3+ $CFSE_{lo}$ cells and whether the cloned cells were CD4+ or CD8+ was not established due to very low numbers of clones. This could also be a technical problem with the method as CD4+ T cells require a longer incubation with target cells in a ^{51}Cr assay. It could also be a result of a very disturbed immune environment from which the cells were taken, which can vary immensely between patients. Therefore, the original cells used to

generate the clones could have been functionally deficient in some ways. It cannot be ruled out that the effects of CFSE itself could have led to impaired functionality of the clones. It has been shown that the dye is toxic to cells at higher concentrations (Last'ovicka et al., 2009), and despite titration experiments carried out to minimise this risk, it is impossible to rule out the possibility that certain functions were impaired due to the presence of CFSE.

These factors may also contribute to very low frequencies of alloresponsive cells, which may be present at levels too low to be reliably detected by this method. The fact that the mouse studies had far greater responses is not surprising as in some cases the donor mice were vaccinated to have alloreactive T cells, which was necessary to demonstrate the principle. Perhaps if human donors were vaccinated against tumour, then patients would have more easily detectable frequencies of alloreactive, tumour-specific T cells. However there are enormous ethical problems associated with this concept. Another major factor is that the mice used have a 'clean' immunological background, so the system doesn't take into account the decades of immunological challenges that shape a human immune system.

There are many clinical variables that cannot easily be accounted for in stem cell transplant patients. The type of conditioning has a profound effect on reconstitution, which could lead to patients having cells with impaired proliferative potential. For example, campath (anti-CD52 monoclonal antibody), which is routinely used in non-myeloablative transplants has been shown to delay T cell reconstitution (Morris et al., 2003). Both patients from whom clones were generated in this study had myeloablative transplant conditioning with TBI and cyclophosphomide. Prophylactic cyclosporine (CsA) therapy was administered to all patients in this study, but the length of time for this treatment was assessed clinically on a case by case basis. In routine protocols for prophylactic immunosuppressive therapy, cyclosporine is given from the time of infusion and the patient is monitored. When it is deemed

appropriate, the dose of CSA is reduced over time until complete withdrawal. If there are signs of GvHD during this 'tapering' period, then the dose may be increased again. However, immunosuppressive treatment could have impaired the ability of the T cells to proliferate in assays. Similarly, if patients were being specifically treated for GvHD at the time of sample collection, then it is possible they would have circulating steroids in the blood. As these assays were carried out on freshly collected samples, it was not always known if the patient was receiving an immunosuppressive therapy at the time of setting up the assay. Where patients were receiving highly suppressive drugs, such as intravenous prednisolone, samples were not used for proliferation assays.

Whilst it was encouraging to have isolated a clone that could recognise and kill tumour cells, the low number of patients in whom alloreactive T cells could be detected was disappointing. Another major drawback to this study was that in order to screen patients, and then expand and test any clones, large amounts of pre-transplant and donor cells are required. Detection could possibly be enhanced by repeated stimulation with allogeneic cells as in some tumour antigen studies. For example, in a recent WT1 vaccine study, T cells were restimulated three times with peptide (Krug et al., 2010). This would require larger quantities of pre-transplant material and donor cells. The ideal source of allo-antigen would be tumour, but this needs to be collected at diagnosis, and not all patients would go on to receive a stem cell transplant (SCT). Those that do may well be managed for some years before transplantation. It was decided that under those circumstances, this aspect of the study should be discontinued and work should be refocused on studying a more specific approach to detecting anti-leukaemic T cells.

Chapter 4: Detection of Cancer/Testis Antigen-Specific T cells following Stem Cell Transplantation

4.1 Introduction

The successful outcome of allogeneic SCT as treatment for haematological malignancies is enhanced by GvL immune response (Collins et al., 1997, Horowitz et al., 1990, Kolb et al., 1995, Porter et al., 1999). Understanding the cellular targets of this response may facilitate development of therapeutic approaches to increase the efficacy of this treatment. A major complication for allograft patients is GvHD which is a major cause of morbidity and mortality (Billingham, 1959, Lee et al., 2002). Characterisation of GvL may allow development of therapies to boost immunity against leukaemic cells without affecting the patient tissues frequently damaged by GvHD. Although GvL effects have been demonstrated in allograft patients, including in those treated for myeloma and AML (Bensinger et al., 1996), identification of specific targets is important to develop effective T cell mediated immunotherapy.

Studies have demonstrated target potential of a number of antigens overexpressed by tumours such as Wilms Tumour 1 (WT1) and PRAME, although expression is not tumour exclusive which could lead to potential side-effects (Ikeda et al., 1997, Inoue et al., 1994, Kapp et al., 2009, Menssen et al., 1995, Miwa et al., 1992, van Baren et al., 1998). There is also interest in other potential GvL targets, for example minor histocompatibility antigens (mHAgs) such as HA-1 which has been shown to be expressed by AML (Faber et al., 1995, Mutis et al., 1999).

One group of tumour antigens that has potential for anti-tumour immunotherapy is the cancer/testis antigen (CTAg) genes. These genes are only expressed in immune privileged germline cells (and retina in the case of RAGE-1) in healthy individuals, but are frequently expressed by tumours (Boon and van der Bruggen, 1996, Gaugler et al., 1996, Old, 2007, Simpson et al., 2005, Stevenson et al., 2007). Such highly restricted tissue distribution marks

them as potentially suitable vaccine targets for GvL with reduced risk of GvHD. There have been numerous studies demonstrating an effective anti-tumour T cell response directed against these antigens (Dhodapkar et al., 2003, Jungbluth et al., 2005, Lim et al., 2001, Meklat et al., 2007, Pellat-Deceunynck et al., 2000, Taylor et al., 2005, van Baren et al., 1999, van Rhee et al., 2005) and our laboratory has previously demonstrated CTAg-specific T cell responses in myeloma and MGUS patients (Goodyear et al., 2005, Goodyear et al., 2008). It has also been shown that RNA expression of some of these CTAg gene families such as MAGE, BAGE, RAGE-1, LAGE, NY-ESO-1 can be detected in AML patients (Guinn et al., 2005). This opens the possibility of enhancing anti-tumour immunity post-allograft to reduce the risk of relapse for these patients. Vaccine trials in metastatic melanoma have demonstrated tumour regression in up to 20% of patients, without toxic effects (Baumgaertner et al., 2006, Coulie and van der Bruggen, 2003, Jager et al., 2006, Lonchay et al., 2004). Given the gene expression of CTAg genes in AML, it was important to address the question of whether an effective T cell response could be induced to the proteins. To date, no CTAg-specific T cell responses have been reported post-transplantation in AML patients, with only one report in a patient with relapsed myeloma (Atanackovic et al., 2007).

In this study 45 patients (table 4.1.1) were screened immediately prior to, and at various time points post-transplantation, for T cell responses to a panel of 25 peptides derived from 10 CTAg families (table 4.1.2) using the IFN-γ cytokine secretion assay in order to determine whether CTAgs could be potential GvL targets.

Patient Characteristics	
Patient age (years): median (range)	48 (22-64)
Gender, number (%)	
Male	22 (49)
Female	23 (51)
Underlying disease, number (%)	
AML	38 (84.5)
MM	7 (15.5)
Transplant type, number (%)	
Myeloablative	
Sibling	14 (31.1)
Unrelated donor	8 (17.7)
Non-myeloablative	
Sibling	10 (22.2)
Unrelated donor	13 (28.8)
HLA-types of patients, number (%)	
A1	20 (44.4)
A2	26 (57.7)
A3	11 (24.4)
A24	4 (8.8)
B7	16 (35.5)
B18	2 (4.4)
B44	17 (37.7)
Cw7	30 (66.6)
Cw16	4 (8.8)
Diseased state, number (%)	
Relapsed	8 (17.7)
Non-relapsed	37 (82.3)

Table 4.1.1 Characteristics of patients screened for CTAg responses

The table shows details of the ages, sex, transplant conditioning, disease status, donor, HLA-type and relapsed disease in the cohort studied for responses to CTAgs.

GENE	POSITION	PEPTIDE	HLA-Restriction	Population frequency (%)	Reference
BAGE					
	8-10	AARAVFLAL	Cw16	7	(Boel et al., 1995)
LAGE-1					
	Alt-ORF	MLMAQEALAFL	A2	44	(Aarnoudse et al., 1999)
MAGE-A1					
	161-169	EADPTGHSY	A1	26	(Traversari et al., 1992)
	96-104	SLFRAVITK	A3	22	(Chaux et al., 1999a)
	135-143	NYKHCFPEI	A24	19	(Fujie et al., 1999)
	289-298	RVRFFFPSL	B7	17	(Luiten and van der Bruggen, 2000)
	114-127	LLKYRAREPVTKAE	DR13	19	(Chaux et al., 1999b)
MAGE-A2					
	157-166	YLQLVFGIEV	A2	44	(Kawashima et al., 1998)
	156-164	EYLQLVFGI	A24	20	(Tahara et al., 1999)
	212-220	EGDCAPEEK	Cw7	41	(Breckpot et al., 2004)
MAGE-A3					
	168-176	EVDPIGHLY	A1	26	(Gaugler et al., 1994)
	112-120	KVAELVHFL	A2	44	(Kawashima et al., 1998)
	97-105	TFPDLESEF	A24	19	(Oiso et al., 1999)
	195 - 203	IMPKAGLLI	A24	19	(Consogno et al., 2003)
	167-176	MEVDPIGHLY	B18	6	(Bilsborough et al., 2002)
			B44	21	
	243-258	KKLLTQHFVQENYLEY	DP4	75	(Schultz et al., 2000)
			DQ6	63	
	149-160	VIFSKASSSLQL	DR4	24	(Kobayashi et al., 2001)
			DR7	25	
	191-205	GDNQIMPKAGLLIIV	DR11	25	(Consogno et al., 2003)
	281-295	TSYVKVLHHMVKISG	DR11	25	(Manici et al., 1999)
	114-127	LLKYRAREPVTKAE	DR13	19	(Chaux et al., 1999b)
MAGE-A4					
	230-239	GVYDGREHTV	A2	44	(Duffour et al., 1999)
MAGE-C2					
	191-200	LLFGLALIEV	A2	44	(Ma et al., 2004)
	336-344	ALKDVEERV	A2	44	(Ma et al., 2004)
NY-ESO-1					
	157-165	SLLMWITQC	A2	44	(Jager et al., 1998)
RAGE-1					
	352-360	LKLSGVVRL	A2	44	(Oehlrich et al., 2005)
	32-40	PLPPARNGGL	A2	44	(Oehlrich et al., 2005)
	11-20	SPSSNRIRNT	B7	17	(Gaugler et al., 1996)
SAGE					
	715-723	LYATVIHDI	A24	20	(Miyahara et al., 2005)

Table 4.1.2 CTAg peptides used in study

Peptide sequences are shown along with the protein names they are derived from and their location within that protein. The columns on the right of the table show the HLA restrictions and the prevalence of those HLA types in the Caucasian population.

4.2 Optimisation of T cell assays

Many assays used for identifying T cell responses rely on detection of an upregulated surface marker or measurement of soluble factors, for example, cytokine production. T cells upregulate CD137 upon activation and therefore antibodies to this marker can be used to detect cells responding to a stimulus by flow cytometry (Wolfl et al., 2007). Similarly, CD154 can be used to detect recently activated CD4+ cells (Chattopadhyay et al., 2006).

Traditional standard assays such as ELISA or ELISPOT, rely on detection of cytokines such as interferon-γ (IFN-γ), secreted in response to a specific stimulus (see chapter 2). The disadvantages of these techniques include being unable to further utilise the cells afterwards and the inability to study surface markers in order to characterise the phenotype of the responding cells. The latter problem can be overcome by use of intracellular cytokine staining which allows surface marker detection by antibodies (see chapters 2 and 5). However, due to the 'fix and permeabalisation' steps, the cells are killed during the assay which makes it impractical for further culture and functional studies.

An assay that uses a surface bound antibody to 'capture' secreted cytokine, therefore maintaining the integrity and viability of the cell, was developed by J Campbell (Campbell, 2003). A second, fluorochrome-conjugated antibody is used to detect the cell by flow cytometry and magnetic beads can be used for enrichment. The detailed method was outlined in Chapter 2 and a schematic diagram is shown in figure 4.2.1. An example of the flow cytometric readout is shown in figure 4.2.2. This assay is known as the IFN-γ Cytokine Secretion Assay (or IFN-γ Capture).

The detection sensitivity can be enhanced to 0.0001% on the basis of the number of cells in the post-selection fraction, although this tends to underestimate the true frequency due to cell loss during selection. For example, if $6x10^6$ PBMCs were stimulated with CTAg peptide

and 17.9% were CD8+, then from these 1 075 800 CD8+ T cells, if 14 were PE-labelled in the positively-selected fraction it would indicate that by this method at least 0.013% ((14/1 075 800)x100) of the CD8+ T cell pool was CTAg-specific.

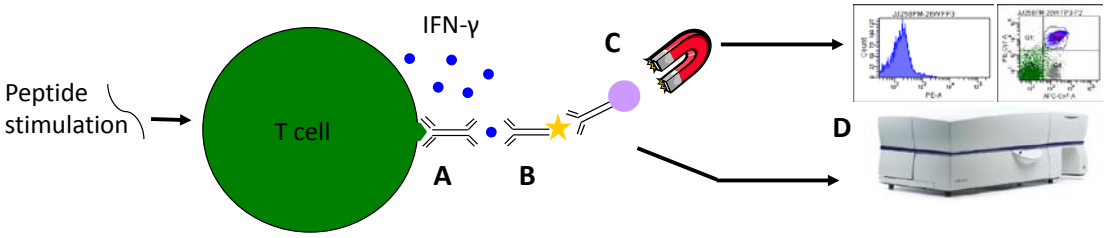


Figure 4.2.1 The IFN-γ cytokine secretion assay (IFN-γ Capture)

Following peptide stimulation the T cell produces IFN-γ. A bi-specific antibody is bound to CD45 on the T cell surface. The other specificity is IFN-γ which 'captures' the secreted cytokine at the cell surface (A). A second IFN-γ specific, fluorochrome conjugated antibody also binds the captured cytokine (B). The cells can then be enriched magnetically by the use of fluorochrome specific magnetic beads (C) and the number of IFN-γ secreting cells can be enumerated using flow cytometry (D).

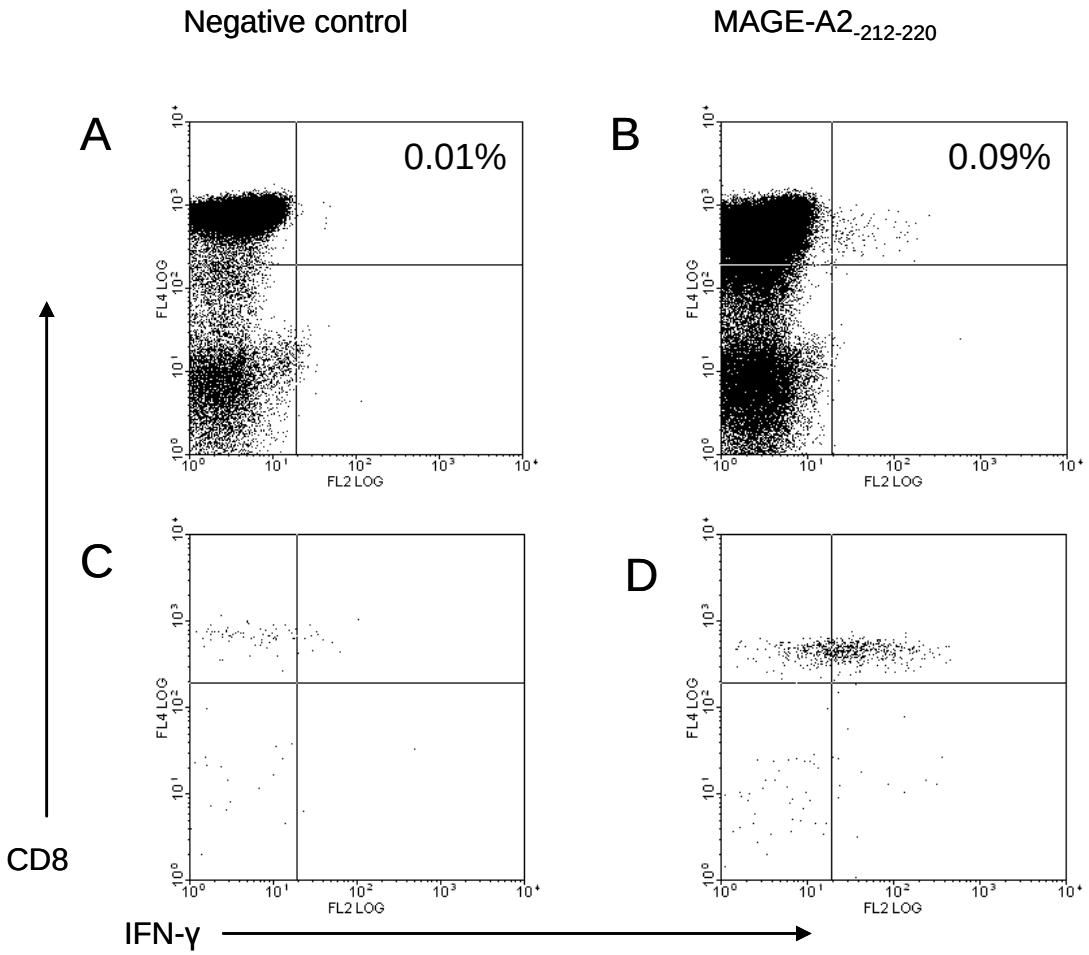


Figure 4.2.2 Example flow cytometric dot plots from IFN-γ capture assay

Background IFN-γ production (x-axis) by CD8+ T cells (y-axis) was established by the negative control where an equivalent volume of DMSO was added instead of peptide (A and C). This was subtracted from any response following peptide stimulation (B and D). In many cases, pre-enrichment frequencies were used to measure responses (A and B) but a magnetic enrichment step was included to increase the sensitivity of detection where the unsorted frequency was low, or in order to isolate CTAg-specific T cells for cloning purposes (C and D).

4.2.1 Detection of viral-specific T cell responses using IFN-γ cytokine secretion assay

The suitability of the IFN-γ capture assay was assessed by enumerating the frequency of T cells specific for known viral peptides in the blood of healthy lab donors. In order to test reproducibility, PBMCs were resuspended at a concentration of $1x10^7$ cells/ml in RPMI supplemented with 10% Human Serum and 0.5ml volumes were aliquoted into wells of a 48 well tissue culture plate. Three wells were stimulated using a viral peptide at a final concentration of 2μg/ml and an equivalent volume of DMSO was added to a negative control well. The assays were analysed on a Coulter XL Flow cytometer. Two healthy donors were screened with one peptide each, on two separate occasions, seven days apart. EBV peptides, CLG and GLC (table 4.2.1.1), were selected because colleagues had previously shown low frequency T cell responses to one of those peptides in these donors, which would be most comparable to the predicted low level tumour responses. Figure 4.2.1.1A shows the mean response frequencies after any background was subtracted. Representative plots of pre- and post-enrichment FACS plots are shown for one donor at one time point in figure 4.2.1.1B. The assay was extremely reproducible between wells. Differences between responses at different times in the same donor were intra-assay fluctuations which were expected.

Peptide	Antigen	HLA restriction	Virus	Reference
FPKTTNGCSQA	IE-$1_{221-231}$	B55	CMV	(Khan et al., 2007)
AGILARNLVPMVATV	$pp65_{489-507}$	DRB5	CMV	(Li Pira et al., 2005)
NLVPMVATV	$pp65_{495-503}$	A2	CMV	(Wills et al., 1996)
GLCTLVAML	BMLF-$1_{259-267}$	A2	EBV	(Steven et al., 1997)
CLGGLLTMV	$LMP2_{173-181}$	A2	EBV	(Rickinson and Moss, 1997)
NPKFENIAEGLRALL	EBNA-$1_{475-489}$	DR11	EBV	(Leen et al., 2001)
HRCQAIRKK	EBNA-$3B_{149-157}$	B27	EBV	(Lautscham et al., 2001)

Table 4.2.1.1 Viral peptides used for control and optimisation assays

Amino acid sequences of CMV and EBV peptides used for optimisation experiments to measure patient or healthy donor responses, the gene they were derived from and the position therein. These peptides were referred to by a code derived from the first three letters of the amino acid sequence (underlined). HLA restrictions are shown for each peptide and the virus they were derived from.

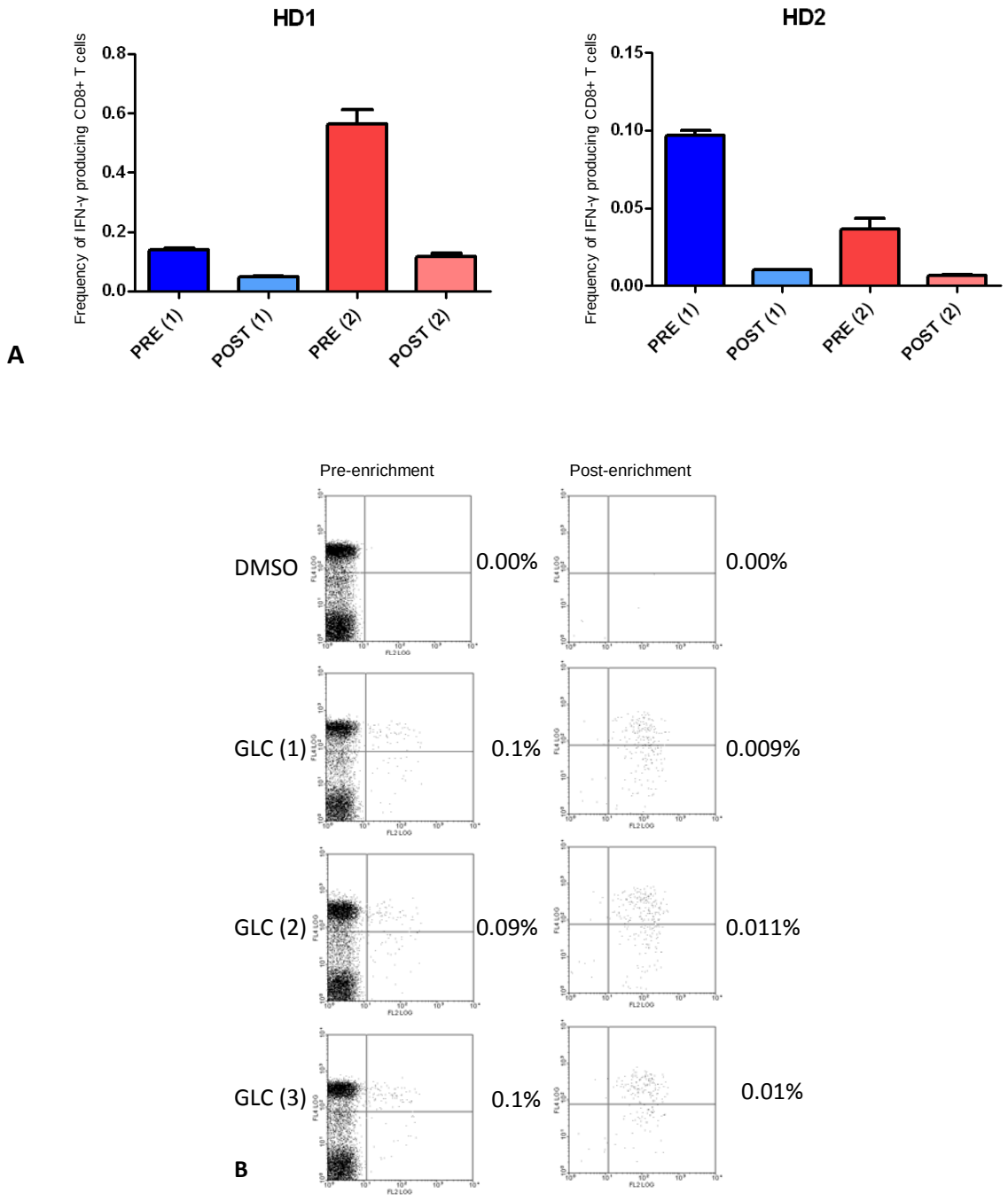


Figure 4.2.1.1 Viral responses can be detected reproducibly by IFN-γ capture

In order to assess the IFN-γ capture assay for reproducibility, healthy donors with known viral responses were screened in triplicate and repeated after seven days. The mean responses (with standard deviation), after any background was subtracted are shown for two donors (A). Week 1 responses are in blue and week 2 in red. Darker coloured bars represent pre-enrichment frequencies and the post-enrichment as lighter bars, and the

frequencies on the y-axis. Sample dot plots showing an assay in triplicate (B) show CD8+ T cells (y-axis) and the frequencies that secreted IFN-γ (a-axis) in response to peptide or DMSO. Pre-enrichment plots (left) and enriched plots (right) are shown. Frequencies shown are percentages of the whole lymphocyte population. Low frequency responses to those peptides, in the selected donors, had previously been detected using ELIspot assays by colleagues.

4.2.2 Optimisation of sample processing prior to screening

One limitation of using the IFN-γ CSA for screening patients for T cell responses is that it does not work well when using cryopreserved PBMCs. The number of samples that can be screened at any one time is limited by the labour intensive and time consuming nature of the assay. As most samples were obtained from a single, weekly clinic, it was sometimes difficult to screen all eligible patients. Therefore to establish whether delaying the assay would have any effect on the results, an experiment was set up to compare the results of an assay being carried out immediately on PBMC isolated at the time of blood collection with another carried out 24 hours after PBMC isolation. The effect of immediate isolation of lymphocytes or leaving the sample as whole blood for 24 hours was also assessed. A summary of the experimental design is shown in table 4.2.2.1 and the frequencies of CD4+ and CD8+ T cells responding to the viral peptides are shown in figure 4.2.2.1. EBV peptide NPK was used to stimulate CD4+ T cells and peptide HRC to stimulate CD8+ T cells. The frequency of responding CD4+ T cells in the non-enriched fraction increased from 0.02% to 0.04% in experiment B when the cells had been incubated without stimulus for an extra 24 hours, and a smaller increase to 0.03% was seen when the blood was left whole for 24 hours. These increases were exaggerated following enrichment. No increase in background was seen and these increased responses were not apparent with CD8+ T cells which showed a small decrease from 0.05% to 0.04%. No conclusions can be drawn from this experiment without further investigation as to the reason for the increased CD4+ effect, although it is interesting to note that there was no background increase in the unstimulated well. It may be that some suppressive factor is involved which decreases with time when in culture or lymphocyte isolation is delayed, or it could simply be due to variability between assays. The majority of the screening of this study was against MHC class I restricted peptides and this experiment did not show a major decrease in frequency, although some increase in

background CD8+ IFN-γ from 0% to 0.01% was seen. When the background is subtracted, the frequency of antigen-specific response decreases from 0.05% to 0.03%. The purpose of this experiment was to establish whether the assay could be delayed and what the preferred method of storage was for the cells. The results suggest that although a delay is not optimal, it would be possible to detect responses two days after the blood was taken although the threshold of detection could be compromised. The CD4+ T cell data indicated that the blood should be left whole and lymphocyte isolation should be performed immediately prior to the assay. This also opened up the possibility of obtaining samples from more distant locations, although very low frequency responses may not be detected.

A further experiment was carried out to investigate the effects of short-term culture on viral response detection by IFN-γ capture. The response to an CMV peptide FPK becomes undetectable after five days of culture at $37^{O}C$ in 5% CO_2 (figure 4.2.2.2). It is possible that without stimulation or adequate cytokines the T cells became anergic or died.

Day 1	Day 2	Day 3
Blood taken		
75% lymphoprep - assays (A) & (B) set up	Assay (A) run	Assay (B) run
25% left whole	25% lymphoprep (assay (C) set up)	Assay (C) run

Table 4.2.2.1 Design of experiment to determine the effect of delaying the assay on detection of antigen-specific T cells

On day 1 a blood sample was obtained from a healthy donor with a known EBV peptide response. The sample was split and PBMCs were isolated immediately from 75% of the sample (fraction 1). The remaining 25% was kept overnight at $4^{O}C$ prior to PBMC isolation (fraction 2). Isolated lymphocytes from fraction A were plated out at $5x10^{6}$ into wells of a 48w plate. Assay A was carried out on Day 2 using wells of PBMCs from fraction 1 and the remaining wells were kept at $37^{O}C$ 5% CO_2 for a further 24 hours before Assay B was carried out. On Day 2, PBMCs were isolated from fraction 2 of the blood sample and plated out in a similar fashion to fraction 1. The lymphocytes were incubated at $37^{O}C$ 5% CO_2 and then used for Assay C on Day 3.

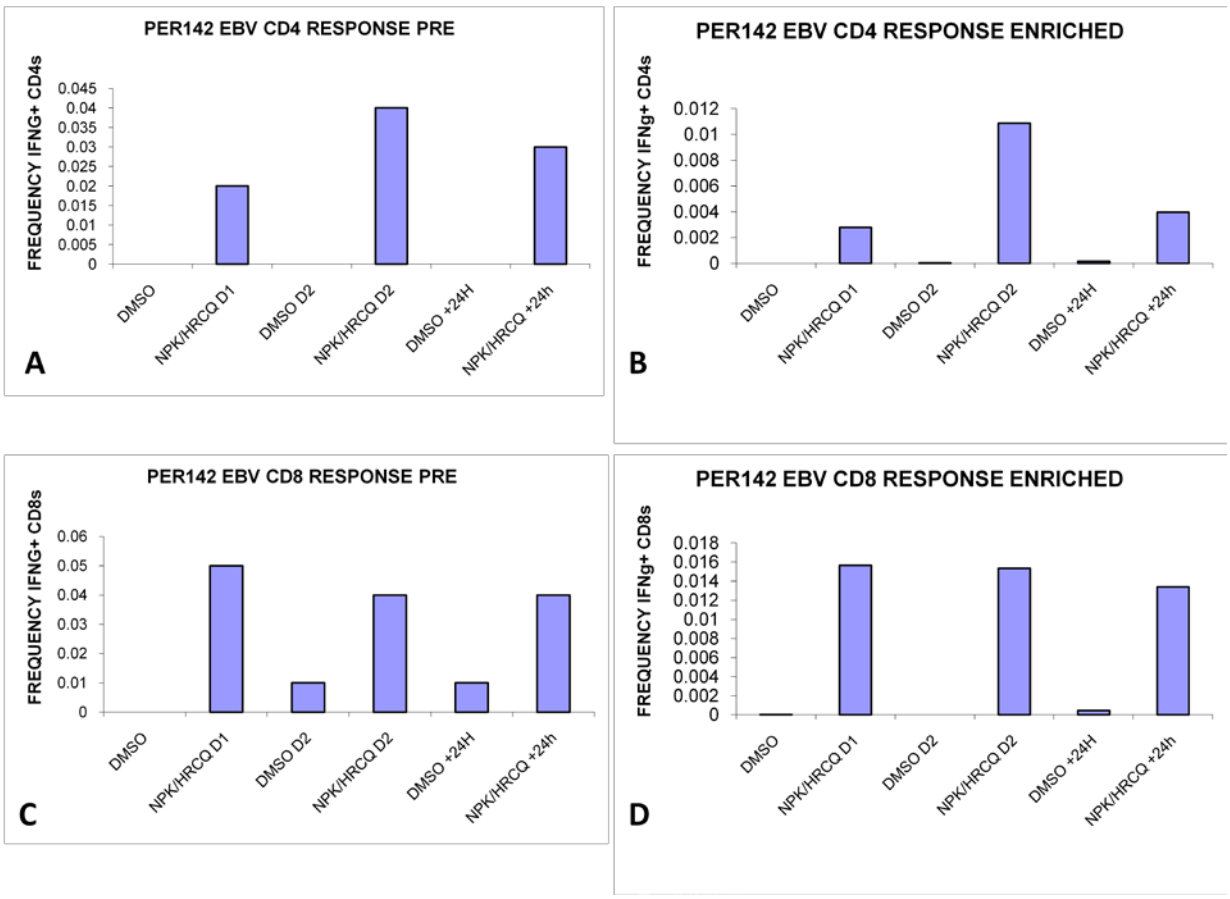


Figure 4.2.2.1 The effect of delaying the assay on detection of antigen-specific T cells

T cell responses to EBV peptides in a healthy donor, screened by IFN-γ capture carried out at different times following blood sample collection according to the schedule detailed in table 4.2.2.1. On the x-axis, D1 corresponds to Experiment A, D2 corresponds to Experiment B and +24h represents Experiment C. Frequencies of T cells producing IFN-γ in response to stimulation by peptide is shown. Pre- and post-enrichment frequencies of CD4+ T cells in response to NPK (A and B respectively) are shown and CD8+ T cells in response to HRC (C and D).

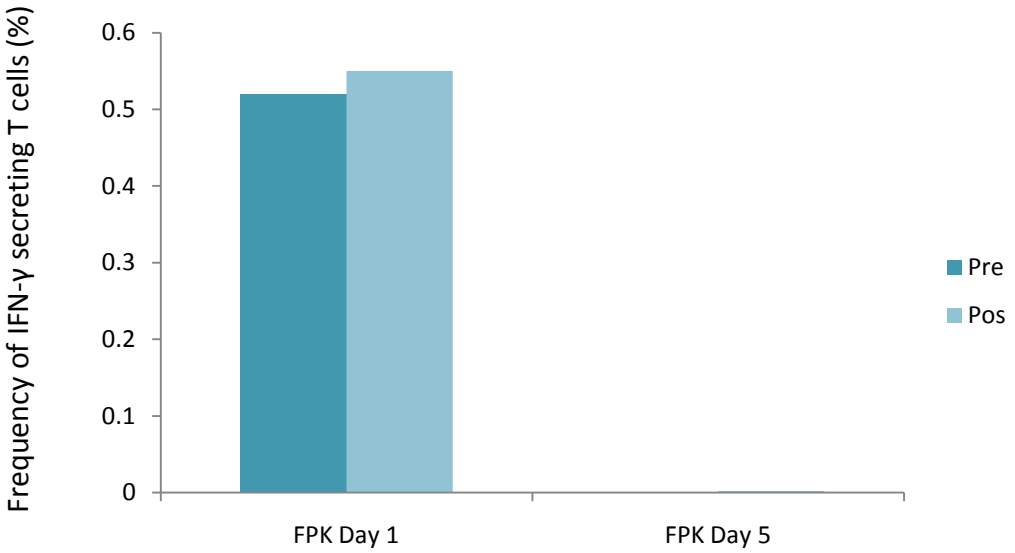


Figure 4.2.2.2 The effect of short-term culture of cells on the detection of viral-specific responses by IFN-γ capture

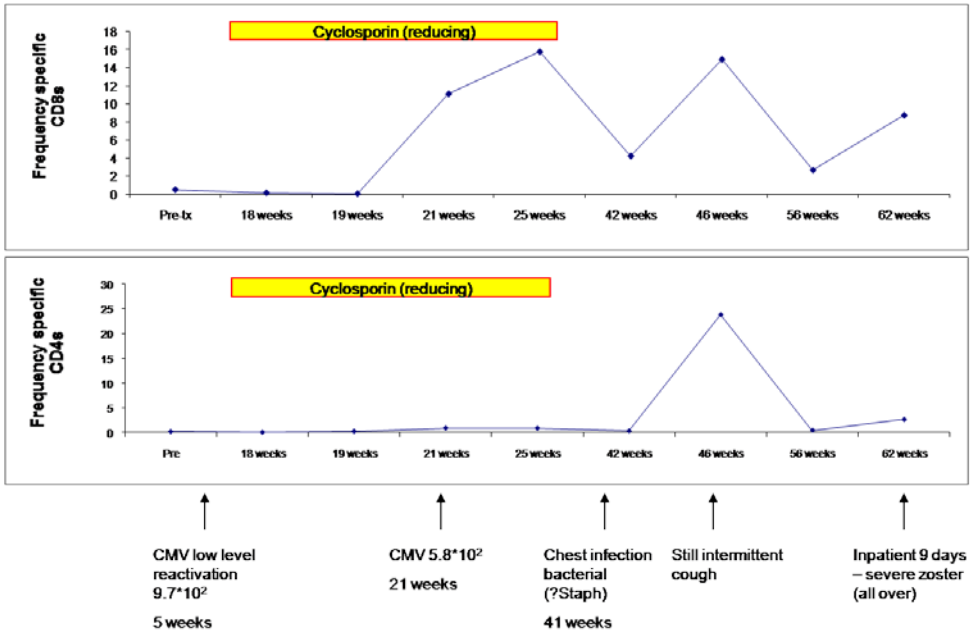
PBMCs were isolated from blood sample and the cells resuspended at $1x10^7$ per ml. The cells were divided into wells of a 48 well tissue culture plate with each well containing $5x10^6$ PBMCs, and incubated at 37^oC in 5% CO_2. An IFN-γ capture assay was carried out on half of the wells on day one and then the other half on day five in order to establish the frequency of peptide-specific T cells (y-axis) that could be detected using this assay, following overnight resting and after culture for 5 days. CMV peptide FPK was used to stimulate the cells to detect viral responses. Appropriate positive and negative controls were used, as described in Chapter 2.

4.3 Isolation, culture and testing of T cell clones derived from allograft patients

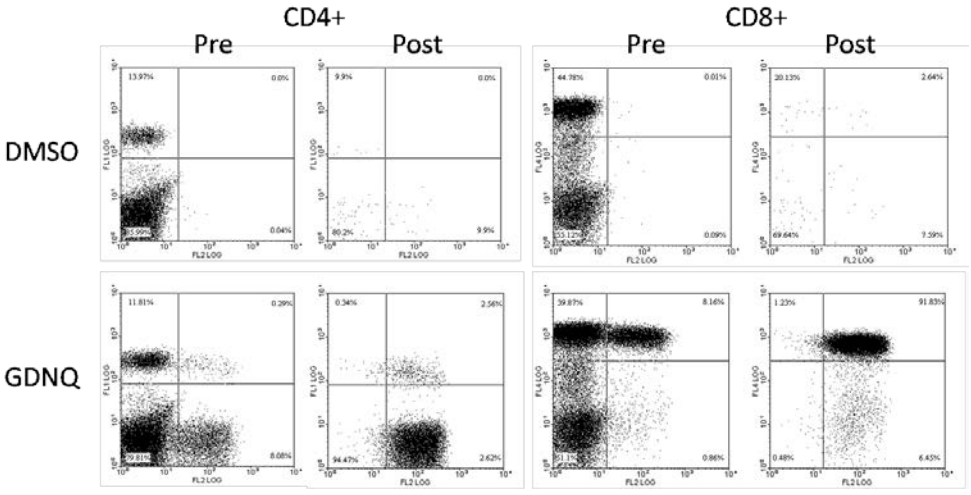
Strong CD4+ and CD8+ T cell responses were detected in AML allograft patient SS238PM when MAGE-A3 peptide GDNQ-batch 1 was included in the screening panel. Substantial numbers of IFN-γ producing T cells could be isolated using magnetic separation and T cell clones were derived using the protocol detailed in the methods chapter. Several batches of T cell clones were generated allowing extensive work to carried out on these cells. However, it was ultimately determined that these were not in fact CTAg responses, but probably the result of contamination of the GDNQ peptide by a viral peptide. Nevertheless, this work has been included to show the principle of the cloning technique being used to generate T cell clones from an allograft patient, and the process by which the probable contaminant was determined.

4.3.1 Frequency and phenotype of responses to peptide GDNQ-Batch1 in patient SS238PM

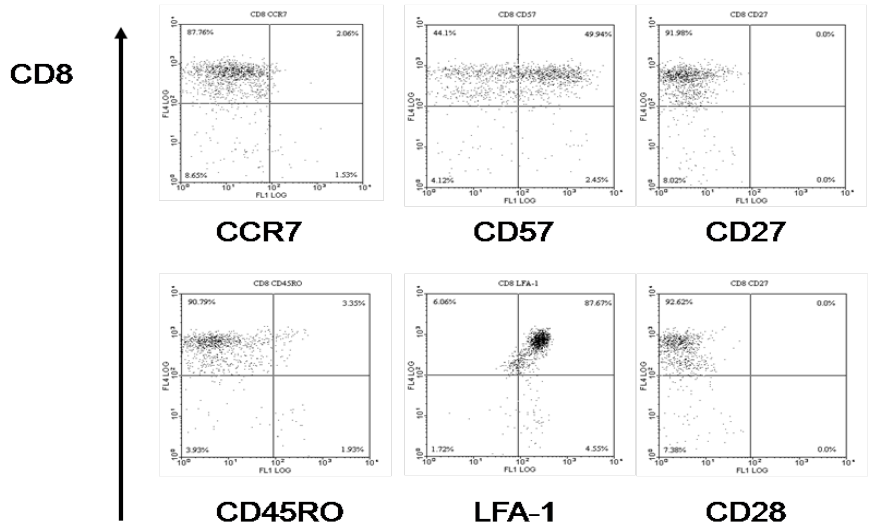
High-frequency CD4 and CD8 responses were detected in AML patient SS238PM on a number of occasions. Responses were detected prior to transplant and then at multiple times post transplant. These are shown, along with associated clinical complications, most notably two CMV reactivations, up to 62 weeks post-transplantation in figure 4.3.1.1a. At 25 weeks a very high frequency CD8+ T cell response was detected and flow cytometry dot plots are shown (figure 4.3.1.1b). Cloning was attempted with 50% of the enriched CD8+ fraction and the rest was split into tubes for phenotyping. Those IFN-γ secreting cells were mainly CCR7- CD27- CD28- CD45RO- LFA1+ and CD57+ which suggests that they were highly differentiated, not unlike CMV-specific CD8+ T cells (Appay et al., 2002).


Cyclosporin (reducing)
Frequency specific CD8s
18
16
14
12
10
8
6
4
2
0
Pre-tx
18 weeks
19 weeks
21 weeks
25 weeks
42 weeks
46 weeks
56 weeks
62 weeks
Cyclosporin (reducing)
Frequency specific CD4s
30
25
20
15
10
5
0
Pre
18 weeks
19 weeks
21 weeks
25 weeks
42 weeks
46 weeks
56 weeks
62 weeks
CMV low level reactivation 9.7*10²
5 weeks
CMV 5.8*10²
21 weeks
Chest infection bacterial (?Staph)
41 weeks
Still intermittent cough
Inpatient 9 days – severe zoster (all over)

A


CD4+
Pre
Post
CD8+
Pre
Post
DMSO
GDNQ

B


CD8
CCR7
CD57
CD27
CD45RO
LFA-1
CD28

C

Figure 4.3.1.1 Time course and phenotype of T cell responses to GDNQ – Batch 1 from patient SS238PM

GDNQ-specific T cell frequencies were monitored over time in samples from AML patient, SS238PM (A). Time points from pre-transplant to 62 weeks post-transplant are shown, with CD8+ responses in the upper graph and CD4+ in the lower. Clinical events are shown beneath the x-axis. Screening was carried out using IFN-γ capture assay. Very high responses were detected at some time points and an example of staining is shown of one such response, seen at 25 weeks (B). Due to the high numbers of cells in the post sort-fraction, 50% was used for cloning by limiting dilution, whilst the remaining cells were further characterised by antibody staining for CD8 (y-axis), CCR7, CD45RO, CD57, LFA-1, CD27 and CD28 expression (x-axis) (C).

4.3.2 Culture, expansion and chimerism of T cell clones

IFN-γ producing T cells from the positively selected fraction were cloned by limiting dilution as per the protocol outlined in Chapter 2 (section 2.8) and where cell numbers permitted, plated at concentrations of 100, 30, 10, 3, and either 1 or 0.3 cells per well. Wells that showed a clearly enlarged pellet after 21-28 days in culture at 37^{o}C 5% CO_2, were transferred to a single 48 well plate. Irradiated PHA-stimulated PBMCs and LCLs were used with PHA, to boost proliferation and half of the media in the wells was replaced twice per week, supplemented with either IL-2 only (20 IU/ml) or along with IL-7 (5ng/ml), IL-15 (2ng/ml), IL-21 (2ng/ml). As the clones became confluent, they were split between further wells of a 48w or 24w plate. Clonal specificity was measured and is detailed in section 4.3.3.

It has been reported that functional mHAg-specific T cells can persist in the recipient despite rigorous transplant conditioning (Shatry et al., 2007). It was interesting to note that this patient had detectable responses to peptides GVYD and GDNQ both prior to transplant conditioning and at 18 weeks following transplantation (figure 4.3.2.1), which raised the question as to whether cells specific for either of these peptides had persisted through conditioning with proliferative capacity to give rise to detectable responses. CD4+ frequencies were 0.058% pre-conditioning and 0.011% at 18 weeks, and CD8+ frequencies were 0.062% and 0.07% respectively. Two clones (IB8SS & IG9SS) isolated from patient SS238PM post-transplantation were expanded using a rapid expansion protocol (see Chapter 2, section 2.9) and sent to the West Midlands Clinical Regional Genetics Laboratory (WMCRGL) to determine whether they were of patient or donor origin. DNA microsatellite analysis was carried out to compare DNA extracted from the T cell clones with stocks of DNA taken from the patient prior to transplantation and the stem cell donor that were held by the WMCRGL. In brief, these clinical standard tests were comparisons of 16 highly-

polymorphic alleles. A section of results are shown in figure 4.3.2.2. Position, height and area of the peaks are compared between samples. One such set of peaks has been highlighted in the figure, demonstrating a difference in position between the patient's pre-transplant DNA and that of the donor's DNA. The origin of the clones can be determined by matching the peaks from clonal DNA to the patient or donor DNA. The highlighted peaks show that both clones match the donor DNA rather than the patient. This pattern was repeated for other polymorphisms and the laboratory report confirms that these clones were derived from donor haematopoietic stem cells, so therefore likely to have been generated post-transplantation. It is unlikely that the healthy donor would have a CTAg-specific T cell response, although this needed to be confirmed by obtaining a sample from the donor and screening for such responses. Therefore, these results suggest that the response was generated post-transplant.

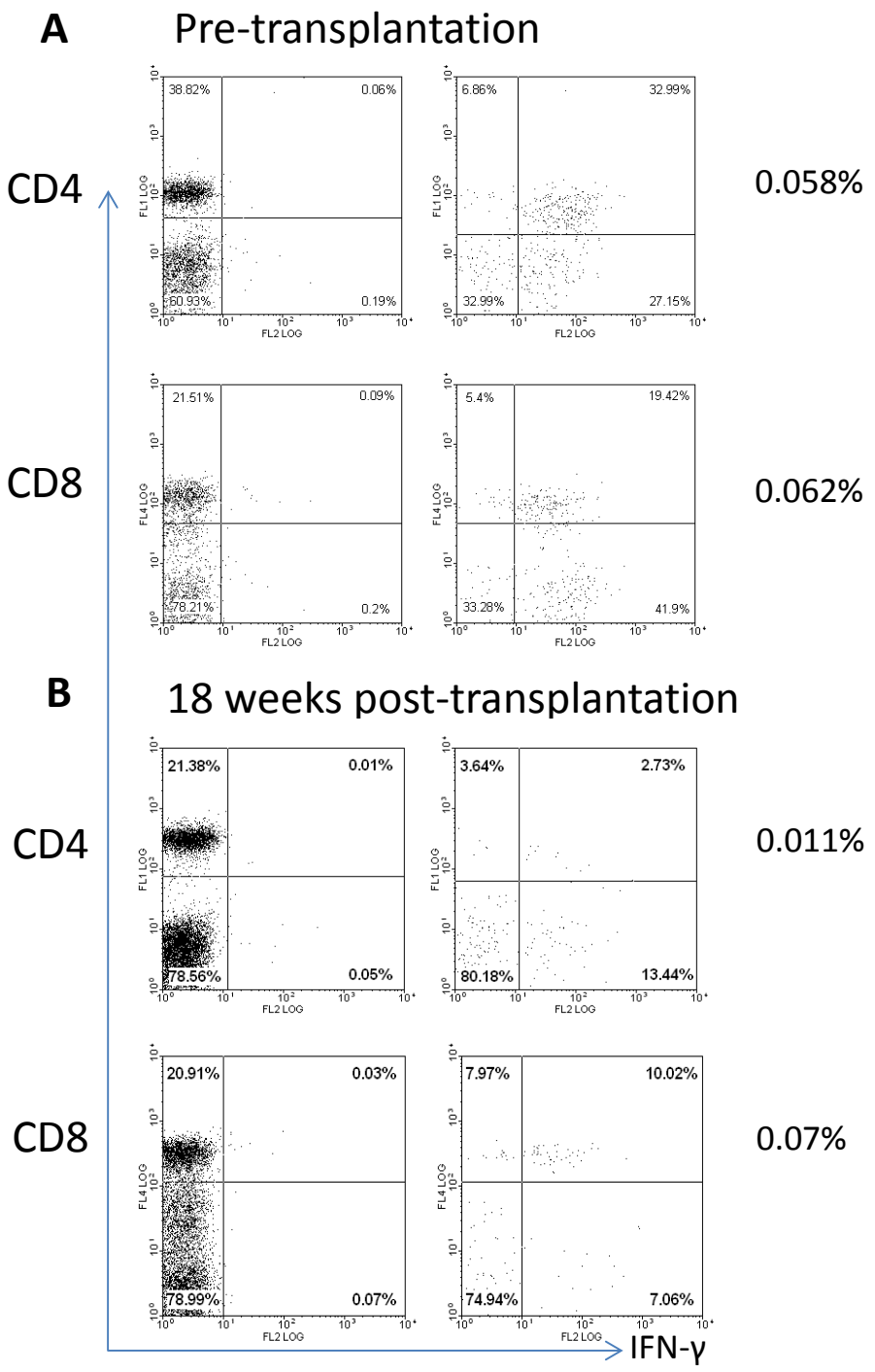


Figure 4.3.2.1 Responses to GVYD/GDNQ pools pre- and post-transplantation

CD4+ and CD8+ T cell (x-axis) responses to pooled peptides GVYD and GDNQ were detected by IFN-γ capture (y-axis) in patient SS238PM, both prior to transplant conditioning (A) and then again at 18 weeks following transplant (B).

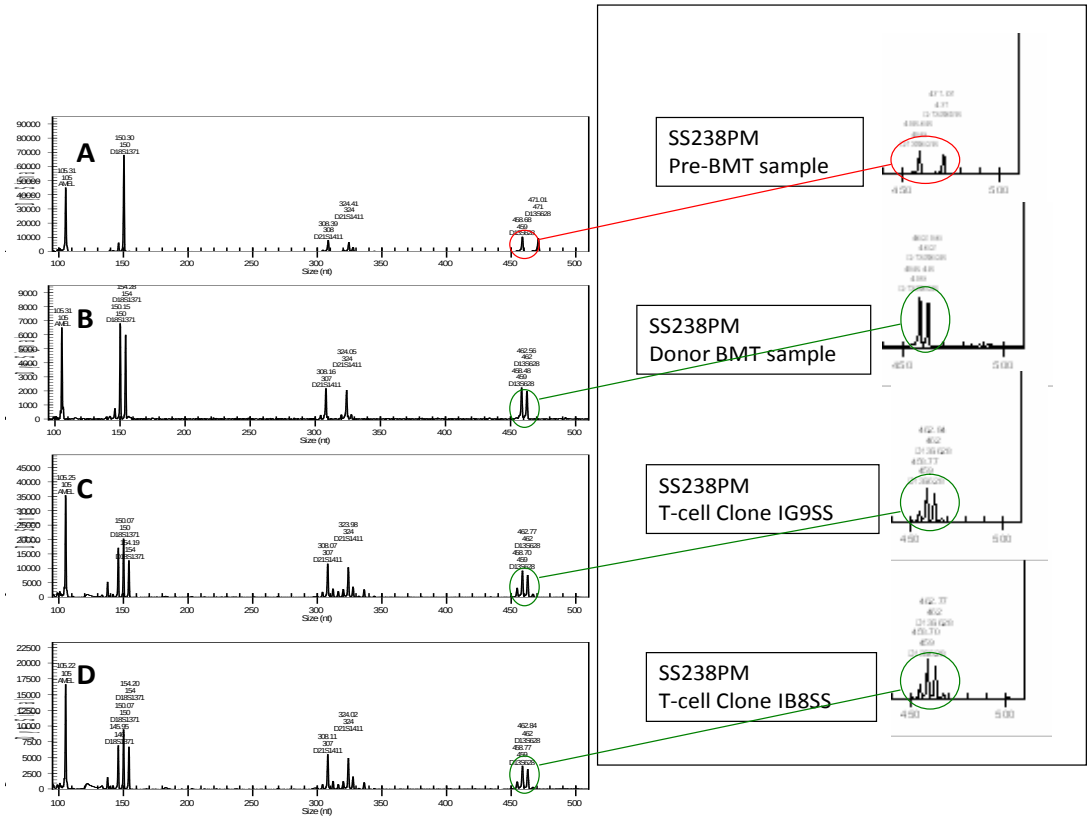


Figure 4.3.2.2 Chimerism analysis to determine origin of clones from SS238PM

GDNQ-Batch 1-specific T cell clones IB8SS & IG9SS derived from SS238PM were sent to West Midlands Clinical Regional Genetics Laboratory to establish whether they had originated from cells of patient or donor lineage. Microsatellite analysis was performed to compare 16 highly polymorphic alleles in DNA samples taken from the patient pre-transplantation (A), from the donor (B) and from two clones isolated from the patient post-transplantation specific for GDNQ peptide (C and D). Position, height and area of peaks were compared between samples. One set of peaks showing similarities between the clones and the donor DNA sample has been highlighted. The figure shows a representative set of peaks and data for all polymorphisms is shown in Appendix A.

4.3.3 Characterisation of specificity and functionality of T cell clones

CD4+ and CD8+ T cell clones were generated from the positively selected fraction following stimulation with GDNQ-batch 1 peptide, at various time points. It was necessary to establish specificity and whether the clones had ability to kill target cells. Initial peptide screening was carried out by IFN-γ ELISA, following the protocol outlined in Materials and Methods (section 2.6). In order to screen large numbers of clones, 50µl of resuspended clone was taken and split into 4 wells of a 96 well U bottomed plate. Two wells were stimulated with peptide and an equal quantity of DMSO was added to the remaining two wells. Figure 4.3.3.1A shows levels of IFN-γ production from samples of clones generated at 19 weeks post-transplantation. The functional ability of these clones to kill target cells was assessed using a chromium release assay (as described in Materials and Methods Chapter 2, section 2.7). Briefly, partially HLA-matched LCLs were loaded with either peptide or an equivalent volume of DMSO, and then labelled with ^{51}Cr. The labelled LCLs were then co-cultured with the T cell clones for 4 hours (or overnight) at 37^{o}C 5% CO_2 and supernatants were harvested to be read on a gamma counter along with those from spontaneous (minimum) and induced (maximum) lysis wells. The machine calculates a percentage of target cells lysed based on comparison with minimum and maximum values. A positive result was considered to be over a threshold of 20% when any background from the negative control (DMSO only) wells was subtracted. Examples of the levels of lysis of targets by the clones are shown in figure 4.3.3.1B. It can be clearly seen that all of the clones shown retained good killing ability, with levels around 50%, at seven weeks following the initial cloning, compared with no killing of the negative control cells, indicating that all lysis was peptide dependent.

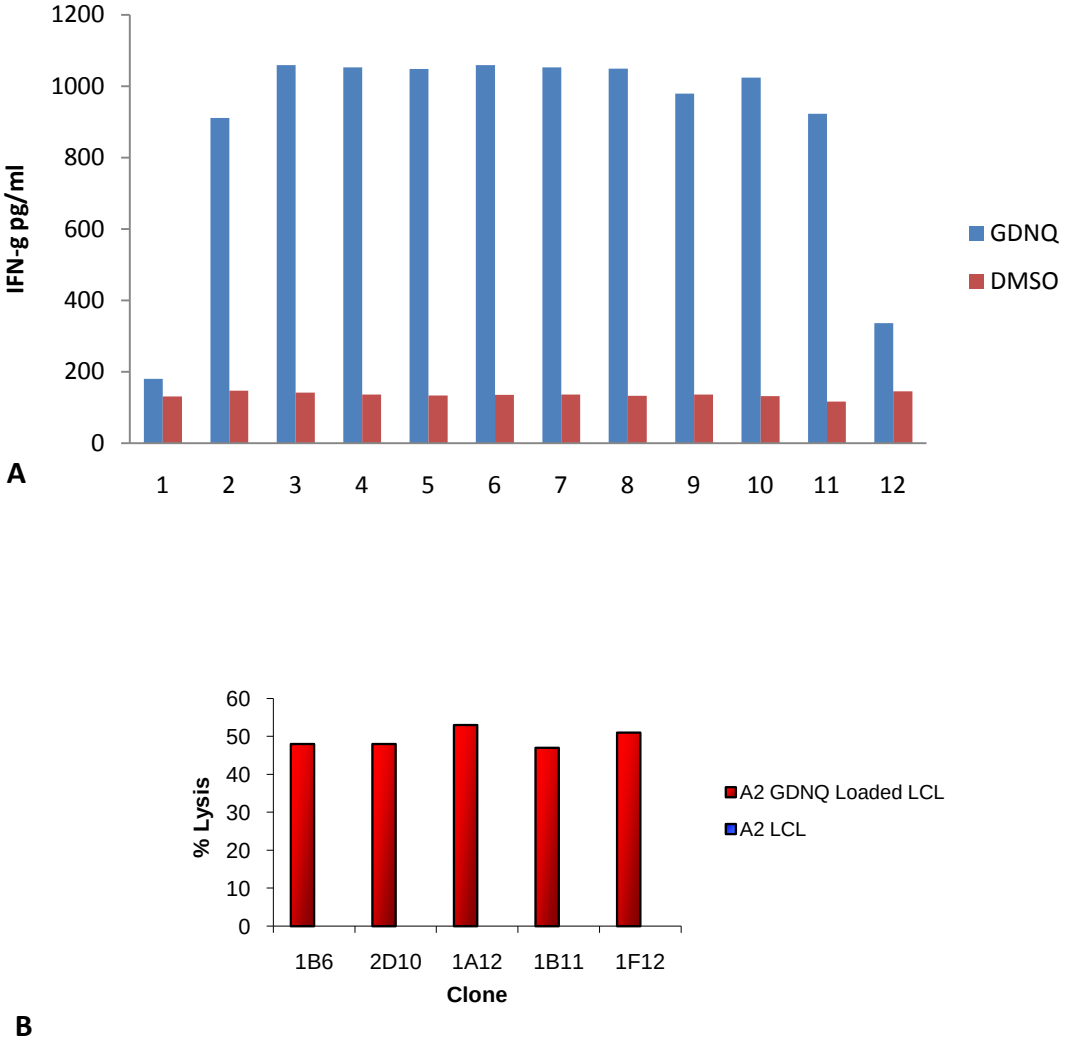


Figure 4.3.3.1 Screening for T cell clone peptide specificity and cytotoxicity

T cell clones were generated at 19 weeks post-transplantation by stimulation of PBMCs isolated from patient SS238PM with GDNQ-batch1 peptide. Specificities of proliferating clones were tested using IFN-γ ELISA (A). Negative control wells had an equivalent volume of DMSO added. Concentration of IFN-γ in pg/ml is shown on the y-axis. Clone 1 is clearly not specific for GDNQ-batch 1 and clone 12 is unlikely to be specific. All other clones are considered to be specific for the peptide and could be selected for further characterisation. Some of the selected clones were used in a chromium release assay to assess cytolytic function (B). Percentage of target cells lysed following co-culture with partially HLA-matched

LCLs which had either been loaded with GDNQ-batch 1 peptide (red bars) or had an equivalent incubation with DMSO as a negative control (blue bars) is shown. Levels of lysis over 20% were considered to be a result of peptide specific recognition and killing.

Although the ability of the clones to kill and recognise peptide had been established it was important to try and show that the clones could recognise endogenously processed antigen. The myeloma cell line, U266, has partial HLA-matching with the patient, and T cell clones from SS238PM can recognise peptide when presented by this cell line. As U266 has previously been shown to express MAGE-A3 (Pellat-Deceunynck et al., 2000), an IFN-γ ELISA was performed to compare U266 recognition by CD8+ T cell clones, with and without loading of GDNQ-batch 1 peptide. A negative control of mismatched LCL loaded with peptide was used to establish the levels of background spontaneous release. Figure 4.3.3.2 shows that the clones strongly responded to peptide loaded U266, and no IFN-γ was produced in response to the mismatched LCL. Low concentrations of IFN-γ were detected in the supernatant of the clones that had been co-cultured with U266 alone. Whilst it would not be surprising if the response to naturally occurring antigen is lower than to a peptide loaded target, the low level of cytokine produced against U266 alone make it difficult to say with any degree of certainty, that this was a result of the T cells recognising and responding to MAGE-A3 expressed by the cell line.

In order to establish the functional avidity of the peptides, a peptide titration experiment was carried out to demonstrate the minimum concentration of peptide recognised by three CD4+ T cell clones (figure 4.3.3.3). Target LCLs were loaded with peptide at 10-fold decreasing concentrations and T cell clone recognition was measured by IFN-γ ELISA.

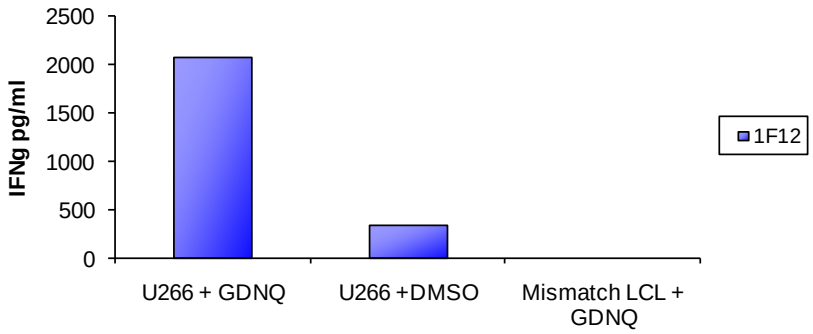


Figure 4.3.3.2 CD8+ T cell clone recognition of myeloma cell line U266 with or without peptide

CD8+ T cell clones isolated from patient SS238PM were co-cultured with the MAGE-A3 expressing myeloma cell line, U266 and response was measured by IFN-γ production. U266 targets were loaded with GDNQ-Batch1 peptide or incubated with an equivalent volume of DMSO in order to establish whether the clones were able to recognise naturally expressed MAGE-A3 or required surface peptide. Spontaneous IFN-γ production was tested by co-culture of clones with a HLA-mismatched LCL loaded with GDNQ-Batch1 peptide.

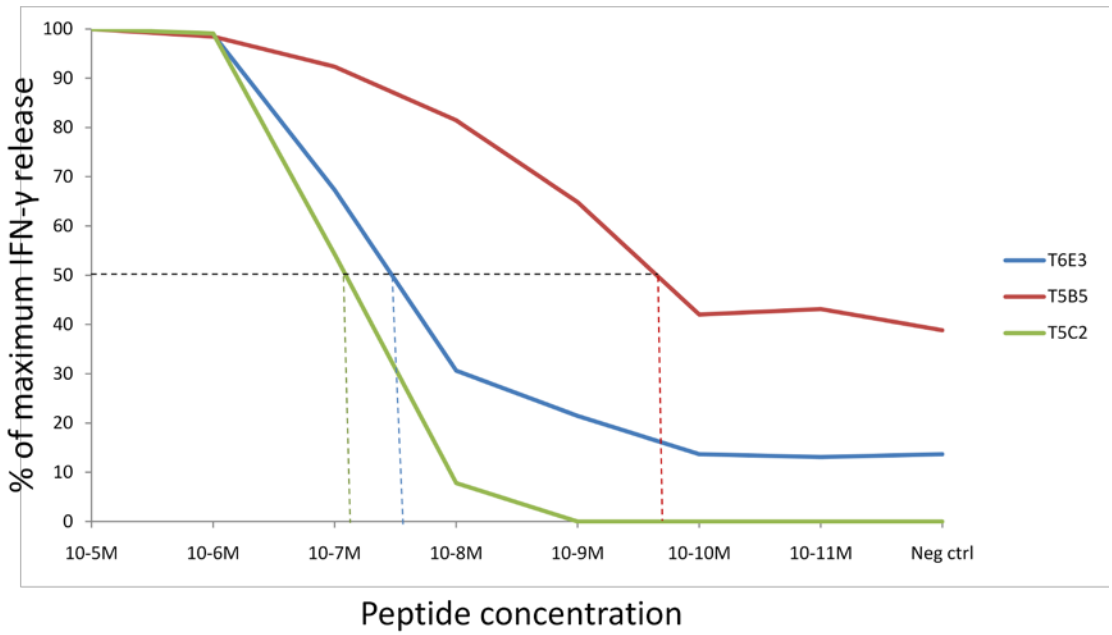


Figure 4.3.3.3 Titration of GDNQ-batch 1 peptide concentration in order to establish avidity of T cell clones

The recognition of decreasing concentrations of GDNQ-Batch 1 peptide by T cell clones with an IFN-γ ELISA readout is shown. Target cells for antigen presentation were partially HLA-matched LCLs loaded with 10-fold diminishing concentrations of peptide. The dotted lines denote the concentrations of peptide that sensitized for half-maximal IFN-γ release for each of the three clones tested and the values were between 10^{-7}M to 10^{-10}M.

4.3.4 T cell responses to overlapping 9mer peptides derived from GDNQ 15mer sequence

Another batch of clones was generated at 42 weeks post transplantation, again following stimulation by GDNQ-batch 1 peptide. Similarly to previous occasions, very strong CD8+ T cell response was generated and cells from the magnetically enriched fraction were expanded by established cloning techniques. As the peptide used was a 15 amino acid peptide which had previously been shown to elicit CD4+ T cell responses (Consogno et al., 2003), it was somewhat surprising to find that such strong CD8+ T cell responses were generated. It was necessary to establish which part of the sequence GDNQIMPKAGLLIIV was causing the CD8+ response. A literature search did not yield any previously described shorter peptides suitable for class I presentation from that sequence. A batch of overlapping 9mer peptides (named E1-7) was synthesised by Alta Bioscience covering the 15mer sequence. Reactivity of a previously characterised CD8+ T cell clone against these peptides was tested by ELISA and compared with GDNQ-batch 1. Stimulation with peptides E2 and E3 led to similar amounts of IFN-γ production as when GDNQ-batch 1 was the stimulus. A lower amount of IFN-γ was produced against E4 but was considerably higher than background, so this peptide was used alongside E2 and E3 in future experiments, but E1, E5, E6 and E7 did not elicit a response (figure 4.3.4.1).

Peptides E2, E3 and E4 were used along with the full length GDNQ-batch 1 peptide and a previously acquired 11mer, also from the GDNQ sequence, to stimulate SS238PM PBMCs *ex vivo* for an IFN-γ capture assay. The sequences and results are summarised in Table 4.3.4.1. As it would not be expected to observe a CD4+ response to the shorter peptides, it was not surprising that CD4+ frequencies are considerably lower. Indeed, the detection of IFN-γ producing CD4+ T cells in the absence of longer peptide may have been due to the large amount of IFN-γ secreted by the CD8+ T cells binding to antibodies on the CD4+ T cells. It

may also be due to other cytokines produced by the responding CD8+ cells inducing IFN-γ production by CD4+ cells, by way of bystander activation. However, the higher frequency of CD4+ T cells secreting IFN-γ in the presence of the full length peptide fits with expected results. One curious observation was that there was absolutely no T cell response to the 11mer peptide, which was surprising as it spanned all three shorter sequences which had generated large CD8+ responses. The suspected reason for this result is discussed later.

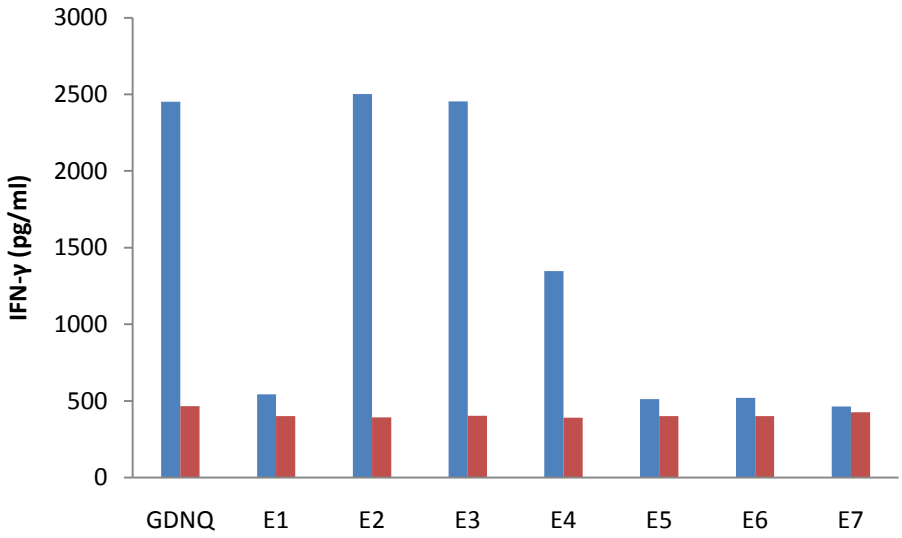


Figure 4.3.4.1 CD8+ T cell clone responses to overlapping 9mer peptides derived from GDNQ

In order to determine the amino acid sequence within the 15mer peptide that was recognised by the CD8+ T cells, a T cell clone which had previously been shown to recognise GDNQ-Batch1 was used to assess reactivity to overlapping 9mer peptides that span the sequence of GDNQ. Peptides were loaded onto T2 cells and specificity was measured by IFN-γ ELISA (γ-axis). Responses to peptide loaded T2s is shown by the blue bars and to unloaded T2 controls indicated by the red bars.

Peptide	Sequence	CD8+ response	CD4 response
Full	G D N Q I M P K A G L L I I V	7.35%	8.54%
11mer	D N Q I M P K A G L L	0%	0%
E2	D N Q I M P K A G	8.23%	1.33%
E3	N Q I M P K A G L	8.16%	1.55%
E4	Q I M P K A G L L	8.65%	0.88%

Table 4.3.4.1 *Ex vivo* T cell responses to overlapping peptides and GDNQ-batch 1

Shorter peptides were made from the sequence of GDNQ in order to establish which part of the sequence was responsible for eliciting CD8+ T cell responses. The amino acid sequences of four shorter peptides is shown along with both CD8+ and CD4+ pre-sort frequencies from *ex vivo* IFN-γ capture screen of patient SS238PM. Peptides E2, E3 and E4 had previously been shown to be the only 9mer peptides recognised by CD8+ T cell clones (figure 4.3.4.1).

4.3.5 Establishing HLA restriction of CD8+ T cell clones

Having established that shorter peptides contained within GDNQ could be responsible for eliciting CD8+ T cell responses, the next step was to further characterise the peptides. Firstly it was important to establish which HLA restriction these peptides had. Comparison of the HLA-typing of the patient and cell lines or LCLs that had previously been successfully used to present the GDNQ-batch 1 peptide showed that the only consistent Class I alleles were A2 and Cw7. CD8+ T cell clones were tested by ELISA, using myeloma cell line U266 (A2+ve, Cw7+ve) and two LCLs from other donors, AS222PM (A2+ve, Cw7-ve) and HD-NK (A2-ve, Cw7+ve). Figure 4.3.5.1 clearly shows that the peptides can only be recognised when presented through HLA-A2, and that in the A2-ve LCL, there is no recognition. It was concluded that CD8+ recognition of peptides within the sequence of GDNQ was HLA-A2 restricted. It was noticeable that higher concentrations of IFN-γ were produced in response to U266 presented peptides than when presented by AS222PM LCLs. This may have been due to the LCLs naturally having lower MHC expression, or the potential reactivity to naturally expressed MAGE-A3 on U266 could have led to a greater response.

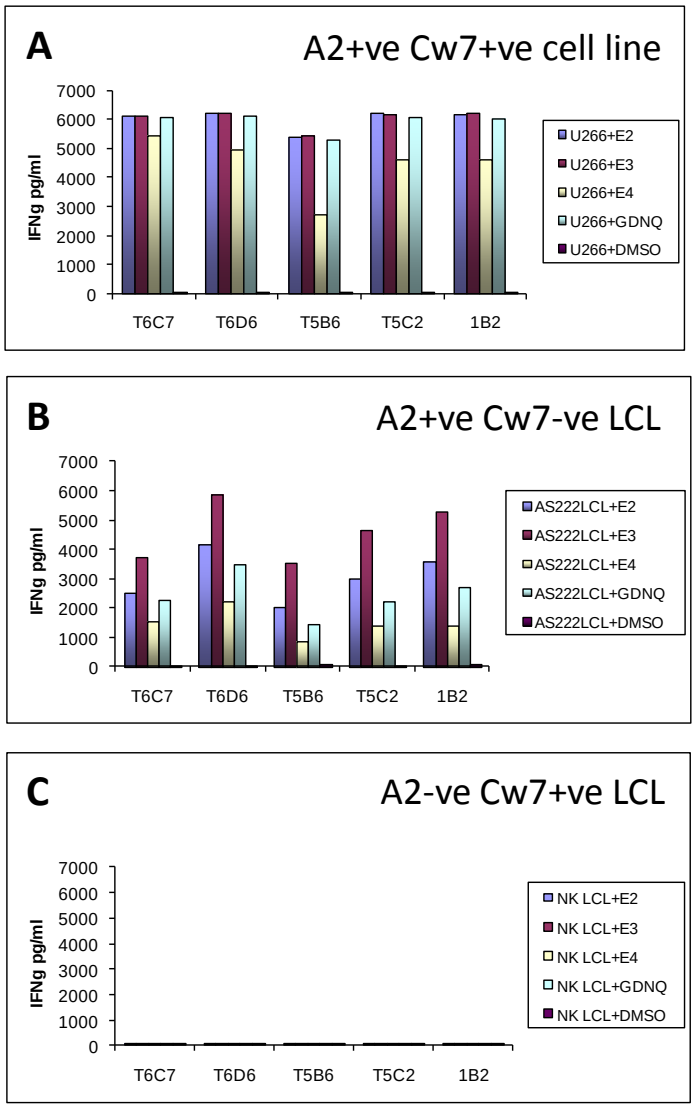


Figure 4.3.5.1 HLA-restriction of CD8+ responses to GDNQ derived peptides

IFN-γ responses to GDNQ and the shorter 9mer peptides derived from that sequence by clones from SS238PM were measured by ELISA, using target cells with differing HLA types in order to establish how the recognition was restricted. Responses to target cells which were double positive for both A2 and Cw7 are shown in A, along with by A2+ve and Cw7-ve LCLs (B) and A2-ve and Cw7+ve LCLs (C). No IFN-γ secretion was observed in the absence of HLA-A2.

4.3.6 Reactivity of clones to MAGE-A3 transfected cell lines

MAGE-A3 transfected LCLs DESA-M3+ and VACH-M3+ (along with empty vector controls - DESA-EBV and VACH-EBV) were kindly provided by Pierre van der Bruggen (Ludwig Institute for Cancer Research, Belgium). An in-house generated LCL, KS114PMd, was also transfected with MAGE-A3 by the van der Bruggen group. These cell lines were used as target cells to test the ability of the T cell clones to recognise endogenously processed antigen as a more physiologically relevant model than peptide loaded targets.

The reactivity of CD4+ clones against a range of transfected and non-transfected targets, with and without peptide, is shown in figure 4.3.6.1. The potential MHC Class II restriction required for GDNQ recognition was uncertain. Although it has been reported that the restriction is HLA-DR11 (see table 4.1.2), the patient is DR11-ve, therefore any recognition must have a different restriction. In this experiment, clones did not respond to DESA, regardless of whether the peptide was present. Only background levels of IFN-γ, close to or below 500 pg/ml, were produced by two clones and around 1500 pg/ml for clone T6E3, in response to all targets including the negative control. Although these were considerably higher than the negative controls for the VACH cell line, it may be due to alloreactivity, and unrelated to the antigen of interest. More intriguing results were seen when VACH was used as the target cell line. Higher levels of IFN-γ were produced to the transfected cell line, with or without peptide, and only to the non-transfected line where peptide was present. Therefore, this raised the possibility that the clones could in fact recognise GDNQ-batch 1 peptide and endogenously expressed MAGE-A3 protein.

CD8+ T cell clones were also screened by ELISA to ascertain whether they could recognise MAGE-A3 transfected cell lines. It was noteworthy that despite large responses, relatively few CD8+ clones grew out following GDNQ stimulation in the IFN-γ capture assay, and none

from E2, E3 or E4 9mer responses. As previously shown, these clones recognised U266 with GDNQ-batch 1 but here there was no recognition of this MAGE-A3 expressing cell line without peptide. There was no evidence of recognition of the HLA-A2+ve LCL KS114PMd with or without peptide, or whether it had been transfected with MAGE-A3 or not. Any IFN-γ produced in response to U266 without peptide was in a similar range, or below levels where the negative control LCL, KS228PM, was the target cell. The donor from whom this particular LCL was generated shares no common HLA type with the patient SS238PM. It was unclear why the clones produced extremely high levels of IFN-γ in response to U266 with GDNQ -batch 1 but not to the HLA matched LCL. It is possible that although the patient SS238PM, cell line U266 and donor KS114PMd are all HLA-A2+ve, the patient and donor may have a different subtype. These clones do not back up the earlier observation that CD8+ clones could recognise U266 without peptide (see figure 4.3.3.2). It may be that these clones have a different affinity.

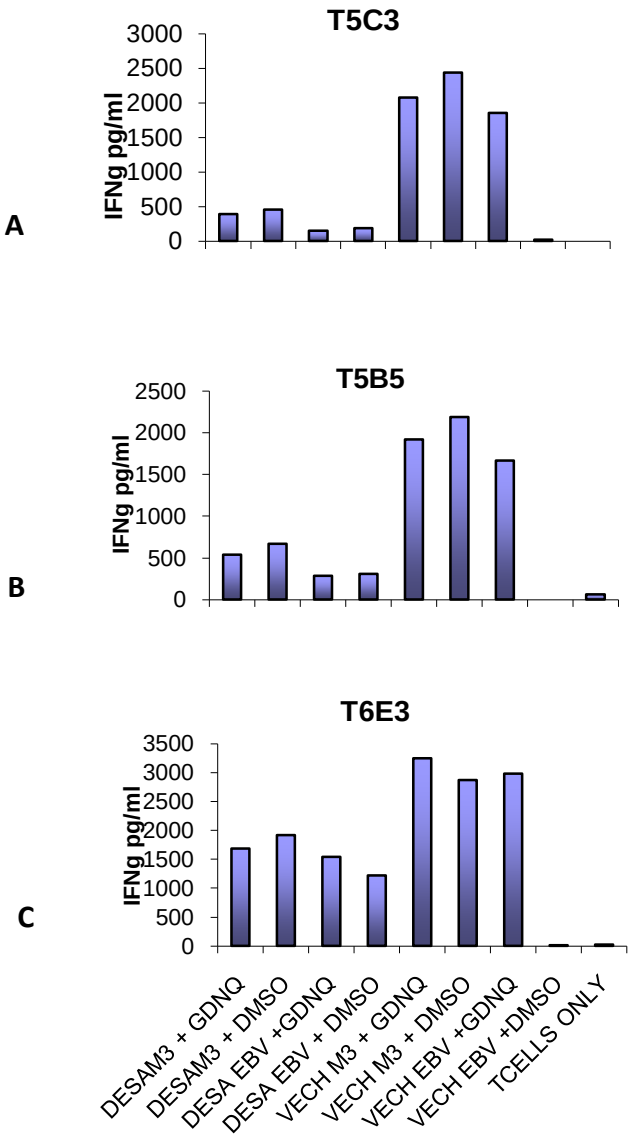


Figure 4.3.6.1 CD4+ T cell clone responses to MAGE-A3 transfected cell lines

In order to investigate whether clones could recognise endogenously produced antigen, three CD4+ clones which had been generated from patient SS238PM after stimulation with GDNQ-batch 1 were screened for reactivity against MAGE-A3 transfected LCLs by IFN-γ ELISA. Concentration of IFN-γ is shown on the y-axis and target cells are on the x-axis. Target cells were non-autologous LCLs, DESA and VACH, which had been transfected with MAGE-A3. Non-transfected DESA and VACH LCLs were used as negative controls. The

capacity for antigen presentation by the target cells to the T cell clones was confirmed by peptide loading target cells with GDNQ-Batch1. IFN-γ production in response to unloaded targets was compared between transfected and non-transfected target cells to establish whether the clones could recognise endogenous MAGE-A3 antigen.

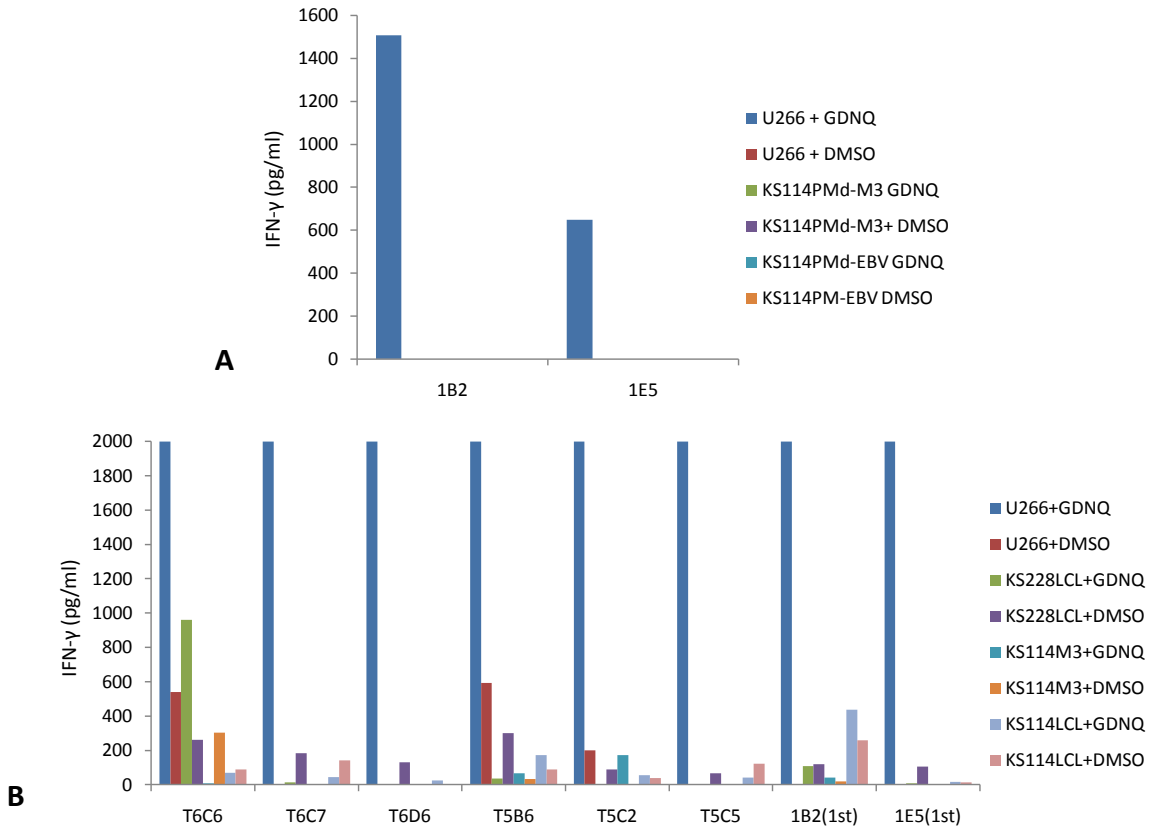


Figure 4.3.6.2 CD8+ T cell clone responses to transfected LCLs and MAGE-A3 expressing tumour cell lines

ELISA screening was used to test specificity of CD8+ T cell clones isolated from patient SS238PM following stimulation with GDNQ-batch1 in IFN-γ capture assays. Clones 1B2 and 1E5 (A and B) were isolated at 42 weeks following transplantation and the remaining clones (B only) were isolated at 46 weeks. Target cells were myeloma cell line U266, HLA-mismatched LCL KS228 and partially HLA-matched LCL KS114, either transfected or non-transfected. Peptide-loaded targets were used to confirm the capacity for antigen presentation to the T cell clones.

4.3.7 Responses to different batches of GDNQ peptide

New batches of some peptides were made to replenish diminished stocks and the new GDNQ peptide was used for further characterisation experiments. However, when this new peptide was used, no reactivity of previously characterised clones was observed. In the light of these observations, clones were tested for response to both batches of peptide by ELISA. Whilst the previous results were replicated when using the old, batch 1 peptide, there was no difference between the new batch 2 peptide and the DMSO controls (figure 4.3.7.1). Therefore, there was no response to the new batch of GDNQ peptide. From this, it was suspected that either the new batch had been incorrectly synthesised, or that the old batch was somehow contaminated with another peptide.

The two batches of peptide were also compared for their ability to stimulate *ex vivo* responses using IFN-γ capture assay. Unsurprisingly, although GDNQ-batch 1 still led to high frequencies of CD4+ and CD8+ T cell responses, GDNQ-batch 2 did not lead to any response (figure 4.3.7.2). Further batches of GDNQ were synthesised and tested, none of which could elicit any response.

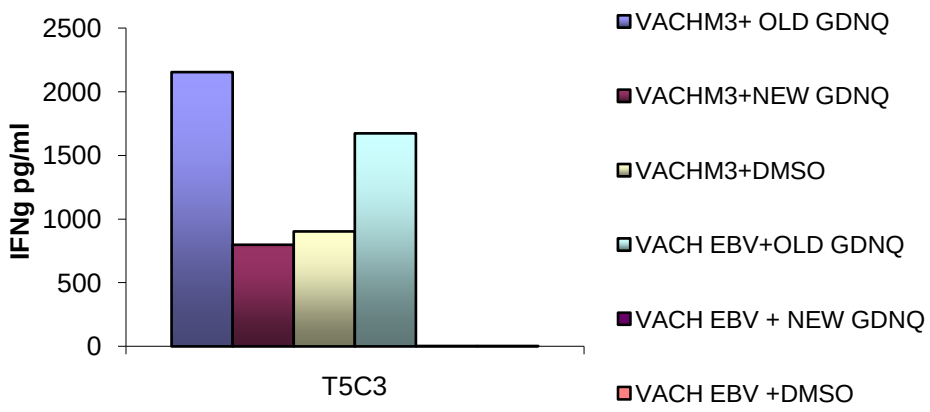


Figure 4.3.7.1 Comparison of responses to new and old batches of GDNQ peptide

CD4+ and CD8+ T cell clones were screened by IFN-γ ELISA for responses to the original GDNQ-batch 1 peptide and newly synthesised GDNQ-batch 2 which had been loaded onto partially HLA-matched VACH LCLs. MAGE-A3 transfected and non-transfected lines were used and cell lines incubated with an equivalent volume of DMSO were negative controls for peptide recognition. A representative CD4+ clone is shown. Responses to GDNQ-batch 1 were in excess of 2000 or 1500 pg/ml against MAGE-A3 transfected and non-transfected LCLs respectively. However, when GDNQ-batch 2 was presented by the same target LCLs the values were similar to the negative control where no peptide was present.

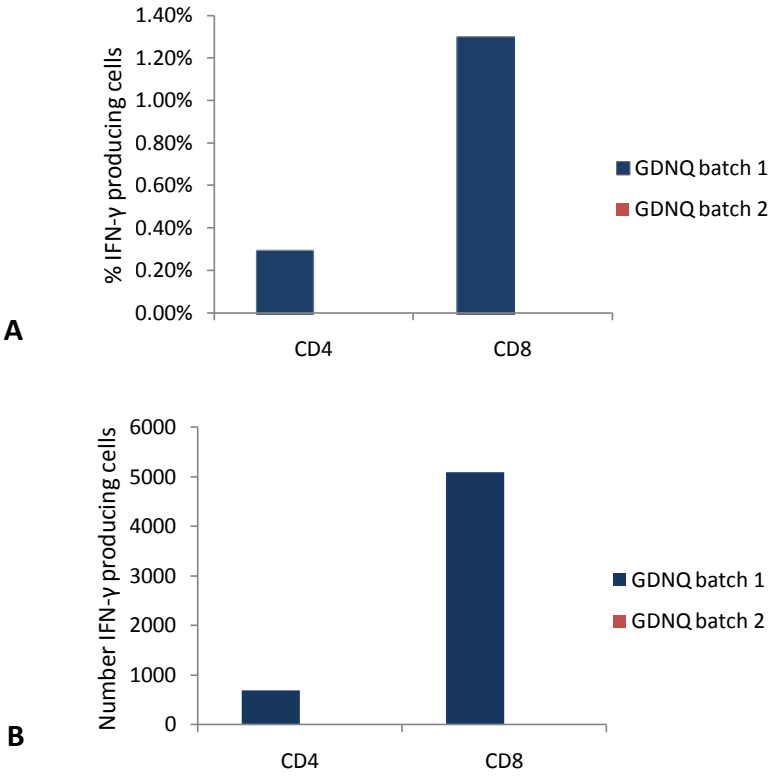


Figure 4.3.7.2 IFN-γ capture assays comparing old and new batches of GDNQ peptide

E*x vivo* responses to batch 1 and batch 2 GDNQ peptides were compared by IFN-γ capture assay. PBMCs freshly isolated from patient SS238PM were incubated with either batch of peptide. Pre-enrichment frequencies (A) and the number of magnetically enriched cells (B) are compared. Blue bars represent responses to GDNQ-batch 1 and the frequency/number of IFN-γ secreting cells following incubation with GDNQ-batch 2 would be in red, but no response was seen GDNQ-batch2

4.3.8 SS238PM patient and donor responses to CMV derived peptides

As in-house tests on the GDNQ-batch 2 by mass spectrometry and the manufacturer's data both suggested that the peptide had been correctly synthesised, attention was given to the possible source and identity of any contaminant. Suspicion fell upon peptides derived from cytomegalovirus (CMV) as they are heavily used in the immediate environment of where GDNQ-batch 1 had been handled initially.

Around this time, a blood sample was obtained from the HLA-matched, but unrelated stem cell donor for SS238PM. Whilst establishing whether similar responses to GDNQ-batch 1 and E2-4 could be detected in the donor, a HLA-A2 restricted CMV peptide seen as a likely candidate for the contaminant was screened. CD8+ responses were generated by this peptide, NLV (see table 4.2.1.1). Responses to the CMV peptide were up to four times higher than to the GDNQ-batch 1 and E2-4 peptides (figure 4.3.8.1), although if it were this peptide causing the contamination, it would be present in much lower concentrations than where the pure CMV peptide was added. Other CMV peptides tested did not elicit responses (data not shown).

Further literature searches revealed that a previously described 15mer CMV peptide AGIL, contained the entire NLV sequence (see table 4.2.1.1). This had also been used in the vicinity of GDNQ-batch 1 and would account for the observed CD4+ and CD8+ responses. As a number of CD4+ T cell clones were growing that had been generated from, and had shown to be reactive to GDNQ-batch 1, they were tested for reactivity to this CMV peptide and also to a third batch of GDNQ. This batch had been made in case the second batch had somehow been incorrect. The data in figure 4.3.8.2 clearly shows that these clones respond to AGIL peptide, and there is no response to the new, higher-purity GDNQ peptide. As two new batches of GDNQ peptide had not caused any reactivity either *ex vivo*, or by T cell clones, it

seemed likely that the original GDNQ-batch 1 had been contaminated with CMV peptide AGIL, and peptides E2-E4 were either also contaminated with this peptide, or possibly with the shorter NLV peptide. This would explain the curious result described earlier (see figure 4.3.4.1) where there was no response to an 11mer peptide which spanned all sequences of E2-E4.

Interestingly, the clones still responded to the MAGE-A3 transfected DESA cell line. An IFN-γ capture assay was set up with transfected and non-transfected DESA cells being used to stimulate PBMCs rather than peptides. T cells produced IFN-γ in response to both cell lines, but the frequency was higher in response to the transfected cell line (figure 4.3.8.3A). It would be as expected to find responses to both because it is likely that the patient would have T cells specific for EBV proteins which are expressed by LCLs. It was encouraging to see a higher response to the transfected line. The enriched fractions were cloned by limiting dilution and then tested for specificity (figure 4.3.8.3B). It was interesting to note that the clones from VACH-M3 responding T cells appeared to have different patterns of recognition to those from cloned from VACH-EBV responses. The non-transfected LCL generated clones recognised LCLs irrespective of peptide, which indicates they were responding to EBV protein. However, the VACH-M3 generated clones responded to the transfected cell line, and also the LCL target cells only when AGIL peptide was present, but not GDNQ (batch 3). It is not surprising that they would recognise the MAGE-A3 transfected line, but it seems that GDNQ is not the peptide responsible for this recognition. However, it is puzzling that they also seem to respond to AGIL peptide. It can also be observed that although the VACH-EBV generated clones recognise the non-transfected LCL, there was a lower concentration of cytokine produced in response to the transfected cell line. The reason for this is unclear.

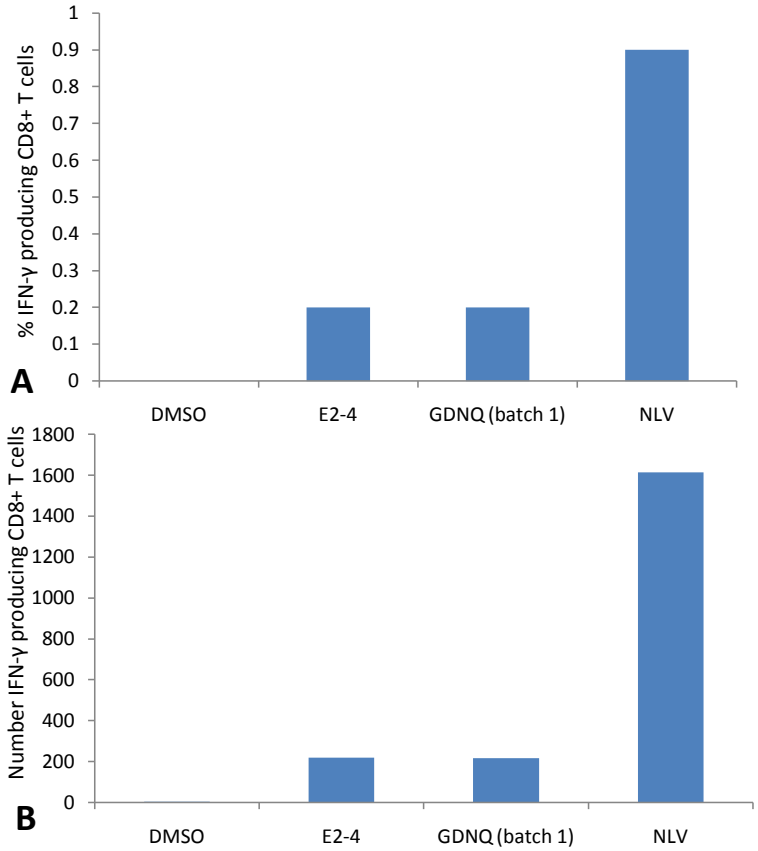


Figure 4.3.8.1 SS238PM stem cell donor responses measured by IFN-γ capture

Freshly isolated PBMCs from the stem cell donor for S238PM were screened for CD8+ T cells specific for GDNQ-batch 1 and E2-4, along with CMV peptide NLV, by IFN-γ capture. The CMV peptide was included as it was suspected that this was the contaminant in the MAGE-A3 peptides. Pre-sort frequencies (A) and the number of magnetically enriched IFN-γ secreting cells (B) are shown (both y-axis).

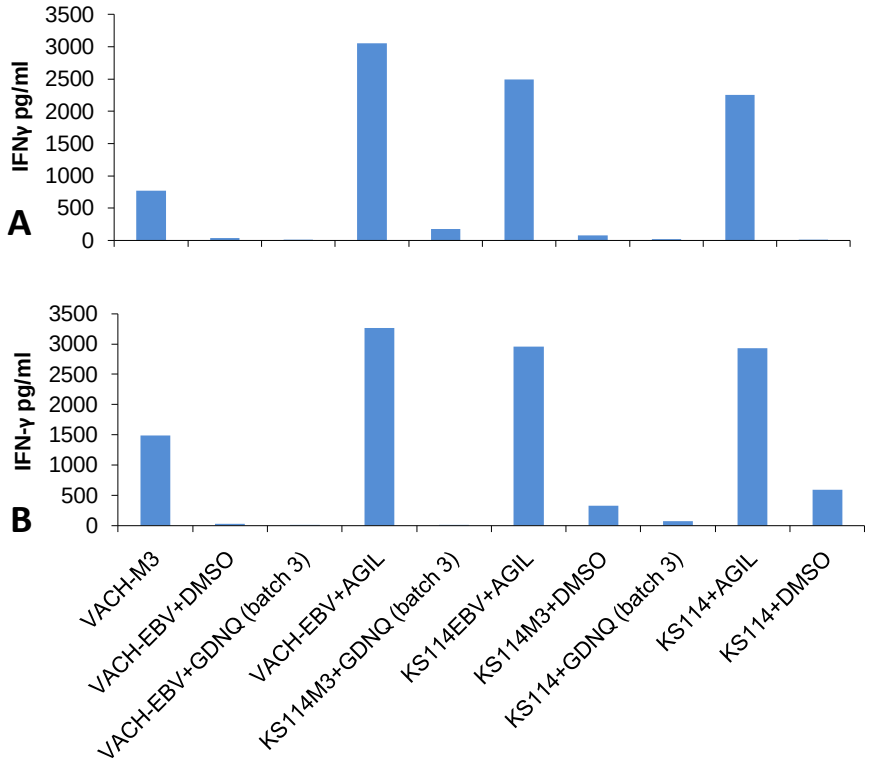


Figure 4.3.8.2 CD4+ T cell clone responses to pure GDNQ-batch 3 and CMV peptide AGIL

CD4+ T cell clones generated by IFN-γ capture following stimulation with GDNQ-Batch1 were stimulated by a newly synthesised GDNQ peptide, and CMV peptide AGIL, which was thought to be the contaminant in earlier batches of GDNQ. VACH LCL which had previously been shown to present GDNQ-Batch1 peptide (figure 4.3.6.1), and a partially HLA-matched LCL (KS114) were loaded with peptide, or incubated with an equivalent volume of DMSO and used to stimulate T cells. MAGE-A3 transfected variants (VACH-M3 and KS114-M3) were similarly loaded for T cell stimulation. Data from two representative clones (A and B) are shown.

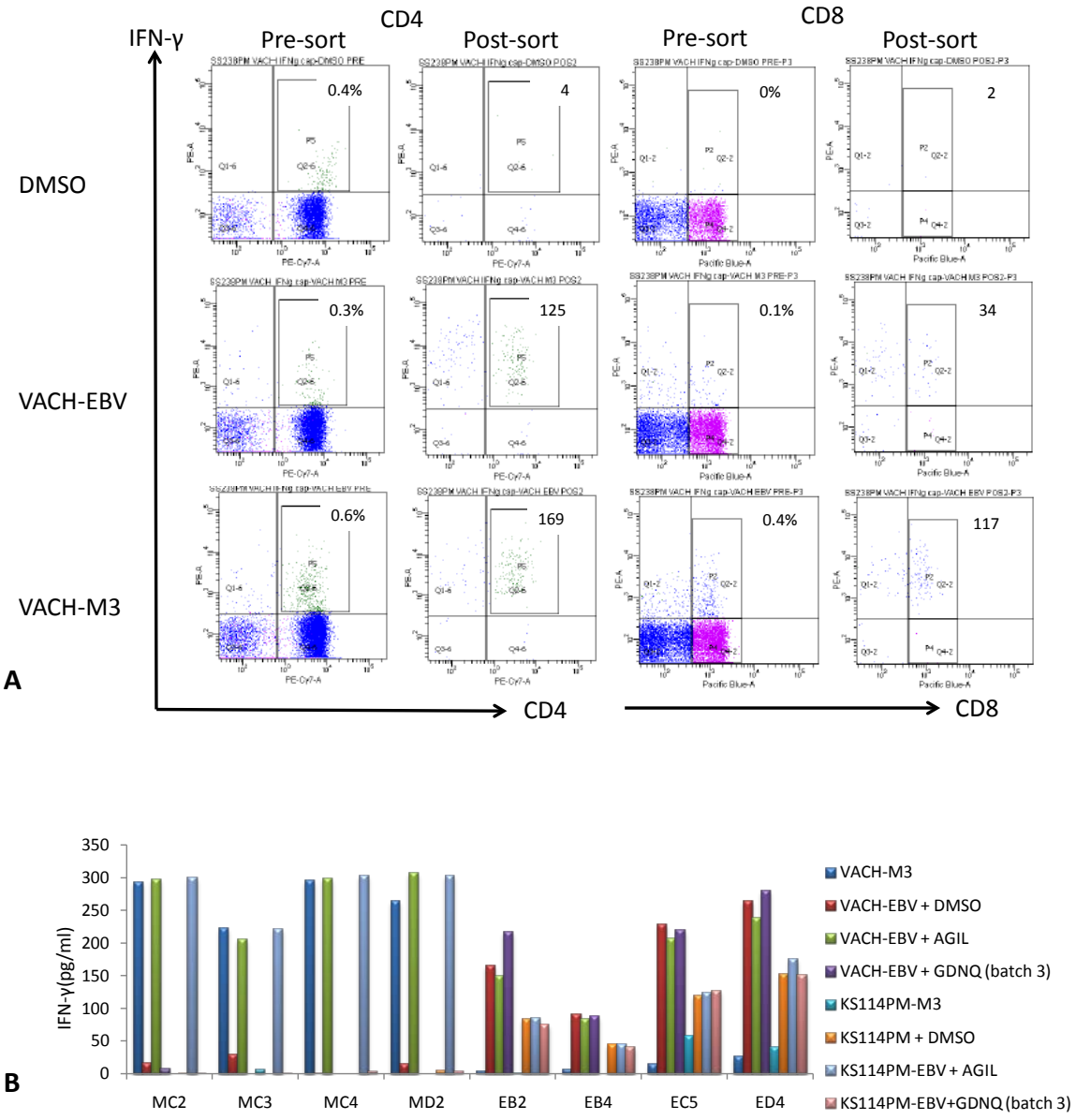


Figure 4.3.8.3 *Ex vivo* responses and T cell clones generated by stimulation with MAGE-A3 transfected & non-transfected cell lines

VACH-M3 and VACH-EBV were used to stimulate PBMCs from patient SS238PM and the frequencies of responding T cells were measured by IFN-γ capture (A). Pre-enrichment frequencies (pre-sort) and the numbers of IFN-γ secreting cells following magnetic selection (post-sort) are shown. CD4 responses are on the left and CD8 on the right. T cell clones were generated from both enriched fractions and reactivity against transfected and non-transfected VACH and KS114PMd cell lines, either with peptides AGIL, GDNQ-batch 3 or

DMSO was tested by IFN-γ ELISA (B). The four clones on the left of the graph were generated from VACH-M3 stimulation and the four on the right, from VACH-EBV stimulation. Representative clones from each group shown.

4.3.9 Investigation of clone responses to MAGE-A3

In an attempt to gauge whether the clones generated from SS238PM did in fact respond to any part of MAGE-A3, they were tested by IFN-γ ELISA, first against overlapping peptides spanning the whole length of the protein, and then against the whole protein. The overlapping 15mer peptides began at every fifth amino acid and were arranged in pools of two peptides per well. A previously characterised clone was used to screen the pools and higher levels of cytokine production would indicate any response (figure 4.3.9.1A). However, no obvious response was seen, with only one possibility being peptide 61. The large error for this result suggested that it may be an artefact, and indeed, when further tests were conducted with that peptide, no response was seen (data not shown).

Full-length MAGE-A3 protein was kindly supplied by Professor Pierre van der Bruggen and was used as a final test for recognition by the clones (figure 4.3.9.1B). In this IFN-γ ELISA, the target cells were the transfected VACH-M3, and non-transfected VACH-EBV loaded either with MAGE-A3 whole protein, AGIL peptide or DMSO as a negative control. As before, there were substantial responses to VACH-M3 and VACH-EBV loaded with AGIL. However, there was no response to the whole MAGE-A3 protein (labelled VACH-EBV+MAGE-A3). Therefore, it is very unlikely that these clones are specific for MAGE-A3. It is not clear why they responded to the VACH-M3 transfected LCL.

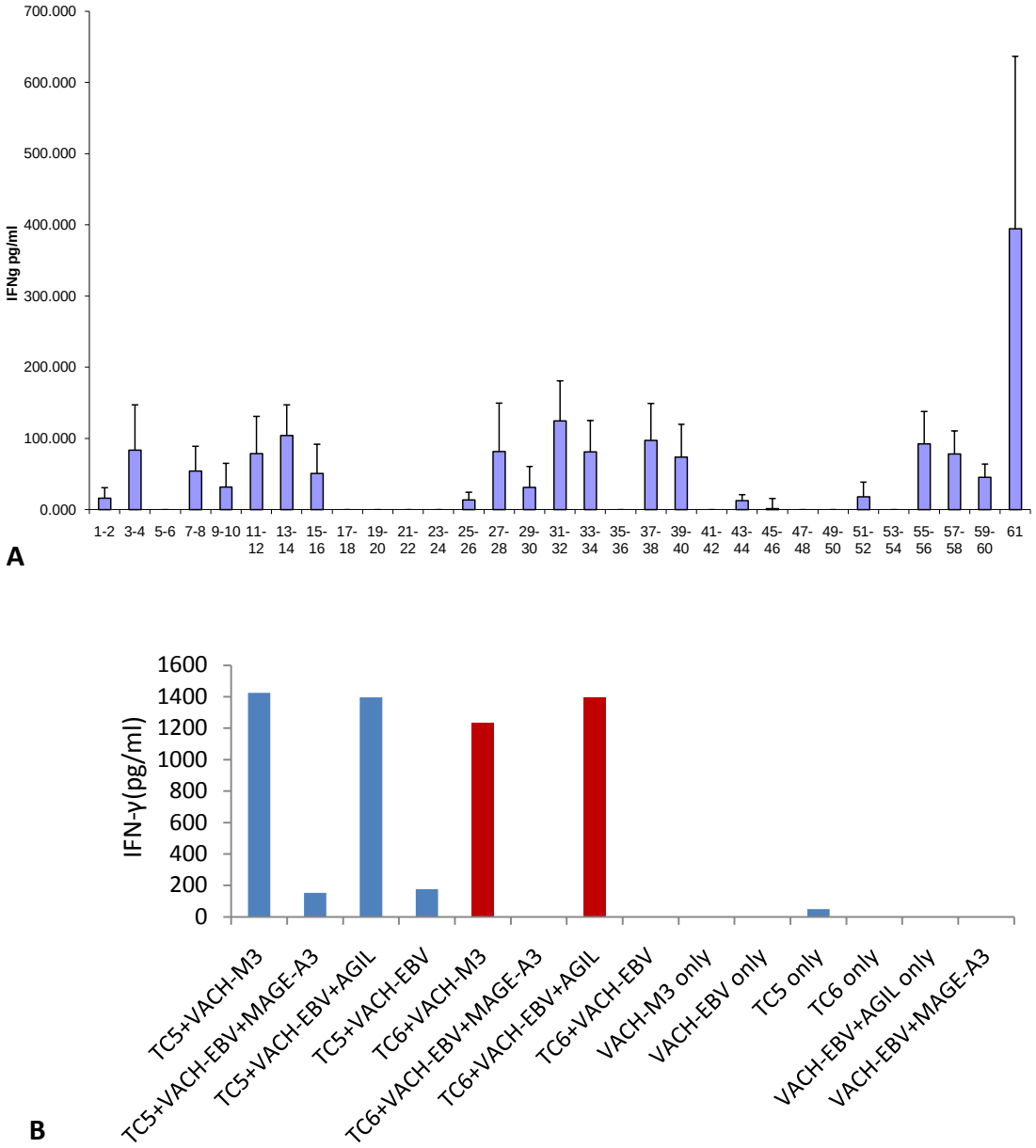


Figure 4.3.9.1 T cell clone reactivity to overlapping peptides and full-length MAGE-A3 protein

In order to investigate whether T cells isolated from patient SS238PM could be specific for a peptide within MAGE-A3, previously characterised clones were tested by IFN-γ ELISA for recognition of overlapping 9mer peptides spanning the entire length of MAGE-A3, which were arranged in pairs (A). Each pair was tested in duplicate and means are shown with

standard error. Two clones were tested for reactivity to VACH-EBV loaded with full length MAGE-A3 protein by IFN-γ ELISA (B). MAGE-A3 transfected (VACH-M3) and non-transfected (VACH-EBV) LCLs were loaded with either full-length MAGE-A3 protein or CMV peptide AGIL, which was the suspected contaminating peptide in early batches of GDNQ peptide. Negative control cells were incubated with an equivalent volume of DMSO.

4.4 T cell responses to cancer/testis antigens can be detected post transplantation

The main purpose of this arm of the study was address the question as to whether there was a detectable CTAg-specific T cell response in allograft patients who were being treated for acute myeloid leukaemia (AML) or multiple myeloma (MM). The presence of these responses may well contribute to a wider graft-versus-leukaemia effect, which, given the highly restricted nature of expression of these antigens, may be a component that could be therapeutically enhanced to improve transplant outcome without GvHD complications.

4.4.1 Cancer testis antigen-specific CD8+ T cell responses can be detected in patients following stem cell transplantation

The interferon-gamma cytokine secretion assay was used to identify CTAg-specific T cell responses in response to a panel of 20 immunodominant peptides derived from 10 CTAg genes (see table 4.1.2). Peptides were selected on the basis that they were derived from proteins for which RNA expression had previously been demonstrated in AML and/or MM, and that immunogenicity has been previously documented in patients with solid tumour malignancies (Boon and van der Bruggen, 1996). Peripheral blood lymphocytes were isolated from patients at several time points post-transplant and were stimulated with CTAg peptides appropriate to the HLA genotype of the patient.

Overall, CTAg-specific CD8+ T cells were identified post-transplantation in five patients. The frequency of the CTAg-specific CD8+ T cell response was variable and ranged between 0.0005% and 0.2% of the total CD8+ T cell pool. The mean frequency of responses was 0.045%, which equates to approximately 1 in 2000 CD8+ T cells and is comparable to the magnitude of many virus-specific immune responses. Of the 20 patients who received reduced intensity conditioning with Campath, four had detectable CD8 responses to CTAg

peptides, whilst of the 21 who underwent myeloablative conditioning only one had CTAg-specific CD8s (figure 4.4.1.1).

The majority of CTAg-specific immune responses could be detected without the requirement for magnetic enrichment, although selection was used to increase the reliable sensitivity of the IFN-γ CSA procedure down to 0.0001%. If a CTAg-specific response was only apparent after magnetic selection then the precursor frequency was calculated on the basis of the post-selection sample, although this approach tends to underestimate the true value due to cell loss during selection. Responses could be detected serially in some cases and the absolute magnitude of the response varied at different time points in the post-transplant period.

Patients were recruited to the study at a range of different time points in the post–transplant period and the association between CTAg-specific immunity and time post-transplant is discussed below.

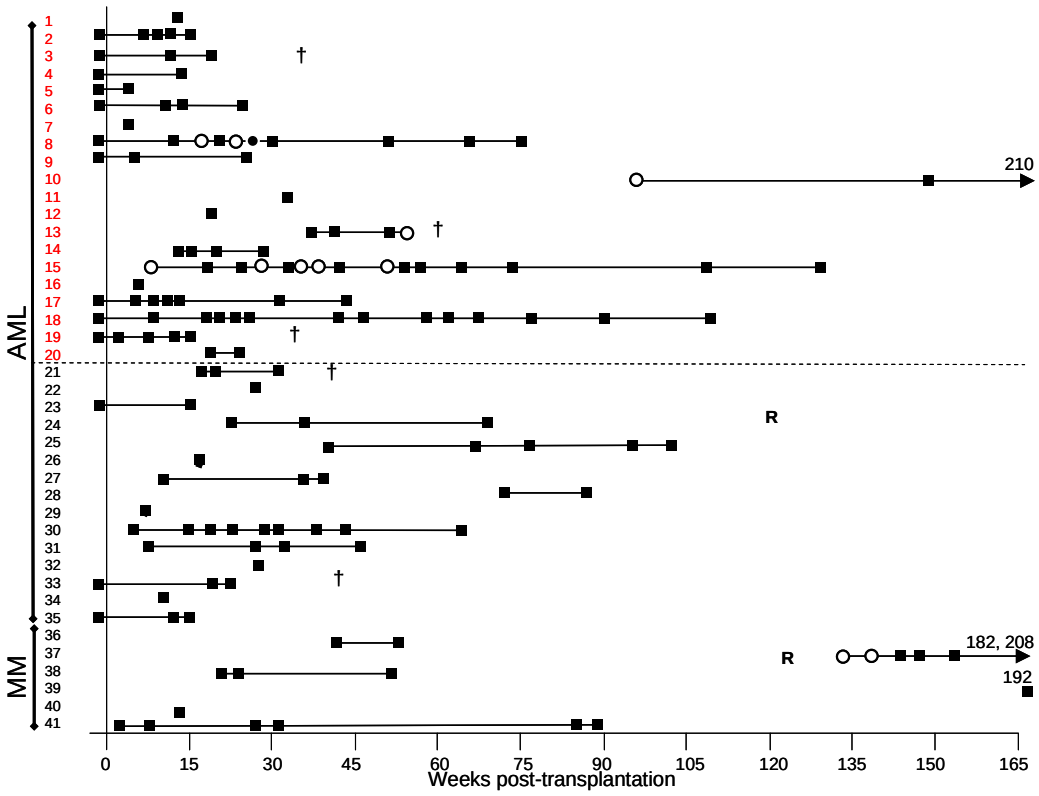


Figure 4.4.1.1 Summary of patient samples screened

The times of all screened samples are shown in weeks post-transplantation (x-axis). Patients 1-35 were treated for acute myeloid leukaemia (AML), and 36-41 for multiple myeloma (MM) (y-axis). Assays where no responses were seen are shown as black squares and positive responses are shown in as open circles. Samples screened after 165 weeks post-transplantation are indicated by number. A cross (†) denotes time of death of patients who died due to relapse, and a letter R shows where patients suffered relapse but were successfully treated and have survived. The patients above the dotted line, with numbers in red, received Campath as part of their reduced intensity conditioning. Patient 15 is shown in more detail in figure 4.4.2.1, patient 8 in figure 4.4.2.2, patient 10 in figure 4.4.2.3, patient 13 in 4.4.3.2 and patient 37 in figure 4.4.3.1. Where the first assay time point is to the left of the y-axis, the patient was screened prior to starting transplant conditioning.

4.4.2 CTAg-specific CD8+ T cell responses were observed in two patients with long term disease free survival

RuH189PM

CTAg-specific CD8+ T cells were detected in two patients within the first year following transplantation and both of these had undergone RIC allograft for AML (figure 3). AML patient RuH189PM had a T cell response to RAGE-1 peptide SPSS and also to the peptide pool comprising ALKD/EGDC. These responses were observed at 9 weeks post transplant which was the first time point of analysis. Of note, the patient had suffered from grade IV graft-versus-host disease (GvHD) of the skin at 3 weeks post transplant and this had been treated with oral prednisolone. The T cell response to SPSS was present at 5 separate time points of analysis until 51 weeks post transplant although the magnitude of the response was variable and was undetectable on 4 occasions. Cyclosporine A (CsA) treatment had been discontinued at 27 weeks post-transplantation and this coincided with a transient increase in the RAGE-1 specific T cell response (figure 4.4.2.1).

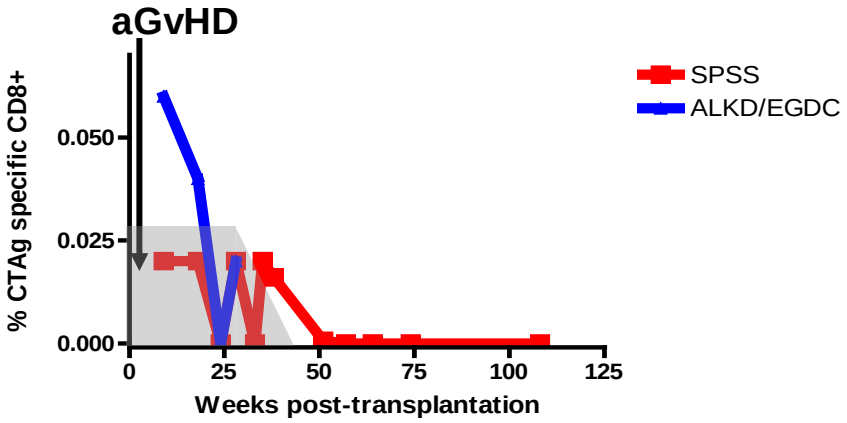


Figure 4.4.2.1 Responses to CTAg antigens in patient RuH189PM

Frequencies of T cells (y-axis) specific for either RAGE-1 peptide SPSS (red) or pools of MAGE peptides ALKD and EGDC (blue) were measured by IFN-γ capture in peripheral blood samples from AML patient RuH189PM. The patient was screened at various time points up to 109 weeks post-transplantation for SPSS responses. An early incidence of grade IV skin GvHD was resolved around seven weeks post-transplantation. The grey area indicates the period during which the patient received prophylactic cyclosporine treatment which was decreased over time until complete withdrawal.

AML patient JB235PM

CTAg-specific T cells were detected in patient JB235PM at 17, 23 and 29 weeks post transplantation and this also coincided with tapering of CsA therapy, which was finally discontinued by 25 weeks. However, at 20 weeks no response was detected (Figure 4.4.2.2). This patient was one of thirteen that were screened prior to starting transplant conditioning, although no pre-transplant responses were detected.

Importantly, patients RuH189PM and JB235PM have remained in remission since transplantation and now remain disease free at 52 and 37 months post transplant respectively. Overall, there were 14 patients who were screened for CTAg-specific immune responses on two or more occasions within the first year post-transplant and who had relapse free survival. As CTAg-specific T cells were observed in two of these, we were able to demonstrate immunity to this relatively limited CTAg peptide panel in 14.3% of this group.

Attempts were made to expand the CTAg-specific T cells from the enriched, peptide-stimulated fraction by limiting dilution cloning (Chapter 2, section 2.8), in order to further characterise the cells. Unfortunately, no clones grew which may be attributable to the cells being more fragile early post-transplantation, and the magnetic selection process may have damaged them further.

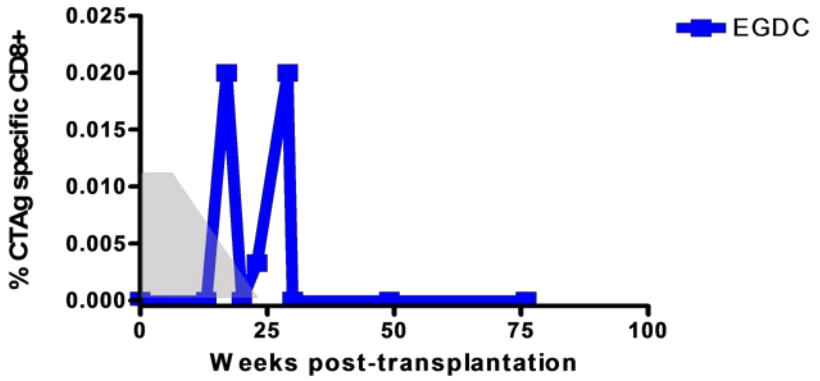


Figure 4.4.2.2 CTAg-specific T cell frequencies in patient JB235PM

The magnitude of the T cell response (y-axis) to MAGE-A2/3 peptide EGDC over time (x-axis) in AML patient JB235PM was measured by IFN-γ capture assay of peripheral blood samples. The grey area indicates the time of prophylactic administration of cyclosporine and the tapering period before complete withdrawal is shown by the slope.

AML patient JT138PM

Screening began on an additional AML patient (JT138PM) at 92 weeks post transplant and a T cell response was observed against peptides ALKD and EADP but not detected thereafter (figure 4.4.2.3). This patient has also remained relapse free during follow up.

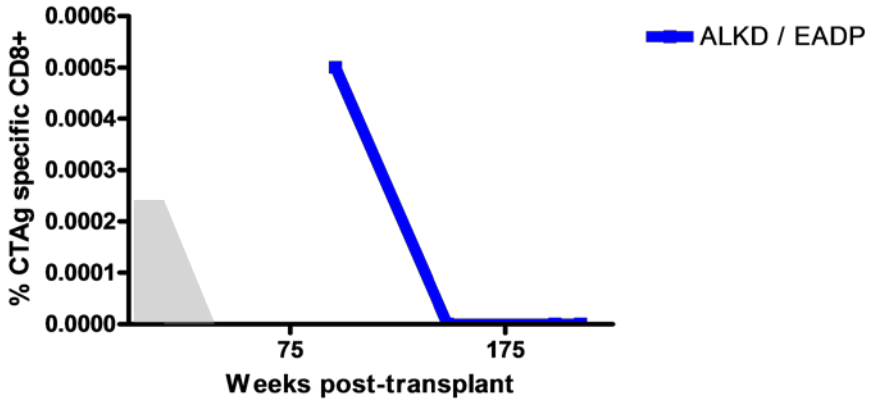


Figure 4.4.2.3 CTAg-specific T cell frequencies in patient JT138PM

Patient JT138PM was screened from 92 weeks post-transplatation for the presence of CTAg-specific T cells by IFN-γ capture. CD8+ T cells responding to MAGE peptides ALKD/EADP were detected, although the response was near the level of detection of the assay, and subsequently, no response was evident. Prophylactic cyclosporine treatment is shown by the grey area, and the tapering of this treatment prior to complete withdrawal is indicated by the slope.

4.4.3 CTAg-specific T cell responses can also be detected at the time of disease relapse MM patient TS125PM

Previous studies of CTAg-specific immunity in patients with myeloma have revealed that CD8+ responses are often observed around the time of disease relapse and probably reflect immune response to increased tumor load (Goodyear et al., 2008). A similar pattern was observed in patient TS125PM with a diagnosis of MM who was initially studied at a time point over 2 years after SCT with a myeloablative conditioning regimen. The serum paraprotein level had risen at 124 weeks post-transplant and indicated the onset of disease relapse. The first CTAg analysis was undertaken 10 weeks after this point and revealed a broad CTAg peptide-specific immune response which included specificity for peptides in three different peptide pools and included reactivity against the peptide EGDC. The patient was treated with thalidomide and the next sample was taken at 143 weeks at which time the magnitude of the EGDC-specific response had fallen by 60% and responses to other peptide pools were not observed. The patient responded well to thalidomide with a reduction in the paraprotein level, and no CTAg-specific immune response could be detected in subsequent analyses. (figure 4.4.3.1).

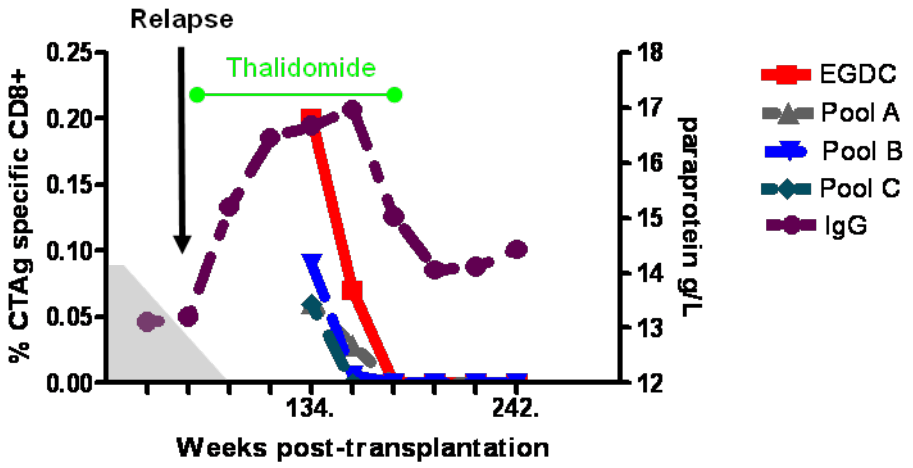


Figure 4.4.3.1 CTAg-specific responses in patient TS125PM

A time course of responses to CTAg peptides and peptide pools (red, grey, blue and green lines) measured by IFN-γ capture assay of peripheral blood samples from myeloma patient TS125PM is shown along with paraprotein concentration (purple line, right y-axis). Duration of thalidomide treatment of the relapse is shown in green. Prophylactic cyclosporine therapy is indicated by the grey area, with the slope denoting the period during which this was tapered prior to complete withdrawal.

AML patient NC172PM

Patient NC172AM with a pre-transplant diagnosis of AML was screened from 38 weeks post-transplant and responses were seen at 53 weeks to peptides SLFR, EGDC and MEVD. At 55 weeks the patient was diagnosed with relapse of AML and died shortly afterwards (figure 4.4.3.2).

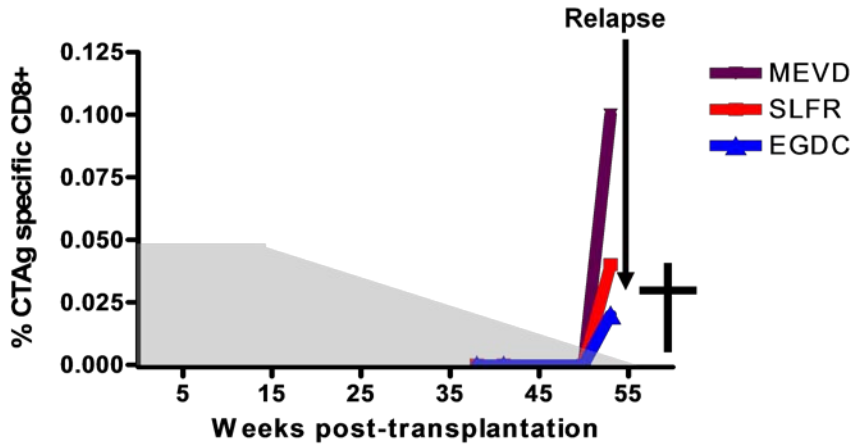


Figure 4.4.3.2 Responses to CTAg peptides in patient NC172PM

CTAg-specific CD8+ responses (γ-axis) were measured by IFN-γ capture assay carried out on peripheral blood samples from AML patient NC172PM. Screening commenced at 37 weeks post-transplantation and responses were detected on one occasion shortly before diagnosis of relapse. The patient subsequently died as indicated by the cross. The period of prophylactic cyclosporine therapy is indicated by the greyed area and the slope denotes the time over which this therapy was tapered prior to complete withdrawal.

4.4.4 RAGE-1 specific T cells can be detected at higher frequency within bone marrow compared to peripheral blood

Paired blood and bone marrow samples from patient RuH189PM were obtained at 35 and 51 weeks post-transplantation. T cells specific for SPSS were detected by IFN-γ cytokine secretion assay and the frequency was found to be up to seven times higher in bone marrow compared to PBMC. In peripheral blood, at both 35 and 51 weeks, 0.0006% of CD8+ T cells were specific for SPSS, whereas in the bone marrow, the frequencies were 0.0021% at 35 weeks and 0.0042% at 51 weeks (figure 4.4.4.1).

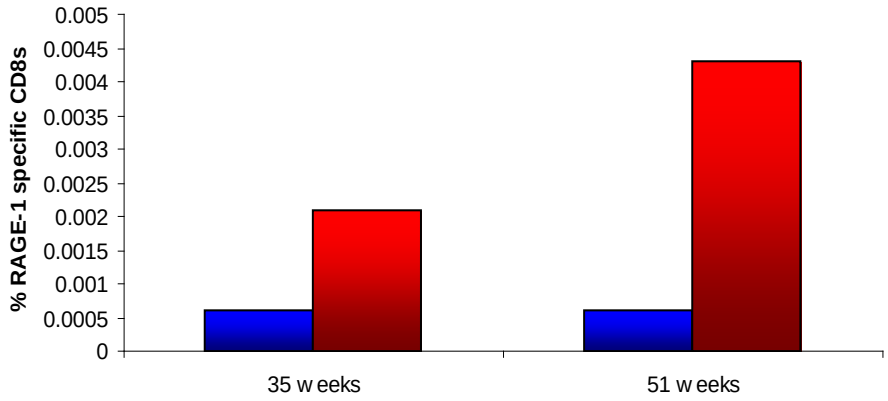


Figure 4.4.4.1 Comparison of CTAg-specific CD8+ frequencies in blood and bone marrow

Paired blood and bone marrow samples were obtained on two occasions from AML patient RuH189PM and screened by IFN-γ capture assay for T cells specific for RAGE-1 peptide SPSS. IFN-γ secreting CD8+ T cell frequencies (y-axis) following peptide stimulation are shown in peripheral blood (blue bars) and bone marrow (red bars) 35 and 51 weeks.

4.4.5 The CTAg-specific CD8+ T cell response post-transplantation is preferentially focussed on peptides derived from the MAGE family of proteins

20 CTAg peptides were used in this study and T cell responses within SCT patients were detected against 6 of these. Interestingly, immunity was focussed on peptides derived from the MAGE family of proteins and four patients showed T cell immune responses against peptide EGDC (figure 4.4.5.1). Overall, CD8+ T cell responses were observed against peptides derived from the MAGE subfamily proteins A1, A2, A3 and C2, and a strong immune response against RAGE-1 was also observed in one patient.

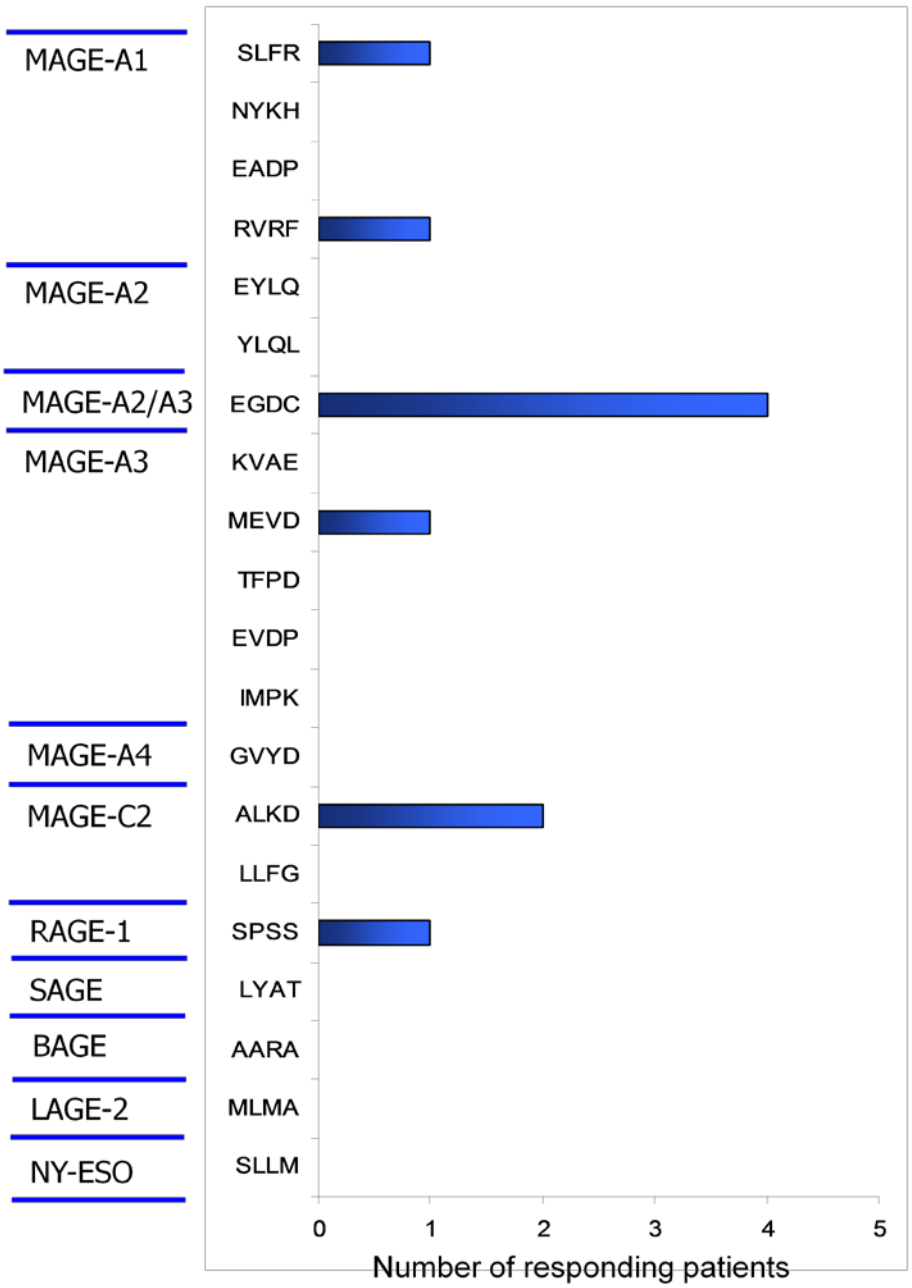


Figure 4.4.5.1 Numbers of patients with responses to each peptide

All peptides used to screen transplant patient responses to selected CTAg proteins (y-axis) are shown. Blue bars indicate number of patients (x-axis) that responded to each peptide, either singly or as a component of a pool, when used in an IFN-γ capture assay to stimulate PBMCs isolated from blood samples taken from AML or MM patients following transplantation.

4.4.6 Detection of CTAg-specific T cells using CD137 assay

Frozen PBMC samples were available for some patients, taken at various times post-transplantation. The IFN-γ capture assay does not work with previously frozen cells and as colleagues had recently been using up-regulation of CD137 as a measure of antigen responsiveness, it was decided to try this assay to test frozen samples for the presence of CTAg-specific T cells. CD137 is transiently up-regulated on T cells following contact with antigen and antigen-specific T cells have successfully been identified and sorted using this marker (Wehler et al., 2008).

The method is described fully in Chapter 2 (section 2.14), but in brief: cryopreserved PBMCs were thawed, washed and resuspended at $1x10^7$ cells/ml in T cell culture medium (RPMI, 10% human serum, 0.5% penicillin/streptomycin, 0.5% L-glutamine). Cells were incubated with CTAg peptide for 24 hours (37^oC, 5% CO_2) and then stained with CD137 antibody, conjugated to PE, along with CD3APC-Cy7 and CD8-AmCyan to identify T cells. CD14-ECD, CD19-ECD, CD56-PC7 and PI were also added to exclude other cell populations and dead cells. Responding cells could either be assessed directly by flow cytometry or enriched using PE-microbeads. Example dot plots showing the gating strategy and results for one patient at one time-point are shown in figure 4.4.6.1.

Samples from two patients with IFN-γ responses were screened in this way. PBMCs isolated from JB235PM at 5,10,13,29 and 40 weeks, along with those from JT138PM at 6, 13, 20, 25 and 33 weeks post-transplantation were screened. In the 10 week sample from JB235PM, 0.31% of CD8+ T cells fell in the CD137+ region against a background of 0.1%. However, this 'population' did not enrich (figure 4.4.6.2). In some IFN-γ capture assays there had been an apparent response that did not enrich, and the assay manufacturer said these were unlikely to be real responses (J. Campbell - personal communication). As the same enrichment

system was used for this assay, this was not considered a positive response. No convincing responses were observed above background

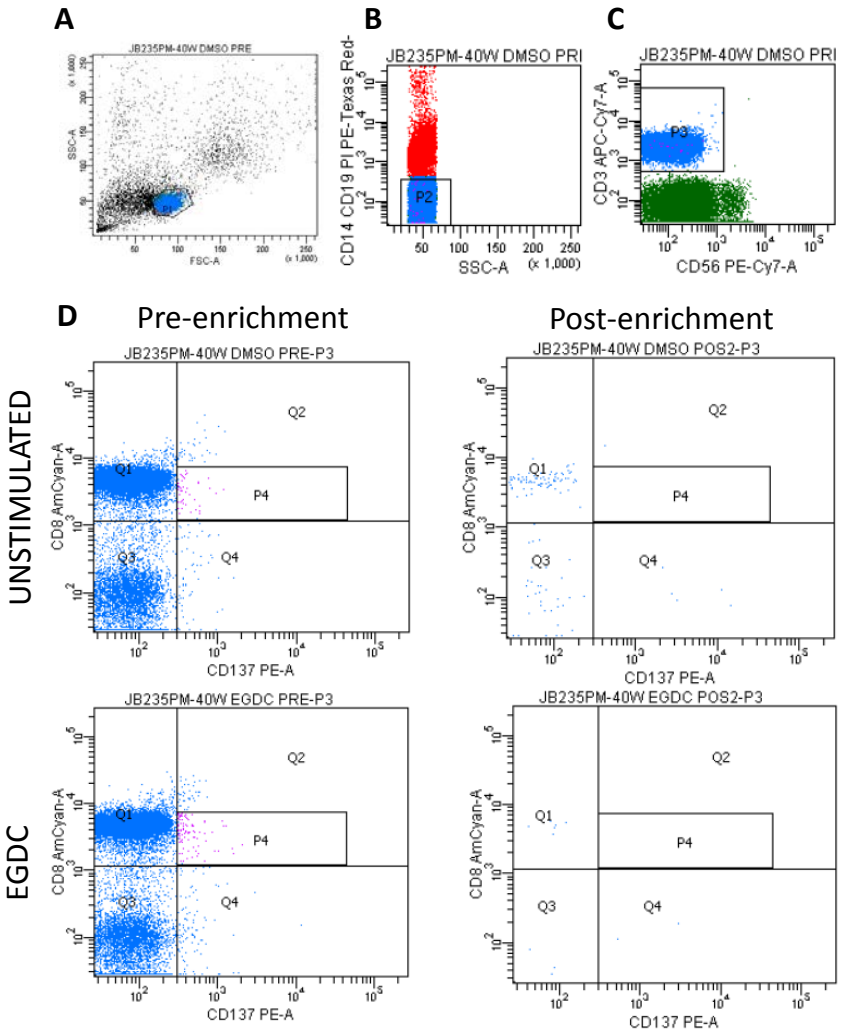


Figure 4.4.6.1 Example of CD137 assay for identification of CTAg specific T cells

A representative example of a CD137 assay, showing the gating strategy and staining. Cryopreserved PBMC isolated from AML patient JB235PM were thawed and stimulated for 20 hours with MAGE-A2/A3 peptide EGDC. Antibodies specific for CD137, CD3, CD8, CD14, CD19 and CD56 were added. The lymphocyte population (A) was gated, then CD14+, CD19+ and dead (PI+) cells were excluded (B). NK cells were gated out by excluding the CD3- CD56+ population (C). CD137 expressing cells CD8+ T cells can be seen in region P4 (D, left) and magnetic beads were used to enrich this population (D, right). The lack of enriched cells casts doubt on the validity of the apparent CD137+ population in the non-enriched fraction.

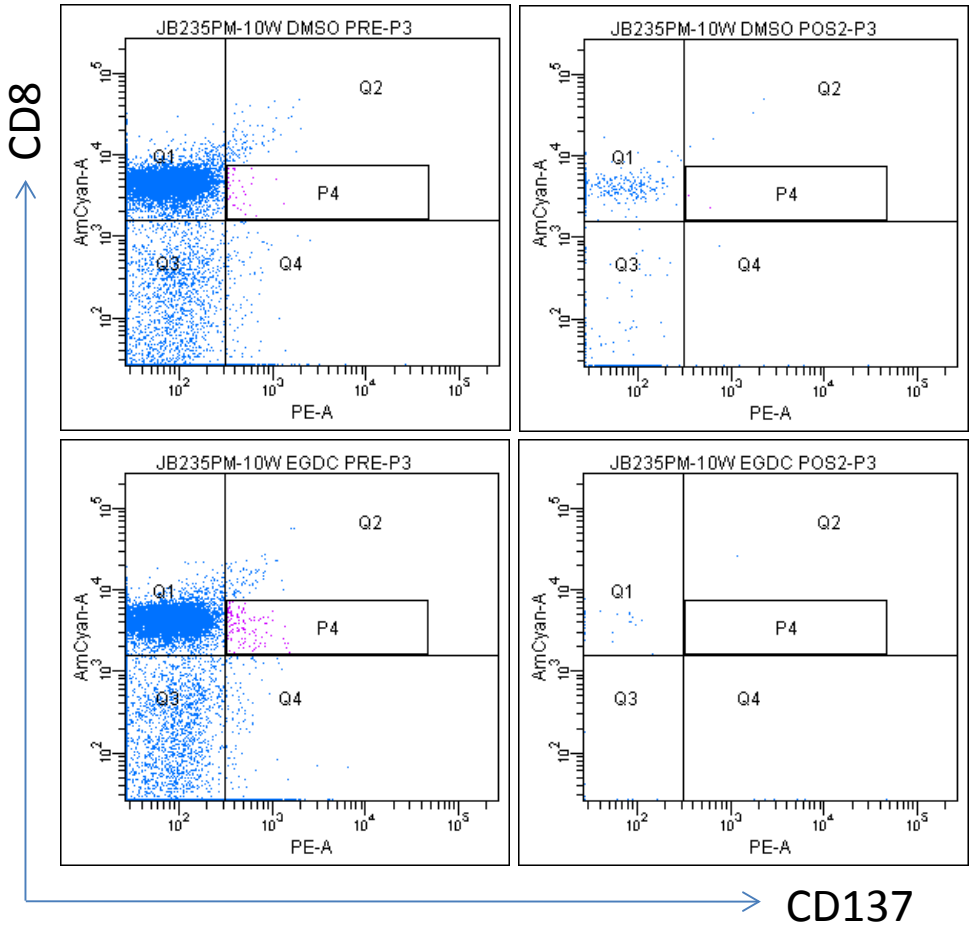


Figure 4.4.6.2 Demonstration of false-positives in non-enriched fraction of a CD137 assay

Cryopreserved PBMCs were thawed and stimulated overnight with CTAg peptide EGDC (lower) or an equivalent volume of DMSO (upper) before being stained with CD137 (x-axis) and CD8 (y-axis) specific antibodies. Magnetic beads were used to enrich the CD137+ population (right). CD8+ CD137+ T cells are shown in the region P4. The lack of enriched cells casts doubt on the validity of the non-enriched fraction.

4.4.7 Peptide restimulation cultures

All screening assays had been conducted *ex vivo* with peptide incubations of sufficient duration to induce specific T cells to respond. In a further attempt to generate CTAg-specific T cell clones, PBMCs from patients RuH189PM and JB235PM were cultured for up to four weeks with weekly peptide restimulation. This approach was not taken with all screening as the potential to influence naive T cells to be responsive to the peptide, and any preferential expansion of specific cells would have prejudiced the frequencies, preventing establishment of the proportion of CTAg immunity *in vivo*.

JB235PM

Fresh PBMCs were isolated from a blood sample taken at 112 weeks post-transplantation. A peptide-TCL line was set up according to the method outlined in Methods (Methods 2.15). EGDC peptide was added to a final concentration of 10µg/ml at weekly intervals until four weeks had elapsed. The cells were then harvested and tested for the presence of EGDC-specific T cells by IFN-γ capture (figure 4.4.7.1). Despite there being no apparent response above background, half of the post-selection fraction was cloned by limiting dilution (Methods 2.8) in case there were specific cells present. No T cell clones grew.

RuH189PM

Cryopreserved PBMCs taken at 11 weeks post-transplantation were thawed and a T cell line was set-up, stimulated with SPSS peptide (as in previous section). A strong response had previously been detected at 9 weeks post-transplant using fresh PBMCs. Peripheral blood had been taken and frozen at 7 weeks, which contained a large number of stem cells, possibly resulting from G-CSF treatment. In order to stimulate the T cell line during culture and for IFN-γ capture, these stem cell rich aliquots were thawed and loaded with peptide.

To ensure their suitability for peptide loading, MHC class I levels were assessed by flow cytometry and judged to be sufficient. Following IFN-γ capture, no clear response was shown. However, attempts were made to clone the post-selection fraction to see if any SPSS specific T cells could be cultured. A few wells grew and were expanded further before testing for specificity by IFN-γ ELISA. The clones secreted IFN-γ at concentrations of up to 900pg/ml, with most in the range of 400-700pg/ml. However, background levels of cytokine production in response to negative controls (LCL without peptide and no stimulation) were greater than 50% of peptide loaded LCLs so no clones were considered to be specific for SPSS (figure 4.4.7.2).

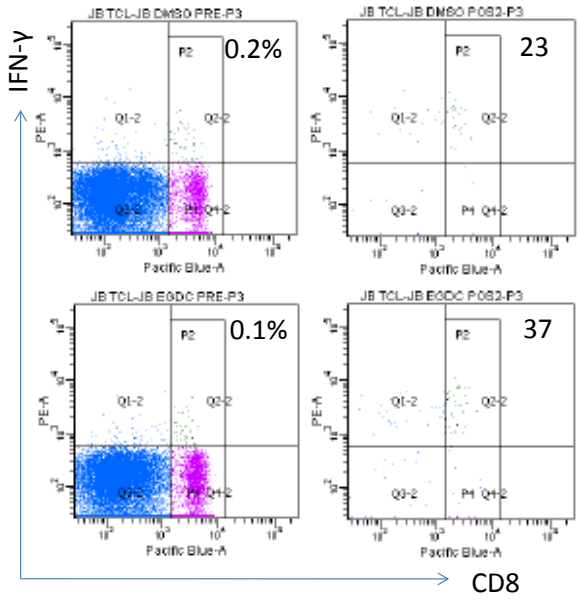


Figure 4.4.7.1 AML patient JB235PM T cell line restimulation experiment

Freshly isolated PBMC from AML patient JB235PM at 112 weeks were cultured for four weeks at 37^{o}C 5% CO_2, and were stimulated by addition of MAGE-A2/A3 peptide EGDC at weekly intervals. The line was harvested and split, before addition of EGDC peptide (bottom row) or an equivalent volume of DMSO to establish background levels of IFN-γ production (top row). The presence of EGDC specific T cells was assessed by IFN-γ capture assay. Magnetic beads were used to enrich any IFN-γ secreting population. Pre-selection dot plots are on the left with frequencies of IFN-γ producing cells, and post-selection on the right with the number of IFN-γ producing cells marked.

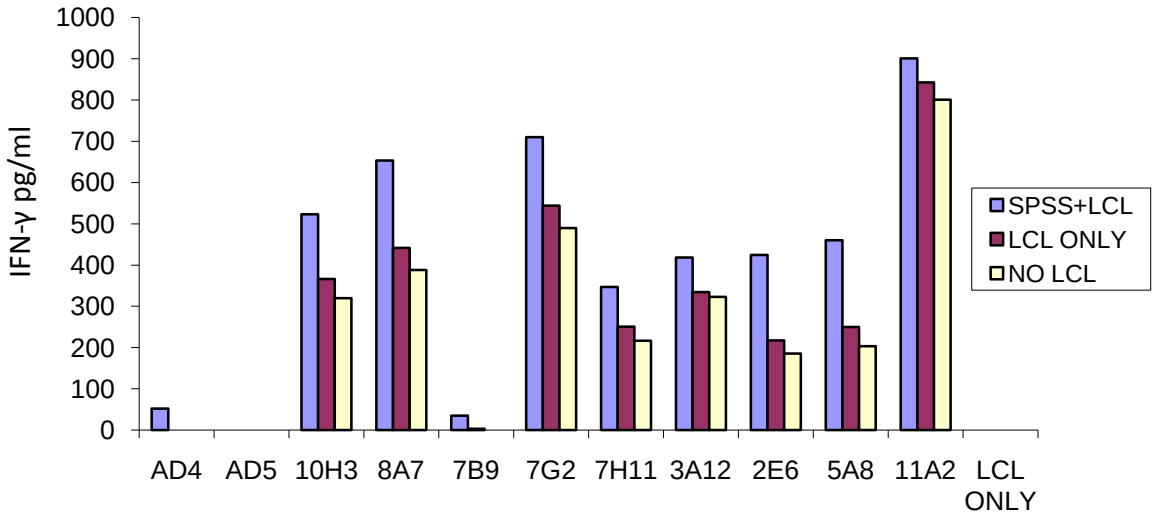


Figure 4.4.7.2 ELISA screen for specificity of clones generated from RuH189PM

T cell clones were generated from cryopreserved PBMC isolated from AML patient RuH189PM at 11 weeks post-transplantation, by weekly stimulation over four weeks with SPSS peptide and IFN-γ capture assay with magnetic enrichment. Specificity of T cell clones was assessed by IFN-γ ELISA. Targets were HLA-matched LCLs loaded with SPSS peptide (blue bars) or unloaded as a negative control (red bars), and T cells alone were used as a further negative control (yellow bars). Concentration of IFN-γ is shown on the y-axis in pg/ml.

4.4.8 Immunohistochemistry

Having established that there was T cell immunity to MAGE family proteins in certain patients, an antibody to MAGE-A was used to investigate expression of these CTAgs in the patients' bone marrow trephines by immunohistochemistry. Sections of diagnosis biopsy were obtained, mounted on slides, from the pathology department of the Queen Elizabeth Hospital, Birmingham. These were stained according to the protocol outlines in Methods Section 2.13, using an anti-MAGE antibody (Invitrogen, Paisley, UK). A positive control of a known MAGE expressing tissue was stained alongside each patient. A sample of one patient and a positive control is shown in figure 4.4.8.1.

The slides were examined under a light microscope and assessed for positive staining. The results were unclear so a second opinion was sought from an expert pathologist at Birmingham Heartlands Hospital. All patient samples were considered negative with little evidence of malignant cells.

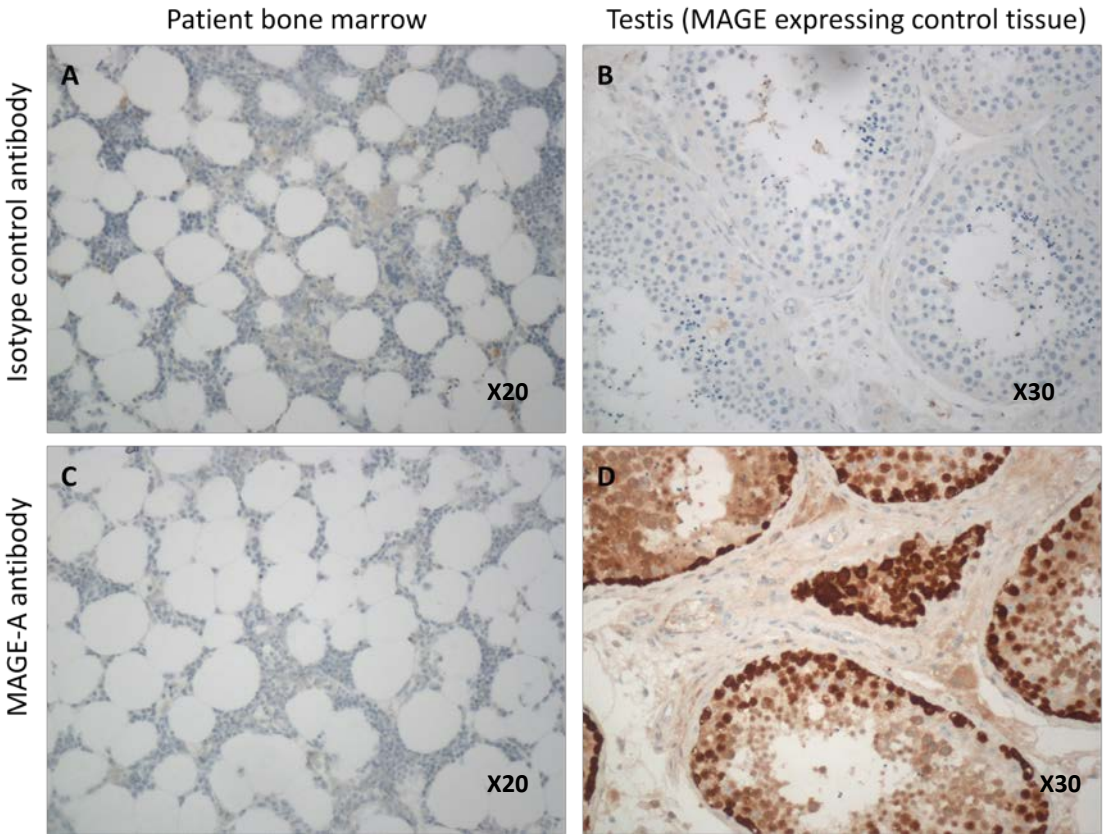


Figure 4.4.8.1Staining for MAGE-A expression by immunohistochemistry

Slide mounted bone marrow biopsy samples from patients with CTAg responses were obtained from the pathology department of the Queen Elizabeth hospital, Birmingham. The samples were stained with an isotype control antibody (A) or an antibody specific for the MAGE-A family of CTAg proteins (C). Testis samples, which naturally express MAGE-A3, were also stained with the isotype (B) and MAGE-A antibody (D).

4.4.9 CTAg immunity in non-transplanted AML patients

Thirteen study patients were screened prior to beginning their transplant conditioning (see figure 4.4.1.1). A further seventeen AML patients were recruited from the Clinical Haematology department of the Queen Elizabeth Hospital, Birmingham. Blood samples were taken either during attendance at a clinic appointment, or from in-patients when lymphocyte counts recovered following chemotherapy. Samples with extremely high levels of blast cells were generally avoided as it was felt that those cells would affect the efficacy of the assay. In some cases, attempts were made to remove these cells by plastic adhesion, or magnetic selection with CD34 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). However, these methods proved to have limited effect. After many adhesion steps, the number of tumour cells was still far too high. The volume of CD34 microbeads required to treat such a high cell concentration was prohibitively expensive, and it was found that less than 10% of blast cells expressed CD34 so making this approach would have had little benefit.

Screening of the non-transplanted cohort was carried out in the same way as the post-transplant group. Only one sample was available from most patients, but in some cases, serial analysis of more than one sample was possible. If the pre-transplant assays are grouped with the non-transplanted cohort then the total number screened was 30. IFN-γ secreting T cells were detected in only one of these patients on one occasion to a pool of 5 CTAg peptides (data not shown). The patient was duly followed up after further chemotherapeutic treatment but no further responses were observed. It was not possible therefore to verify the response and establish which peptide elicited the response in that patient.

4.5 DISCUSSION

Allogeneic stem cell transplantation is a widely used procedure for treatment of haematological malignancies and other bone marrow disorders which is increasingly being considered as a strategy for treatment of solid tumours such as breast cancer (reviewed in (Bregni et al., 2004)). Success of the transplant is thought to require an effective graft versus leukaemia (GvL) effect (Collins et al., 1997, Horowitz et al., 1990, Kolb et al., 1995, Porter et al., 1999) which also carries the risk of graft versus host disease (GvHD) complications (Billingham, 1959, Lee et al., 2002). Allograft patients have a decreased risk of relapse compared to stem cell transplant patients who receive their graft from an identical twin, although the latter group are less likely to experience GvHD. This suggests that both GvHD and GvL could be T cell mediated, and the presence of tumour reactive T cells has been shown previously (Montagna et al., 1998). However, the targets of anti-tumour T cells have not been well characterised, so identifying these may enable tumour-directed immunotherapy to boost anti-tumour immunity without the risk of increasing GvHD. Cancer testis antigens (CTAgs) could be attractive targets for anti-tumour immunotherapy due to their highly restricted tissue expression. In healthy individuals, they are only expressed on germline cells which lack MHC molecules and are therefore considered immune privileged sites. 45 allograft patients were studied for the presence of CTAg-specific T cells and these responses were found in 11.1%. The frequencies ranged from 0.0005% to 0.2%, with a median 0.024% and mean of 0.045%, although the responses were not always detectable.

In two AML patients (RuH189PM and JB235PM), CTAg-specific T cells could be detected just after the cessation of immunosuppressive therapy. Following immune suppression, a general increase in immune activity occurs. It is possible that if these T cells were present at such a low frequency as to be normally below the level of detection of the assay, then such

an increase in immune activity could result in a transient expansion of this cell population, making detection possible. In CTAg vaccine trials for metastatic melanoma, tumour regression has been demonstrated even though it was not always possible to detect tumour-specific T cells, leading to the idea that very low numbers of T cells are required for an effective anti-tumour response (Boon et al., 2006, Lonchay et al., 2004). This may explain why the T cells were not seen at all time points.

CTAg-specific T cells were observed in an AML patient (NC172PM) 2 weeks prior to a diagnosis of relapsed disease, and in a myeloma patient (TS125PM) shortly after relapse. It may be that the increased presence of tumour in these patients resulted in a higher frequency of T cells, which were then detectable. Patient NC172PM did not survive following relapse, but had also suffered a number of complications including graft failure. Although the relapsed myeloma patient has remained stable since, definitive conclusions cannot be drawn about whether such responses could aid survival or be an indication of disease as the patient was treated with thalidomide for the relapse, and it is not known if CTAg-specific T cells could have been detected before relapse. A previous study in 106 myeloma patients detected humoral responses to CTAgs post-stem cell transplantation, and found in one relapsed patient, T cell responses to NY-ESO (Atanackovic et al., 2007). This study has found CD8+ T cells which respond to MAGE-A2 and pools of peptides from MAGE-A1 and MAGE-C2 in one relapsed myeloma patient. It may be the case that following relapse, where there is an increased number of tumour cells, there could be more antigen present to stimulate an immune response.

Paired blood and bone marrow samples were obtained from one of the responding AML patients, and frequencies of CTAg-specific CD8+ T cells were found to be up to seven times higher than in peripheral blood. If there are residual tumour cells present, they are most

likely to reside in the bone marrow cavity and therefore within this site, there would be more antigen to elicit T cell responses.

This study, nevertheless, has shown for the first time, T cell responses to CTAgs in AML patients following allogeneic stem cell transplantation. Due to the nature of the screening assay, we could only screen for a limited panel of CTAg peptides, which may have resulted in underestimating the number of AML patients who could have CTAg-specific T cells. Reliance of IFN-γ secretion to detect responding cells may have led to an incomplete picture of the breadth of responses and further underestimation of the frequencies of responses. Future studies would perhaps utilise another marker such as CD137 upregulation which would detect cells responding by means other than IFN-γ secretion. This may be particularly important when establishing the role of CD4+ T cells in the GvL response.

Previous studies have reported different findings when investigating RNA expression of CTAgs in AML (Adams et al., 2002, Chambost et al., 2001, Martinez et al., 2007). This may be due to the function of these genes. It has been hypothesized that CTAgs have a role in tumour progression closely related to their reproductive system function. Their expression is transient in spermatogonia and is lost on differentiation into sperm cells. Similarly it may be that cancer stem cells express CTAgs which are lost as cancer cells differentiate (Old, 2007). This could explain huge differences between the numbers of myeloma patients who have CTAg-specific T cells as shown in previous studies by colleagues (Goodyear et al., 2005, Goodyear et al., 2008) and the number of non-transplanted AML patients. Another study in this institute has found that AML cell lines express fewer CTAgs than myeloma cell lines (Goodyear et al., 2010). Therefore, CTAg expression may be more transient in AML due to the acute nature of the condition compared to myeloma. If leukaemic stem cells express CTAgs whereas AML blasts are less likely to, then it is tempting to speculate that this is

reflected in the observation of higher frequencies of CTAg-specific T cells in the bone marrow of AML patients. Transient expression on selected cells may also account for the lack of MAGE antibody staining in the immunohistochemistry study.

These data raises the possibility that following transplantation, if there are low numbers of residual leukaemic stem cells present expressing CTAgs, immune responses can be directed against these antigens and contribute to a graft versus leukaemia effect.

The experience with the contaminated GDNQ peptide shows the importance of keeping an open mind about unexpected results, in this instance the implausibly high frequency responses that well out of the range of 'usual' tumour antigen responses. It was tempting to speculate that it was an extraordinarily good finding, but the frequencies were very much higher than reported responses to other tumour antigens, and the thorough investigation into the nature of those responses strongly suggested that there was a contamination, which when identified was disappointing. However, it is satisfying that erroneous results were not reported.

Somewhat surprisingly, the majority of T cell responses were specific for peptides derived from MAGE proteins. Although some studies found that MAGE was not expressed in AML (Chambost et al., 2001, Meklat et al., 2007), mRNA expression was demonstrated (Adams et al., 2002, Martinez et al., 2007). The fact that MAGE expression is highly restricted to germline cells and malignant tissue is likely to dictate that MAGE-specific immune responses are manifest as a GvL response rather than GvHD.

In conclusion, CTAg-specific CD8+ T cells have been demonstrated in patients following reduced intensity stem cell transplantation. The possible association between the early detection of such an immune response and prolonged disease remission provides support

for the concept that CTAg-specific immunity plays an important role in GvL. Further studies are now indicated to explore the potential for enhancing CTAg-specific immunity in the post-transplant setting.

Locally managed clinical trials to investigate the anti-tumour, and GvL enhancing potential of treating AML (non-transplanted or transplanted) patients with a demethylating agent, 5-azacytidine, with a histone deacetylase inhibitor (HDACi) in order to upregulate tumour antigens such as CTAgs, is being carried out. Early monitoring of the non-transplanted cohort of patients for responses to CTAg antigens was carried out as part of this study, although no responses were seen. However, monitoring was subsequently taken over by the dedicated trial team, and after several rounds of treatment, an increased frequency of patients developed CTAg-specific responses (Goodyear et al., 2010). This provides further evidence that CTAgs can elicit immune responses and for the exciting possibility that their expression can be pharmacologically enhanced for the benefit of patients.

Chapter 5: CD4+ T cell Subsets in Graft-versus-Host Disease

5.1 Introduction

A recently defined subset of CD4+ T cells, principally characterised by secretion of the cytokine IL-17A, has been shown to form part of the normal inflammatory response (Harrington et al., 2005, Langrish et al., 2005, Park et al., 2005). This subset, known as Th17 cells, has been implicated in a number of inflammatory conditions (Barczyk et al., 2003, Kirkham et al., 2006, Krueger et al., 2007, Matusevicius et al., 1999, Molet et al., 2001, Pene et al., 2008) and there is much interest as to whether manipulating the role of the Th17 subset in inflammation could be a viable treatment strategy for such conditions.

Graft-versus-host disease (GvHD) is a continuing clinical challenge to patients undergoing allogeneic stem cell transplantation (SCT) to treat haematological malignancies, and there have been attempts to ascertain the potential role of this Th17 lineage in the pathogenesis of the disease. Studies carried out in mouse models have reported conflicting findings. In some models the Th17 subset was found to have a causative role in the development of GvHD (Carlson et al., 2009), whereas others have shown that continued IL-17A production is not required to sustain GvHD (Kappel et al., 2009). Another study proposed that the presence of Th17 cells from the donor in the allograft could in fact have a protective effect and lead to suppression of GvHD (Yi et al., 2008).

There has been recent literature challenging the traditional notion that once a T cell's lineage has been determined, then it is irreversibly committed. It has been shown that it is possible for a Treg to differentiate into Th17 (Koenen et al., 2008) and this raises the possibility that it could happen in reverse. This may be the basis for the presence of Th17 cells in the allograft leading to reduced GvHD in the previously highlighted mouse model. Such a change may well depend on the immune environment within the recipient. It has

been proposed that a key factor in the development of both acute and chronic GvHD is a decreased frequency of Tregs (Chen et al., 2007).

The purpose of this study was to investigate the presence of Th17 lineage cells in human allograft patients, to characterise their chemokine profiles and relate the frequencies to those of Th1 and Treg cells during and before/after episodes of GvHD. I also investigated the serum levels of these cytokines in relation to GvHD.

5.2 Optimisation of Foxp3 and Th17 intracellular staining

The samples used in this study had been collected from patients attending a bone marrow transplant clinic at the Queen Elizabeth Hospital, Birmingham. Ethical approval had been received for the collection of 50ml peripheral blood every two weeks from allograft patients. Lymphocytes were isolated from the fresh samples, on the day of collection, according to the protocol outlined in Chapter 2 (see Methods 2.1.3). The cells were cryogenically preserved in a liquid nitrogen storage facility. Clinical information about whether a diagnosis of GvHD had been made was recorded. This was a retrospective study and in many cases only a small number or single samples were available. Therefore, in order to prevent wasting precious samples, the assays were tested using cryogenically preserved PBMCs from healthy donors.

The two intracellular staining protocols used were described in detail in Chapter 2 (see 2.3 & 2.4). Foxp3 staining was carried out using a Foxp3 staining and buffer kit (eBioscience, Hatfield, UK) and the Th1 / Th17 experiments used an established protocol with monensin as a protein transport inhibitor, paraformaldehyde as a fixative and

saponin for permeablisation. The antibody panels are summarised here in table 5.2. All analysis was carried out using a BD LSRII flow cytometer with BD FACS DiVa software (BD Biosciences, Oxford, UK). CD4+ T cells were selected by gating on the CD3+ CD8- population in order to avoid results bias resulting from CD4 downregulation following activation. Live/Dead Fixable Red dye (Invitrogen, Paisley, UK) was used to exclude dead T cells.

FoxP3 Panel Ab	Manufacturer	Th1&17 Panel Ab	Manufacturer
CD127-FITC	eBioscience	CXCR3-PE	BD Bioscience
Foxp3-PE	eBioscience	CCR4-PC7	BD Pharmingen
CD25-PC5	Beckman Coulter	IL-17A-Pacific Blue	Biolegend
CD4-PC7	eBioscience	CD8-AmCyan	BD Biosciences
CD8-Pacific Blue	eBioscience	CCR6-APC	R&D Systems
CD45RA-AF 700	Biolegend	IFN-γ AF-700	Biolegend
CD3-APC-Cy7	Biolegend	CD3 APC-Cy7	BD Biosciences

Table 5.2 Antibody panels used for Treg / Th1 / Th17 staining

Antibody panels used for identification and characterisation of Foxp3+ Tregs (left), or Th1 and Th17 cells (right).

5.2.1 Testing of assays on healthy donors

The basic antibody panel and kit used to identify Tregs had already been established in this laboratory, but minor modifications were verified with healthy donor PBMCs, prior to use with patient samples (figure 5.2.1.1). The conjugated antibodies used for the Th1 & Th17 panels were selected so as to avoid high degrees of spectral overlap. Antibodies were titrated, and then tested in combination to optimise concentrations and build an analysis protocol for the LSRII (figure 5.2.1.2). The Th17 gating strategy was to first select the lymphocyte population, then exclude cells stained with the dead cell exclusion marker. A gate was then used to select the population that was CD3+CD8-. The relative proportions of Th17 cells were established by IL-17A secreting cells, and Th1 by those producing IFN-γ. Cells that secrete both cytokines have been described in literature as Th17-1 (Dhodapkar et al., 2008) and were double-positive in this staining. Gating on each of those populations allowed the frequencies of chemokine receptors CCR4, CCR6 and CXCR3 to be established for each subset. In some experiments, CCR6-FITC was used in place of CCR6-APC.

The transcription factor Foxp3 is widely accepted to be the gold standard marker used to recognise Tregs by antibody staining, and the equivalent transcription factor responsible for IL-17A production has been shown to be RORγt (Ivanov et al., 2006). During the course of this study an antibody to this protein became commercially available. Despite the manufacturer's statement that they had only been able to stain *in vitro* generated Th17 cells, a trial sample was available so it was decided to try the antibody on *ex vivo* samples to see if it could be used to back up IL-17A cytokine staining. However, it proved to be ineffective in this situation (figure 5.2.1.3). CD4+ IL-17A+ T cells were not positive for RORγt when using this antibody.

Some protocols suggest that PBMCs should be 'rested' in culture medium for a few hours or overnight before use in an experiment. However, this may lead to cells dying or becoming anergic. In order to investigate this, an experiment was carried out to compare intracellular cytokine staining for IL-17A and IFN-γ, between a sample rested overnight and one used immediately after thawing. Both samples were aliquots of one donation from a healthy donor, and had hitherto been treated identically. There was an apparent decrease in detection of IL-17A secreting cells from 0.6% to 0.4% and the frequency of IFN-γ secreting cells was reduced by almost half from 13.4% to 7.6% when cells were rested. The proportion of cells secreting both cytokines was 0.2% without resting, but fell to <0.1% when the assay was delayed (figure 5.2.1.4).

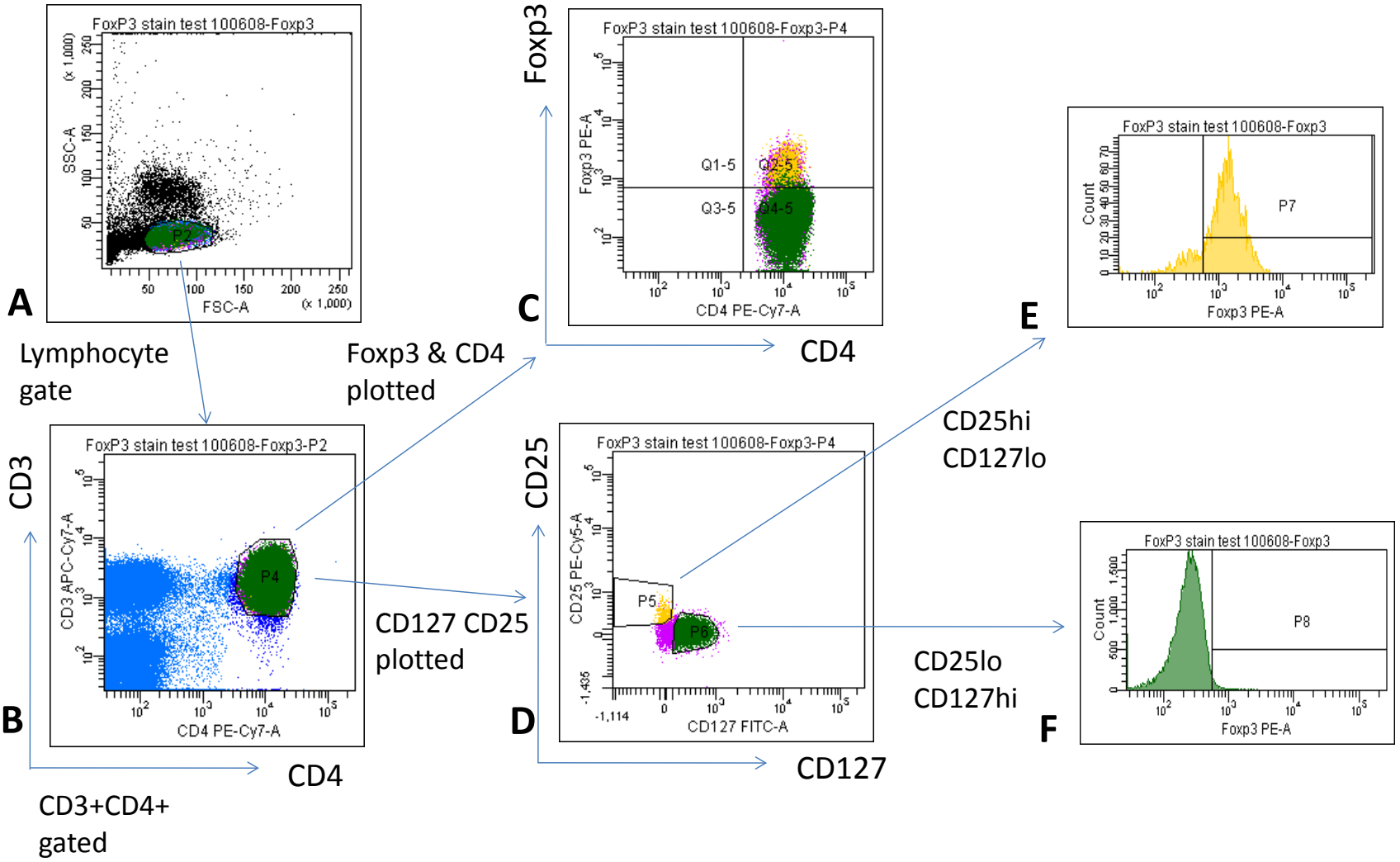

A
FoxP3 stain test 100608-Foxp3
SSC-A
FSC-A
Lymphocyte gate
B
FoxP3 stain test 100608-Foxp3-P2
CD3
CD4
CD3 APC-Cy7-A
CD4 PE-Cy7-A
P4
CD3+CD4+ gated
Foxp3 & CD4 plotted
C
FoxP3 stain test 100608-Foxp3-P4
Foxp3
CD4
Foxp3 PE-A
CD4 PE-Cy7-A
Q1-5
Q2-5
Q3-5
Q4-5
CD127 CD25 plotted
D
FoxP3 stain test 100608-Foxp3-P4
CD25
CD127
CD25 PE-Cy5-A
CD127 FITC-A
P5
P6
CD25hi CD127lo
E
FoxP3 stain test 100608-Foxp3
Count
Foxp3 PE-A
P7
CD25lo CD127hi
F
FoxP3 stain test 100608-Foxp3
Count
Foxp3 PE-A
P8

Figure 5.2.1.1 Gating strategy for identification of Foxp3+ CD4+ T cells by flow cytometry

Cryopreserved healthy donor PBMCs were thawed and stained using a Foxp3 staining kit (eBioscience). The lymphocyte population was identified on a foward-scatter (FS) / side-scatter (SS) plot and gated (A). All subsequent plots were descendents of this gate. The CD3+ CD4+ population was then gated (B) and the percentage of Foxp3+ T cells in the whole CD4+ T cell population could be determined (C). CD25 and CD127 were plots were drawn from the CD3+CD4+ population (D) and the frequency of Foxp3+ cells in $CD25^{hi}$ $CD127_{lo}$ (E) and $CD25_{lo}$ $CD127^{hi}$ (F) populations could be measured.

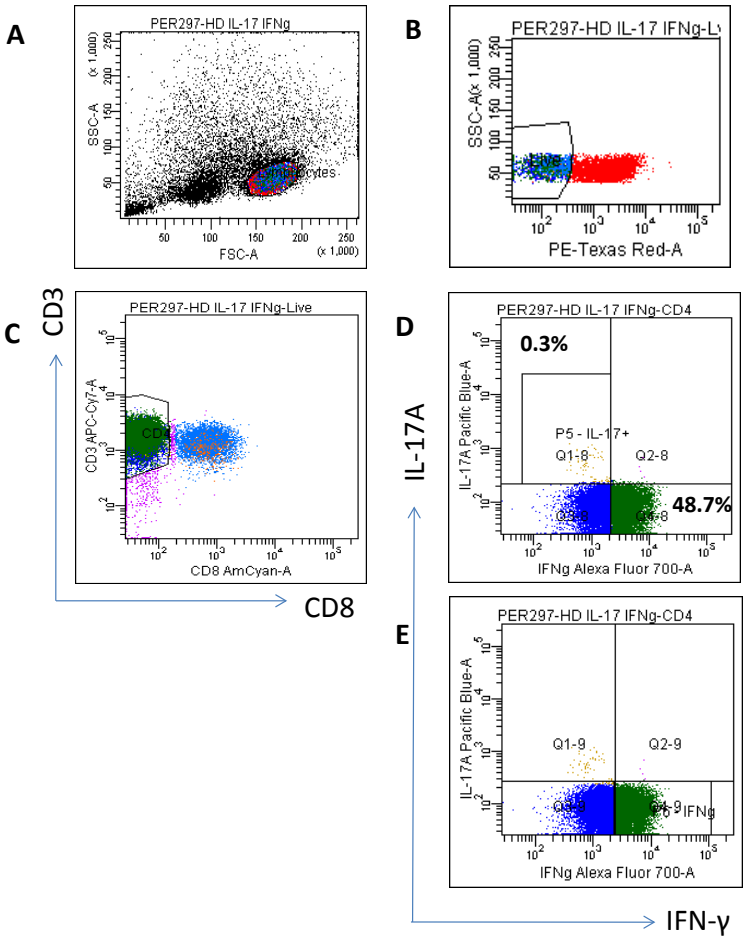


Figure 5.2.1.2 IFN-γ and IL-17A staining of healthy donor PBMCs

Intracellular cytokine staining for IL-17A and IFN-γ was carried out on PMA/Ionomycin stimulated, cryopreserved PBMCs from a healthy donor. The lymphocyte population was gated using a forward scatter (FSC) / side scatter (SSC) plot (A) and then dead cells were excluded by gating on the population negative for Live/Dead Red stain (B). All further gating was carried out on this population. CD4+ T cells were selected by gating on CD3+ CD8- cells (C). IFN-γ and IL-17A were plotted against each other and then gates were drawn around Th17 (D) or Th1 cells (E) for further characterisation.

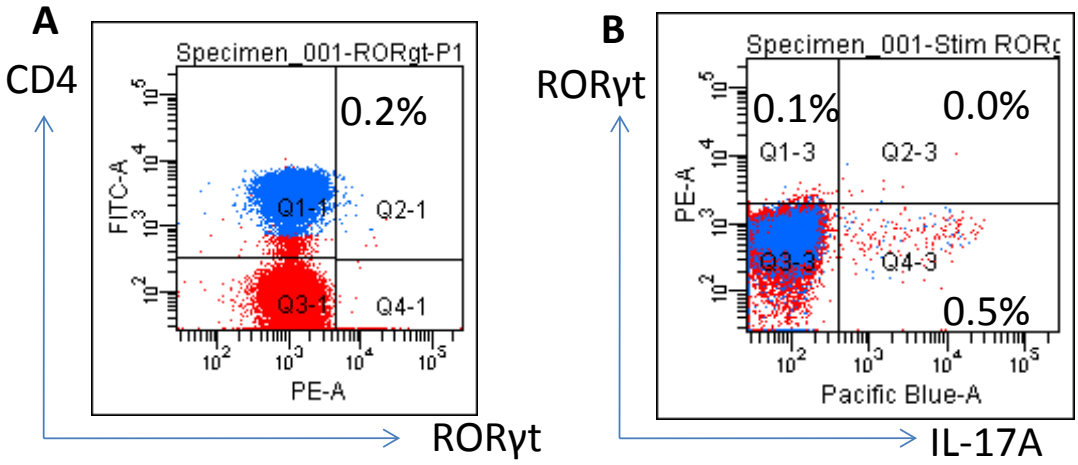


Figure 5.2.1.3 Commercially available RORγt antibody does not stain IL-17A secreting cells

Intracellular cytokine staining was carried out on healthy donor PMA/Ionomycin stimulated PBMCs, which were stained with CD4-FITC and either RORγt-PE antibody (A) or a combination of RORγt-PE and IL-17A-Pacific Blue (B) to investigate whether this commercially available RORγt antibody could co-stain IL-17A secreting cells.

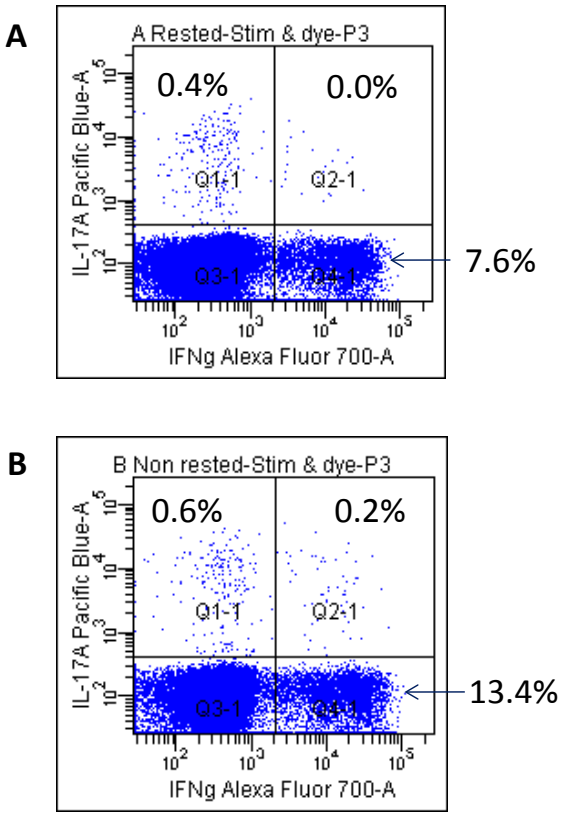


Figure 5.2.1.4 Effect of 'resting' thawed PBMC samples prior to intracellular cytokine staining

Flow cytometric plots of IL-17A and IFN-γ intracellular cytokine staining (ICS) of gated CD4+ (CD3+ CD8-) T cells. PBMCs were isolated from a healthy donor and cryopreserved. One aliquot was thawed and incubated overnight at 37^{o}C 5% CO2 prior to ICS (A), and another was thawed immediately prior to ICS (B). Both assays were carried out concurrently. Frequencies of IL-17A (upper left quadrant), IFN-γ (lower right quadrant) or IL-17A/IFN-γ (upper right quadrant) are shown.

5.2.2 Testing of compensation beads as a replacement for cell compensation controls

The BD LSRII flow cytometer has an autocompensation facility for effective compensation of large antibody panels. Some drawbacks to using cells to compensate are that precious cells that could be used for analysis are diverted to set-up the equipment, and rare antigens may not lead to sufficient fluorescence for accurate compensation. To overcome these problems, compensation beads may be used, which are polystyrene microparticles which are designed to have similar fluorescent properties to lymphocytes. They bind any kappa light chain-bearing immunoglobulin. Therefore, they overcome the problem of low antigen expression to provide a sufficiently bright staining control. The relatively stable binding of antibodies to these beads means they could potentially be used on more than one occasion.

Tests were carried out to determine the stability of these antibody/bead complexes over time (figure 5.2.2.1). The compensation was sufficiently comparable between beads and cells and the positions of the peaks for compensation beads did not change over 10 days. After 19 days, it was observed that there were shifts in peaks for some fluorochromes, and some conjugates had deteriorated. It was decided that one set of compensation beads could be used for multiple experiments for up to one week. A problem did occur with the negative population shifting away from the axis leading to autocompensation errors for some fluorochromes. However, after discussions with the software manufacturers, a gating strategy was developed to accurately define the negative population which overcame this.

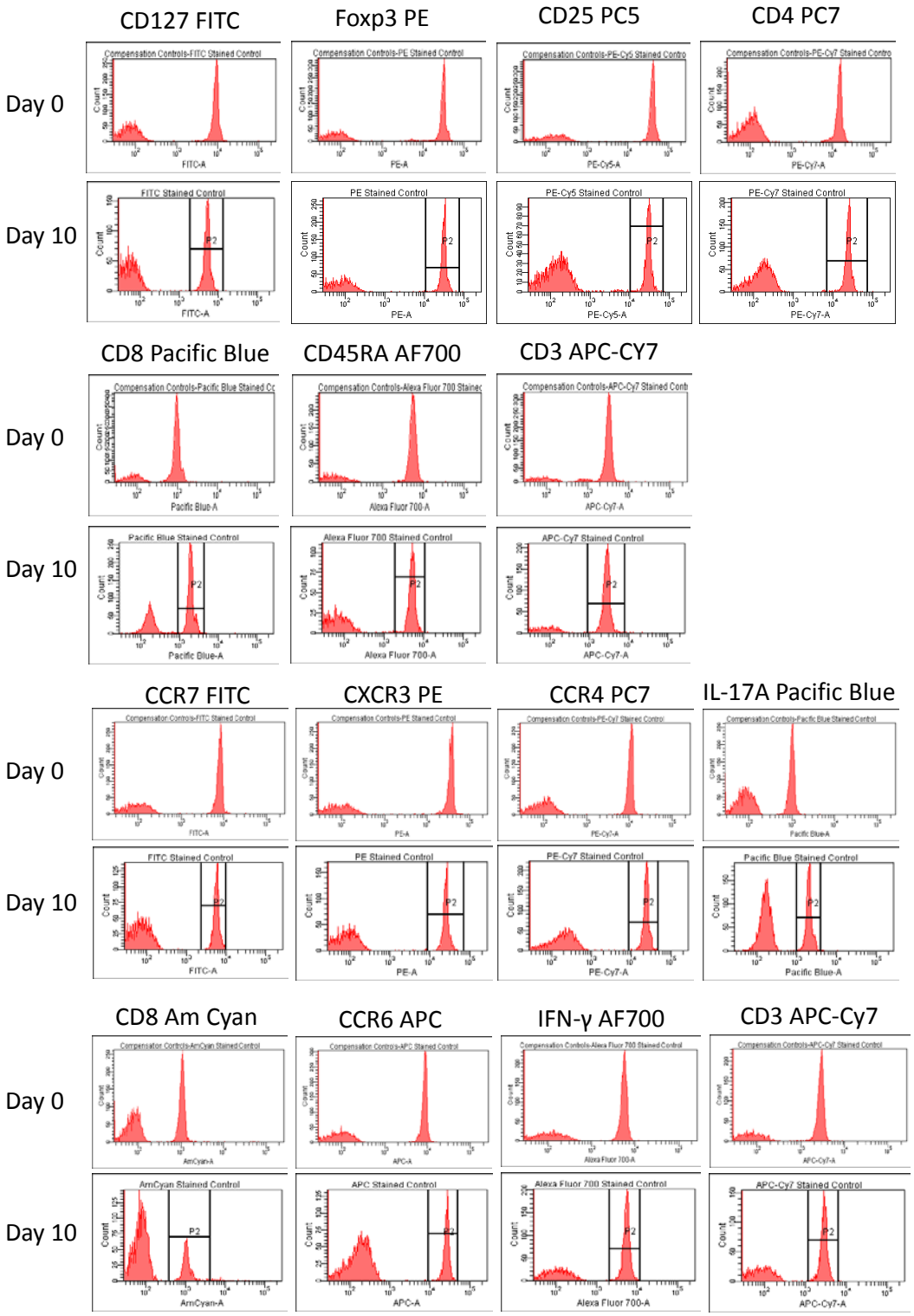


Figure 5.2.2.1 Compensation bead/antibody complex stability over time

Comparison of peak fluorescence (x-axis) between compensation beads run fresh and on day 10 of refridgerated storage, for all fluorochromes used in staining panels.

5.3 Determination of T cell subsets in GvHD patients by intracellular cytokine staining

The main purpose of this study was to establish whether there was any relationship between frequencies of Tregs, Th1, Th17 T cell frequencies and the occurrence of GvHD. Three cohorts of patients were studied in total: those with acute GvHD, with chronic GvHD or no GvHD. Diagnosis and grading of GvHD was made by clinicians at the Queen Elizabeth Hospital. Retrospective analysis of cryopreserved PBMCs was carried out on samples collected during, prior to and/or following GvHD. T cell subset frequencies in samples taken during GvHD were compared with those when there was no active GvHD (before or after). Samples from the cohort of patients who had not had any diagnosis of GvHD were selected to match time-points of samples screened from GvHD patients. Control patients had undergone similar transplant conditioning regimens.

The protocols used for staining were described fully in Methods (sections 2.3 and 2.4). Samples were thawed and then split to be stained immediately for Foxp3 or for IL-17A and IFN-γ staining. Surface antibody staining was carried out initially and then the Foxp3 staining protocol commenced. The IL-17A/IFN-γ cohort was further split for stimulated and unstimulated experiments. PMA/Ionomycin was added to the stimulated experiment tubes, followed by 2ng/ml monensin to all tubes. Both stimulated and unstimulated tubes were incubated for a total of 6 hours at 37^{o}C 5% CO_2. All tubes were then moved to 4^{o}C overnight before intracellular cytokine staining.

Acute GvHD patients' samples were available before or during GvHD. Chronic GvHD patients were screened: (A) before, during and after GvHD; (B) during and before; or (C) during and after GvHD. Control samples were selected to reflect the time-points from cGvHD patients,

and are shown as early, mid or late time-points. These are arbitrary categories due to the varied ranges of time-points of the GvHD cohort patients.

5.3.1 Treg frequencies in GvHD and control patients

Samples were stained using the Foxp3 staining kit (eBioscience, Hatfield, UK) according to the manufacturer's instructions. The frequency of Foxp3+ve T cells in the CD4 populations was established. Tregs are generally considered to be $CD127_{lo}$ and $CD25^{hi}$ so these markers were included as confirmation. A CD8 antibody was included to determine whether any CD8+Foxp3+ve cells could be detected, although none were observed.

The frequencies for each patient are shown as linked time-points (figure 5.3.1.1A and B). This study was primarily interested in how the frequencies changed between time-points where the patients were suffering GvHD and those where they were not. In some cases, there were elevated levels of Tregs during GvHD, but in other cases frequencies were stable or decreased. The frequencies of Tregs in samples taken during GvHD episodes are shown (figure 5.3.1.1C) alongside controls samples taken at similar times from patients who had not suffered GvHD. Median frequencies of Tregs for samples in each group were 5.9% (acute GvHD), 7.5% (acute controls), 6.4% (chronic GvHD) and 4.3% (chronic controls), but there were no significant differences between any groups.

The frequencies of Tregs for all patients in each cohort and all time-points were plotted (figure 5.3.1.2) in order to see whether any increases could potentially be due to reconstitution. However, there was no significant relationship between either elevated or lowered Treg frequencies, and time post-transplantation. There was a slight trend towards increasing Treg frequencies in control patients, but this was not observed with patients. It may be related to GvHD, but that could not be confirmed with this data.

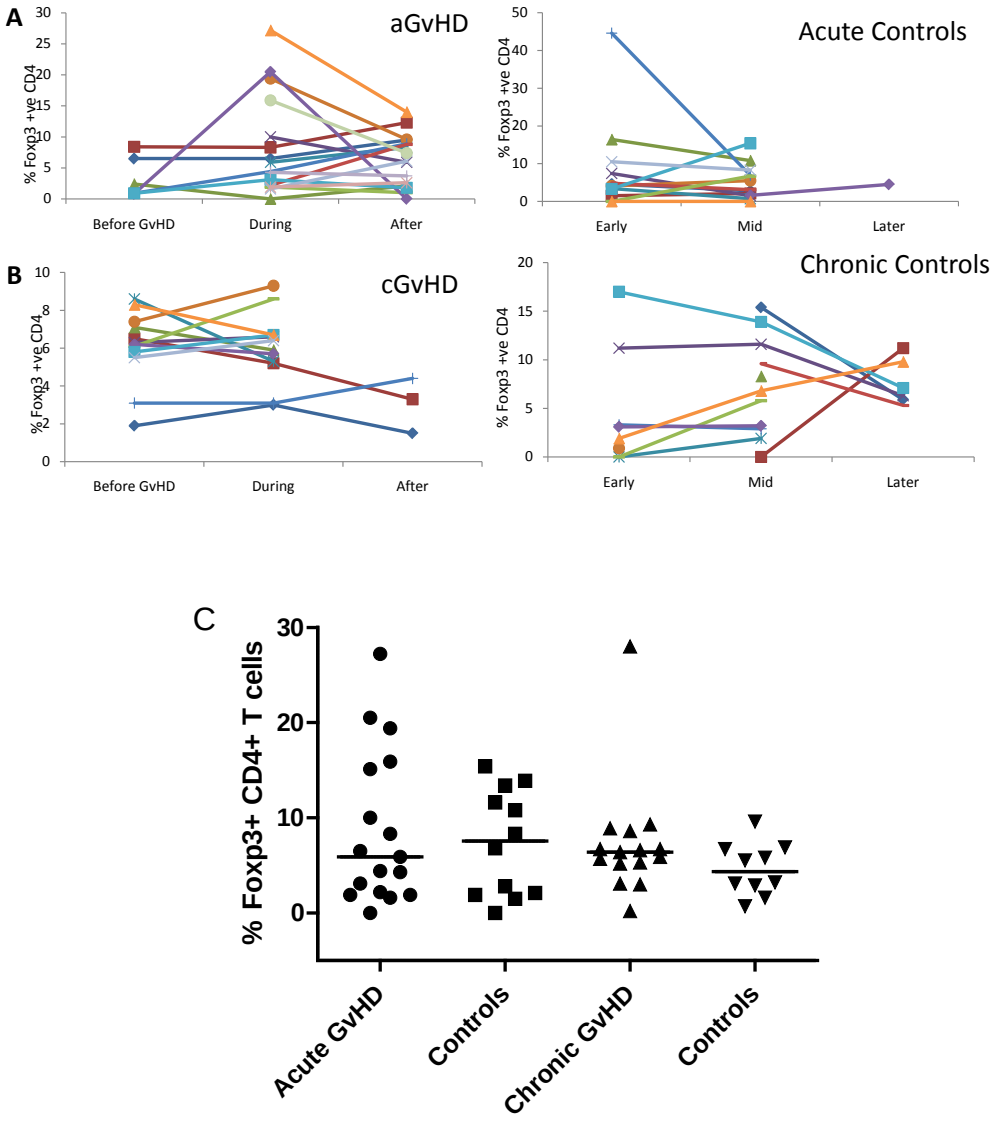


Figure 5.3.1.1 Frequencies of Foxp3+ve Tregs in GvHD patients and controls

Intracellular cytokine staining was used to determine the frequencies of Tregs (y-axis) in peripheral blood samples taken from acute GvHD patients and transplant patients who had not had GvHD (A) or chronic GvHD and patients who had not suffered GvHD (B). Samples were taken during GvHD episodes and before/after disease. Different time-points from the same patient are linked with an individually coloured line. The frequencies of Tregs in GvHD samples are shown for acute or chronic GvHD patients compared to samples taken at similar times post-transplantation from patients who had not suffered GvHD (C). Lines represent median values.

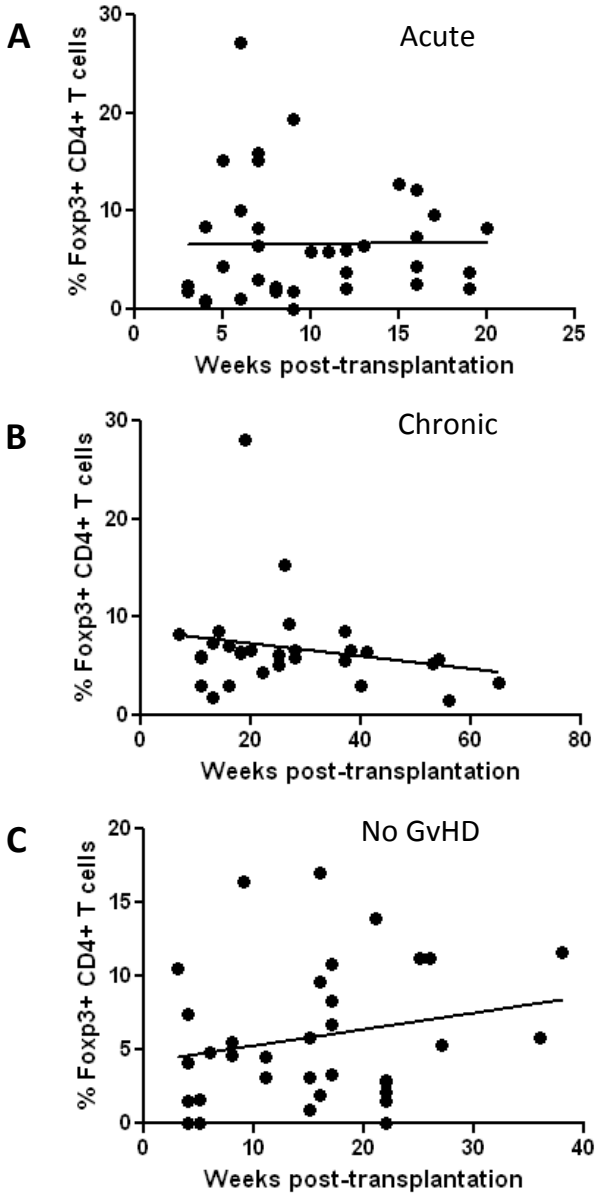


Figure 5.3.1.2 All patient Treg frequencies over time

All frequencies of Foxp3+ CD4+ T cells (y-axis) at each time-point (x-axis) were plotted for all acute GvHD (A), chronic GvHD (B) patients and non-GvHD sufferers (C), irrespective of GvHD episodes. Frequencies from samples taken at the same time-point are shown aligned on the graph.

5.3.2 Determination of Th1 and Th17 subsets in peripheral blood of GvHD patients

The same samples used for Foxp3+ T cell enumeration were used to establish the frequencies of IFN-γ secreting Th1 cells and IL-17A producing Th17 subsets. PMA/Ionomycin was used to stimulate cytokine production. The non-specific nature of this stimulus meant that the frequencies of all cells present would be revealed rather than preferentially stimulating one type. Stimulated and non-stimulated fractions were used, however it was felt that if a cell produces IL-17A in the non-stimulated fraction, then it was still a Th17 lineage cell so that to subtract frequencies in the unstimulated fractions as background was not appropriate. Therefore, all data presented is based on the frequencies of the stimulated fractions.

The frequencies of Th1 and Th17 subsets in samples taken before/during/after acute GvHD episodes (figure 5.3.2.1) or chronic GvHD (figure 5.3.2.2) are shown alongside time-matched controls. Samples taken at different times from the same patient are linked. Median frequencies of T cell subsets in samples during GvHD episodes were compared to median frequencies of these subsets in control samples taken from control patients at similar times (figure 5.3.2.3). No significant differences were found between GvHD and control sample median frequencies. A trend towards decreased Th17 frequencies in acute GvHD samples was observed, this proved to not be significant (p=0767).

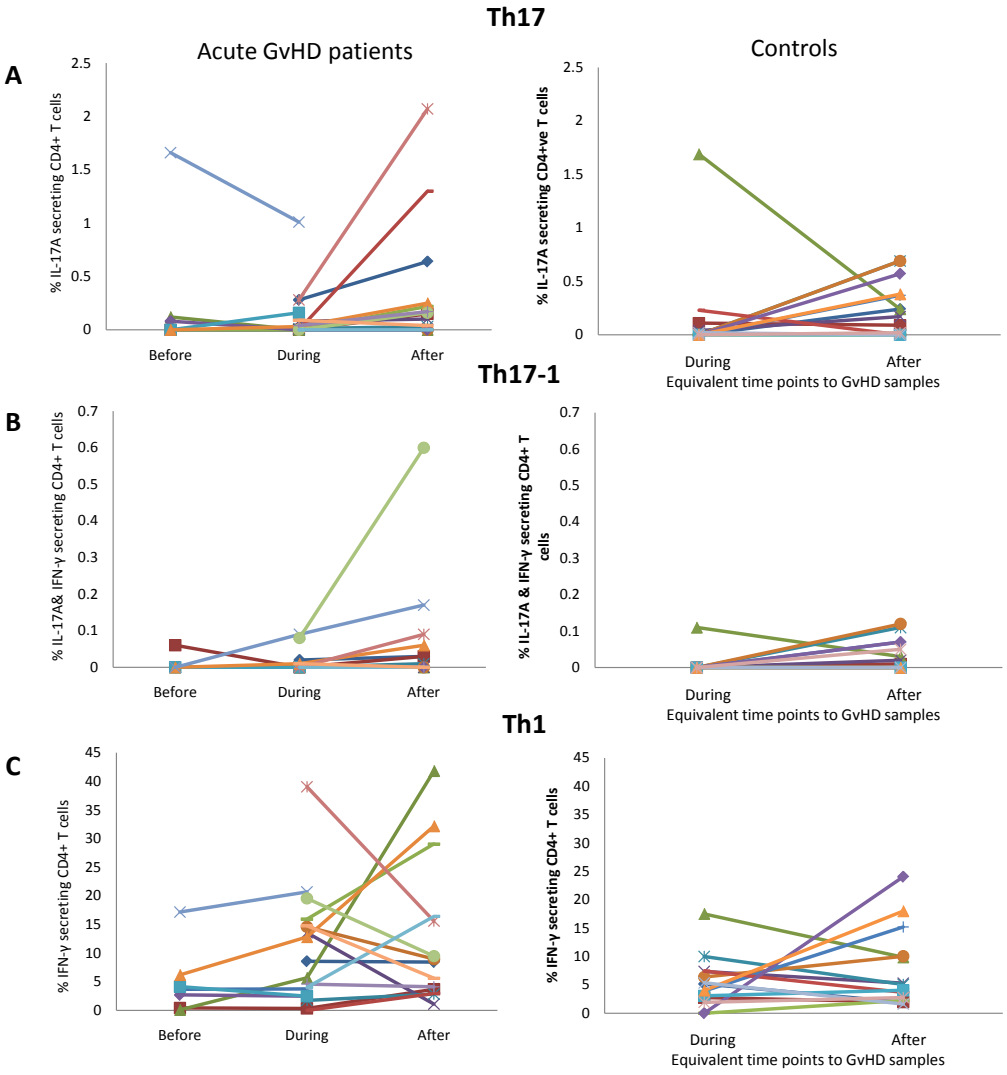


Figure 5.3.2.1 Frequencies of Th1, Th17 and Th17-1 cells in acute GvHD patients before during and after disease episodes

Frequencies of T cell subsets (y-axis) in acute GvHD patients as measured by IFN-γ and IL-17A production following PMA/Ionomycin stimulation. Those cells secreting IL-17A only were considered to be Th17 lineage (A), IFN-γ and IL-17A together were Th17-1 (B) and IFN-γ only were Th1 (C). Samples were taken before/during/after GvHD episodes (x-axis). Different time points from the same patient are shown by linked points and each patient is represented by a different coloured line. Control samples were taken at equivalent times post-transplantation from patients without GvHD and are shown on the right. Controls were not available for all samples.

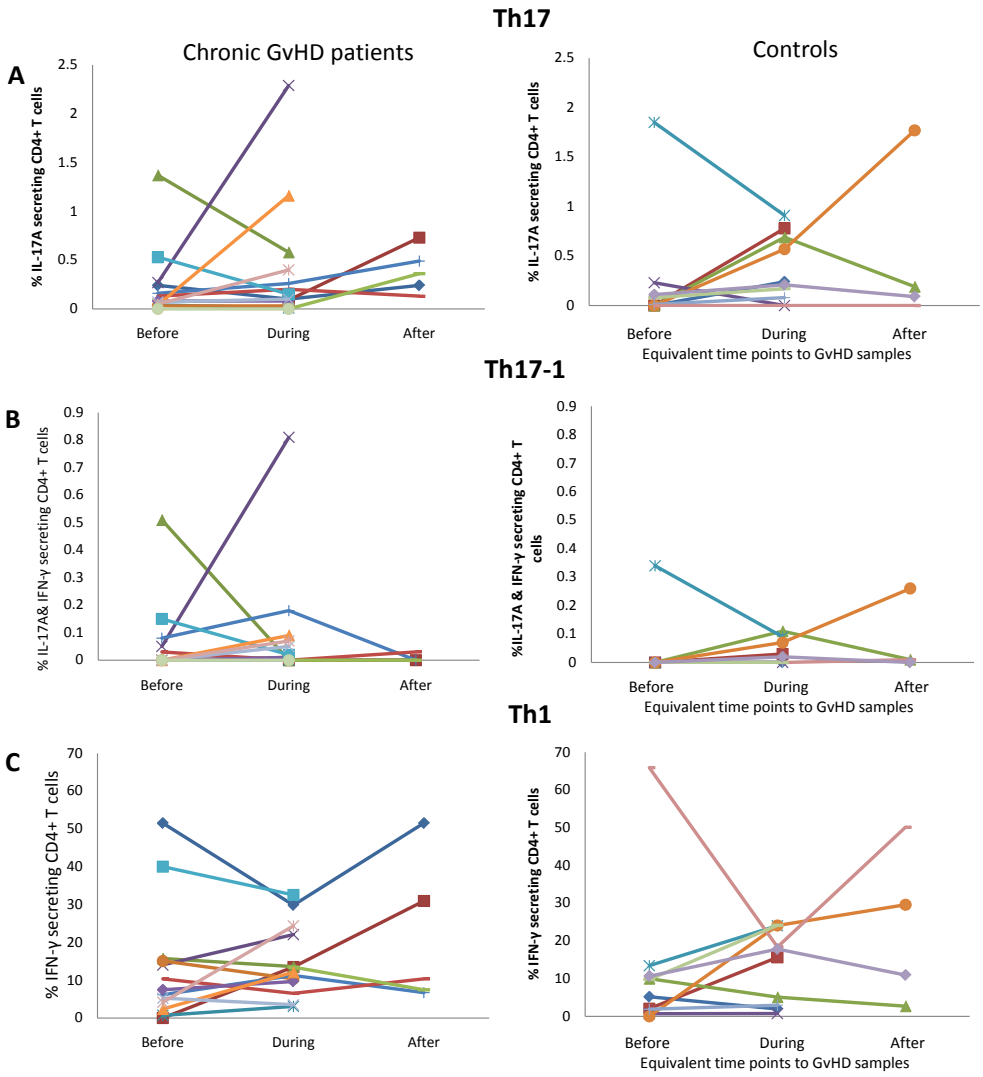


Figure 5.3.2.2 Frequencies of Th1, Th17 and Th17-1 cells in chronic GvHD patients before, during and after disease episodes

Frequencies of T cell subsets in chronic GvHD patients as measured by IFN-γ and IL-17A production following PMA/Ionomycin stimulation. Those cells secreting IL-17A only were considered to be Th17 lineage (A), IFN-γ and IL-17A together were Th17-1 (B) and IFN-γ only were Th1 (C). Samples were taken before/during/after GvHD episodes (x-axis). Different time points from the same patient are shown by linked points and each patient is represented by a different coloured line. Control samples were taken at equivalent times post-transplantation from patients without GvHD and are shown on the right. Controls were not available for ell samples..

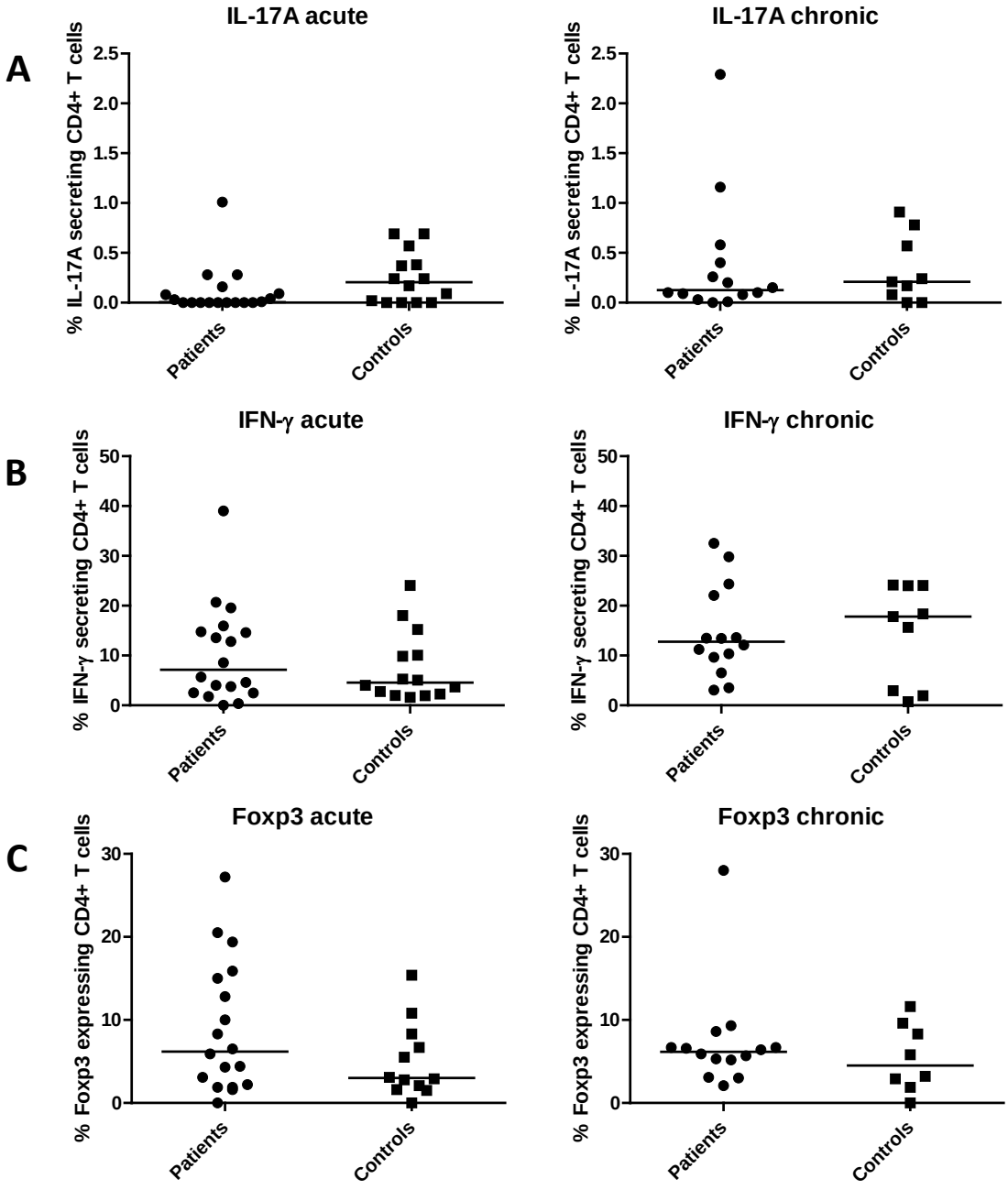


Figure 5.3.2.3 Median T cell subset frequencies in GvHD samples and time matched controls

Frequencies of Th17 (A), Th1 (B) and Treg (C) cells were determined by flow cytometry in SCT patients during episodes of acute GvHD (left column) or chronic GvHD (right column) and compared to time matched samples from SCT patients without GvHD. Values for frequencies of subsets are shown for each patient and median values are indicated.

Trends in overall frequencies regardless of GvHD

As previous figures have shown no clear association between GvHD and frequencies of Th1 or Th17 cells, every time-point for all patients in each cohort was plotted in order to investigate any trends that may be due to general reconstitution. There was no significant relationship between Th1 or Th17 frequencies and time post-transplantation in the acute GvHD cohort (figure 5.3.2.4), and Th17 frequencies did not significantly increase with time in the chronic GvHD cohort or controls. However, there was a significant increase in Th1 frequencies over time in both the chronic GvHD ($p=0.0146$) and all controls cohorts ($p=0.0229$) (figure 5.3.2.5 & figure 5.3.2.6). As there was a considerable range of frequencies at many time-points, the data was split into groups based on transplant type, to establish whether these frequency differences could be related to conditioning regimens. There appears to be little difference overall except that the chronic Th1 increase is not significant in patients who have undergone reduced intensity transplants, as opposed to the myeloablative transplant patients ($p=0.0041$). However, this is probably due to a single high frequency at 13 weeks in one patient. Nevertheless, there is a distinct upward trend in that cohort. Campath conditioning has been associated with delayed reconstitution (Morris et al., 2003) but that does not appear to be the case in these patients.

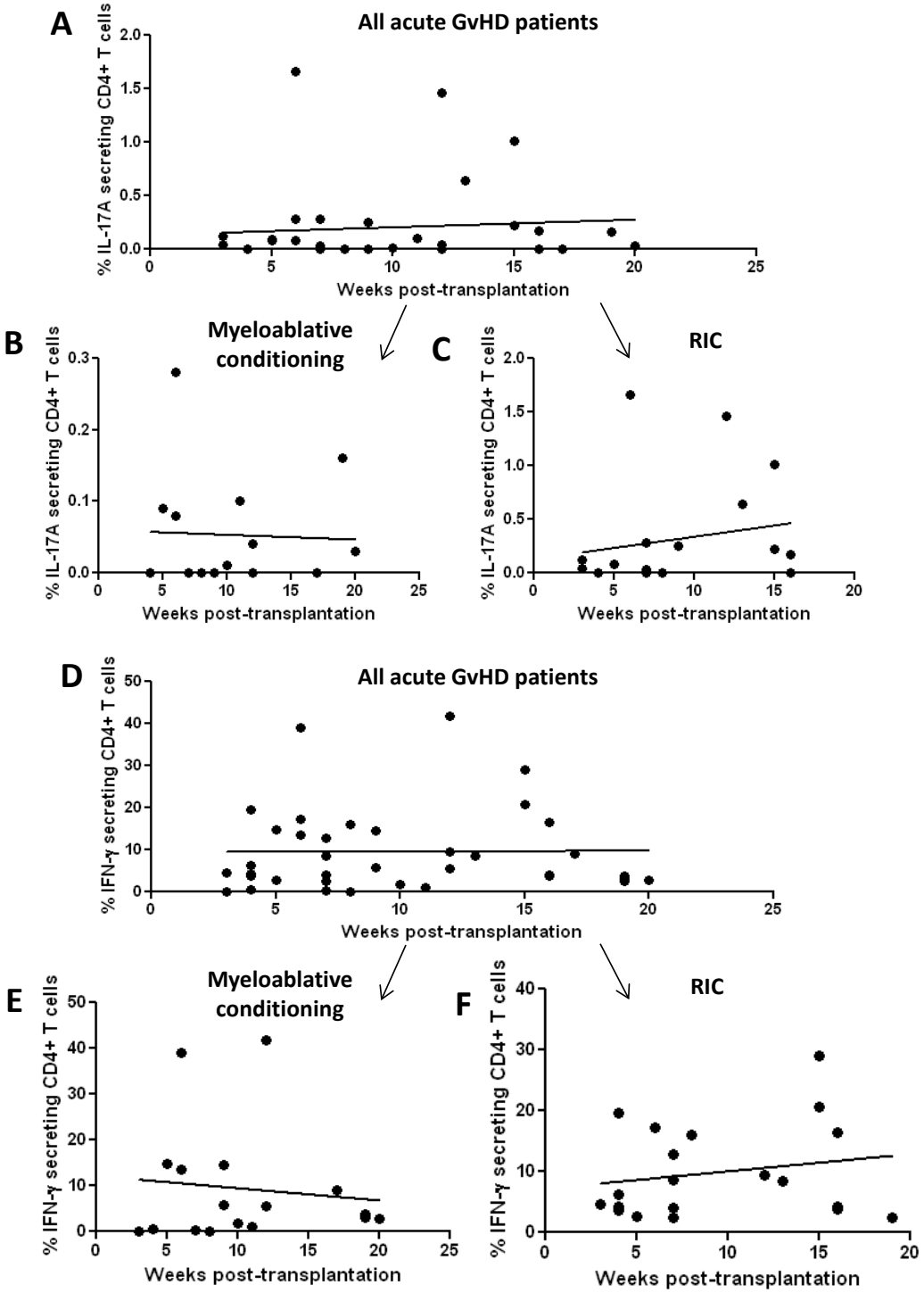


Figure 5.3.2.4 Frequencies of Th1 and Th17 subsets over time in all acute GvHD patients

Frequencies of Th17 cells (A-C) and Th1 cells (D-F) in blood samples from acute GvHD patients were measured by intracellular cytokine staining. Th17 levels shown in A are split into frequencies for patients who had myeloablative conditioning (B) and reduced intensity

conditioning (RIC) (C). Similarly, Th1 frequencies shown in D are divided into myeloablative (E) and reduced intensity (F) transplant conditioning regimens. T cell subset frequencies are shown on the y-axis and the numbers of weeks post transplantation are indicated on the x-axis.

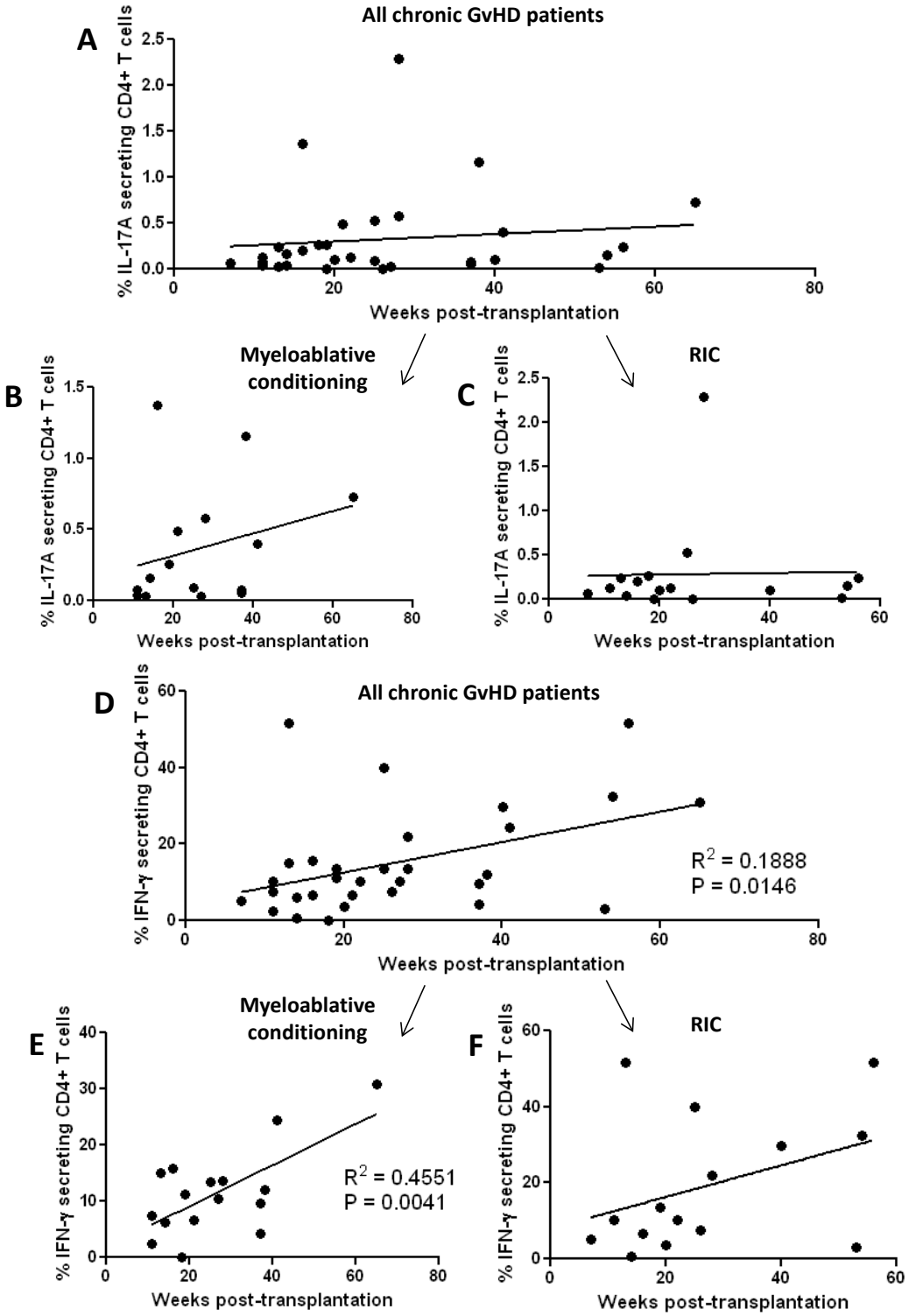


Figure 5.3.2.5 Frequencies of Th1 and Th17 subsets over time in all chronic GvHD patients

Frequencies of Th17 cells (A-C) and Th1 cells (D-F) in blood samples from chronic GvHD patients were measured by intracellular cytokine staining. Th17 levels shown in A are split

into frequencies for patients who had myeloablative conditioning (B) and reduced intensity conditioning (RIC) (C). Similarly, Th1 frequencies shown in D are divided into myeloablative (E) and reduced intensity (F) transplant conditioning regimens. T cell subset frequencies are shown on the y-axis and the numbers of weeks post transplantation are indicated on the x-axis.

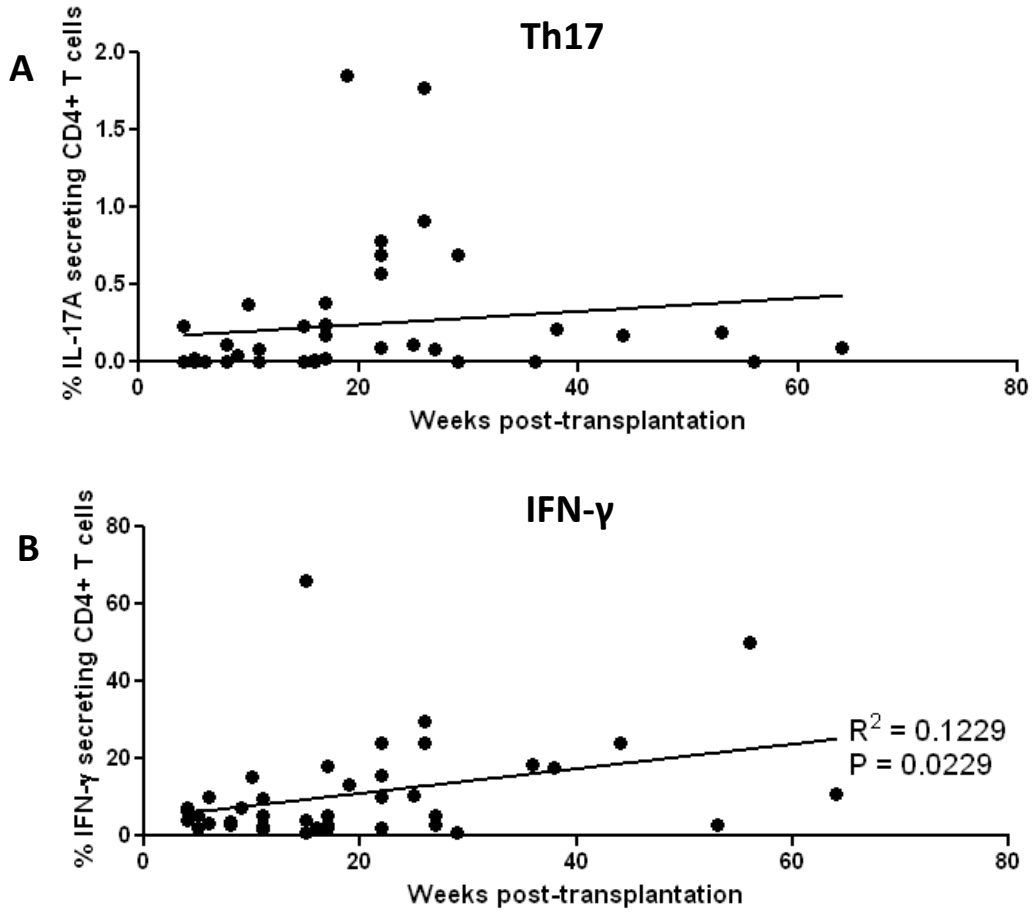


Figure 5.3.2.6 Frequencies of Th1 and Th17 subsets over time in all control patients

Frequencies of Th17 cells (A) and Th1 cells (B) in blood samples from stem cell transplant patients who had not had GvHD were measured by intracellular cytokine staining. T cell subset frequencies are shown on the y-axis and the numbers of weeks post transplantation are indicated on the x-axis.

5.3.3 Relationship of T cell subset frequencies to site and grade of GvHD

GvHD site

The main sites affected by GvHD are skin, liver and gut but any other tissue may be affected, which often includes eyes, mouth, lungs and joints. The most common sites in this study were skin and gut, with many patients affected in both.

T cell subsets in patients with GvHD of either skin or gut only were compared between samples taken during GvHD and those taken before/after disease (figure 5.3.3.1). Whilst there are differences in the changes in frequencies within patients in the same cohort, the most common trend is for Th17 and Th1 levels to be reduced in skin GvHD patients, but raised in gut GvHD patients. However, there are insufficient numbers of patients in each cohort to draw any definite conclusions. Patients affected in both sites were excluded from this analysis. Patients were not discriminated on the basis of acute or chronic GvHD. The cohorts consisted of six acute with seven chronic skin GvHD patients, and five acute with two chronic gut GvHD patients.

Severity of GvHD

Acute GvHD is graded clinically with increasing severity from grade 1 to grade 4. The relationship of GvHD severity on T cell subset frequencies was considered (figure 5.3.3.2) to establish whether increased severity could result from increased frequencies of Th17 of Th1 T cells, or a diminished Treg population. Of the 18 acute GvHD patients studied, eight had grade 0 or I, six had grade II and four had grade III disease. No definite associations could be made as some patients in all categories had increased levels or pro-inflammatory subsets whilst in others, frequencies were decreased.

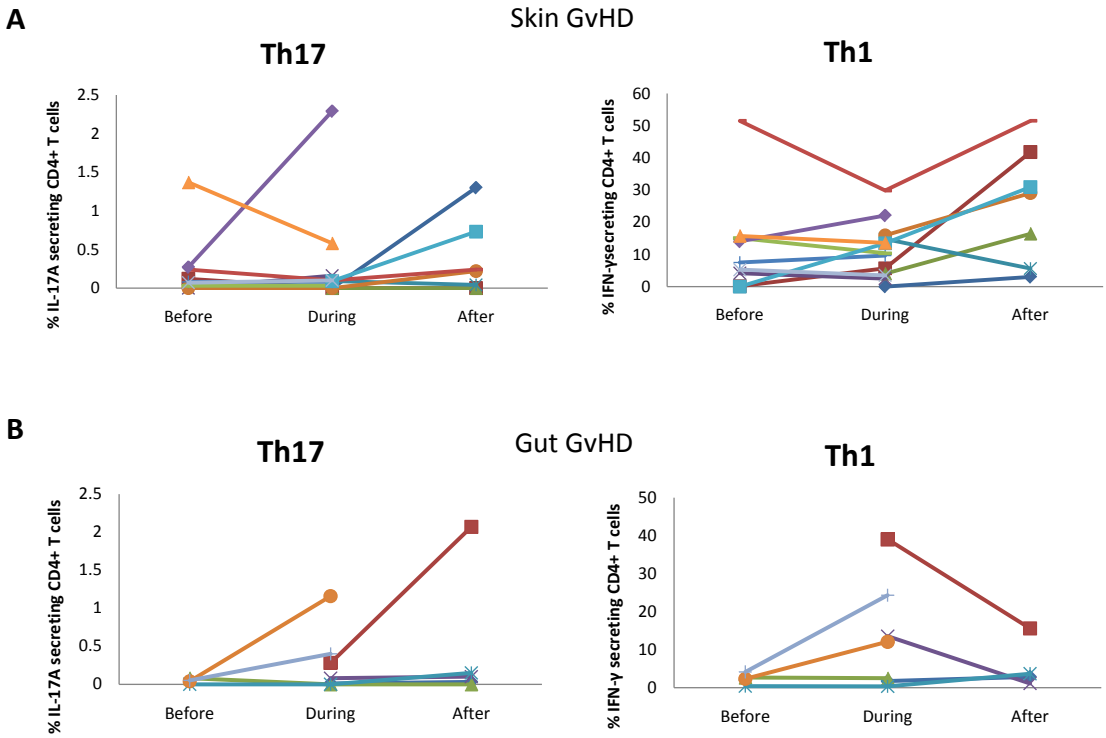


Figure 5.3.3.1 Differences of T cell subset frequencies at sites of GvHD

Frequencies of Th1 and Th17 subsets (γ-axis) in allograft patients who suffered GvHD manifested in skin (A) or gut (B) as measured by intracellular cytokine staining on PBMCs isolated from whole blood. The levels of subsets as determined by IL-17A or IFN-γ secretion are shown in samples taken during GvHD and before and/or after GvHD (x-axis).

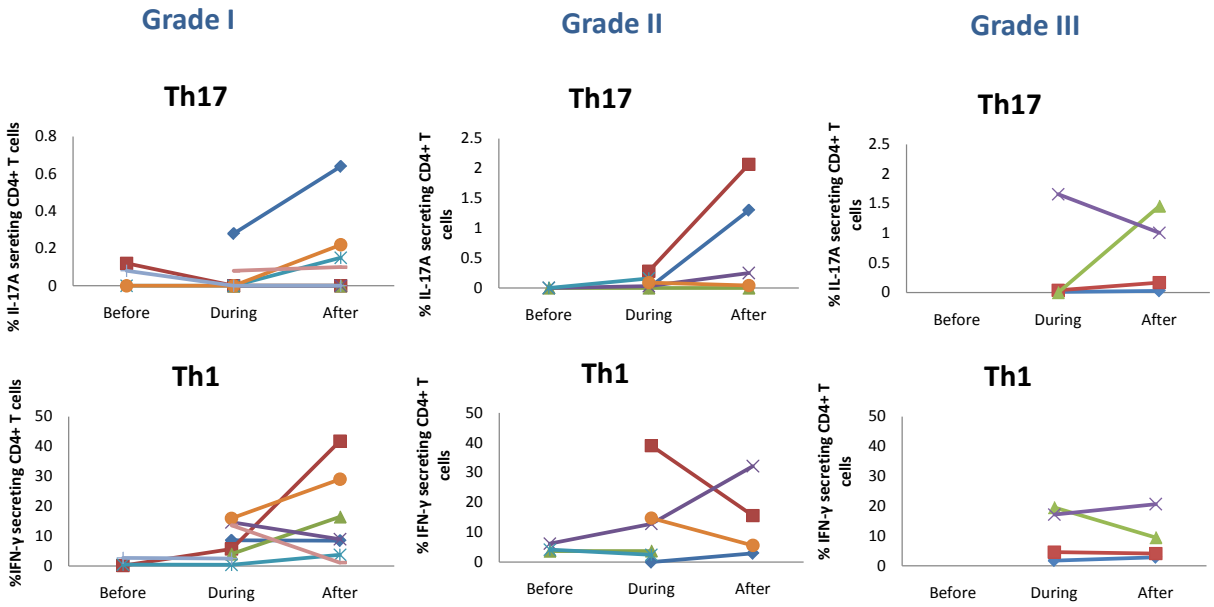


Figure 5.3.3.2 Relationship between Th1 and Th17 subsets with acute GvHD severity

T cell subset frequencies (x-axis) as determined by intracellular cytokine staining for IL-17A and IFN-γ in acute GvHD patients divided into groups based on severity of GvHD. Samples were collected during, and before/after GvHD episodes (y-axis). Clinical assessment of the grade of GvHD was made in clinic.

5.3.4 Expression of chemokine receptors by T cell subsets

T cell subsets, including Th17 and Th1, have been shown to express varying levels of the chemokine receptors CCR4, CCR6 and CXCR3 (Lim et al., 2008). Antibodies to these receptors were included in the staining panel for Th1 and Th17 cells with a view to comparing expression in the different subsets between normal conditions and chronic or acute GvHD. However, one complication of studying cells from allograft patients is that there is a considerably disrupted immune environment and reconstitution can vary vastly between different individuals even when having undergone similar conditioning. These difficulties are exacerbated when investigating relatively low frequency subsets such as Th17. An example of CD3/CD8 and IL-17A/IFN-γ staining of cells from a patient at 8 weeks post-transplantation who had an extremely low frequency of CD4+ (0.6%) and CD8+ (0.24%) T cells, is shown in figure 5.3.4.1.

This patient was excluded from all analysis due to the prohibitively low level of T cells. However, even in patients with considerably more robust T cell counts, the frequency of Th17 (and sometimes Th1) cells was often very low, and Th17-1 proved to be extremely rare. Very low numbers of cell in a particular subset gate could lead to high frequencies from very small numbers of cells. For example, two 'dots' either side of a quadrant arm on the FACS plot would suggest that 50% of cells for that subset are positive for a certain chemokine receptor. But with such small numbers, this would be very unreliable.

As such, a lower limit of 20 events per subset gate was used. This is still quite a low number of events and much of the data for the Th17 and Th17-1 is based on number of cells not much greater than that threshold, so caution should be exercised with interpreting these data. Where data was available, comparisons between GvHD and non-GvHD samples

(before or after) were made. In other cases, the median levels of chemokine receptors are shown for all samples regardless of GvHD status.

Most data is available for Th1 subsets and can be split into acute (figure 5.3.4.2) and chronic patients (figure 5.3.4.3), with equivalent controls. When considering the mean frequencies of cells expressing these chemokine receptors, there is no apparent difference between those samples taken at the time of GvHD and those taken when no disease was evident. However, this perception of no change in expression proves to not necessarily be the case, as the frequencies of cells expressing CCR4 and CXCR3 between these samples can vary considerably. Frequencies of CCR6+ Th1 cells did not vary dramatically in acute GvHD patients or any controls. Chronic GvHD samples did not reflect this, although CCR6+ cells both increased and decreased in different patients leading to no overall change in the medians. Similar bidirectional changes were observed in other chemokine receptor expression, both within patient groups and control groups.

As there does not appear to be a tendency to increase or decrease, and that similar patterns are evident in controls would suggest that whatever the reason for the changes, they are unlikely to be related to GvHD. There may be natural fluctuations in chemokine receptor expression due to the disordered immune environment of these patients, or perhaps infections. A colleague's data on chemokine receptor expression in a healthy donor cohort also showed considerable variation between individuals (O Goodyear - personal communication).

It is apparent that the frequency of cells expressing CCR6 in Th1 cells is generally higher than of those expressing CCR4 and CXCR3. This is similar for the Th17-1 cells as compared to the Th17 population. However, it should be noted that there is a wide range of expression of both receptors in this cell population. Perhaps this is related to the apparent plasticity of

Th17/Th17-1/Th1 cells, although the potential for error due to small numbers of events could be the explanation.

Median receptor expression does appear to decrease during GvHD, but conclusions cannot reliably be made about this as these frequencies are not in samples taken from the same patients. The only linked samples do not show any change. More samples with higher frequencies are needed to investigate this.

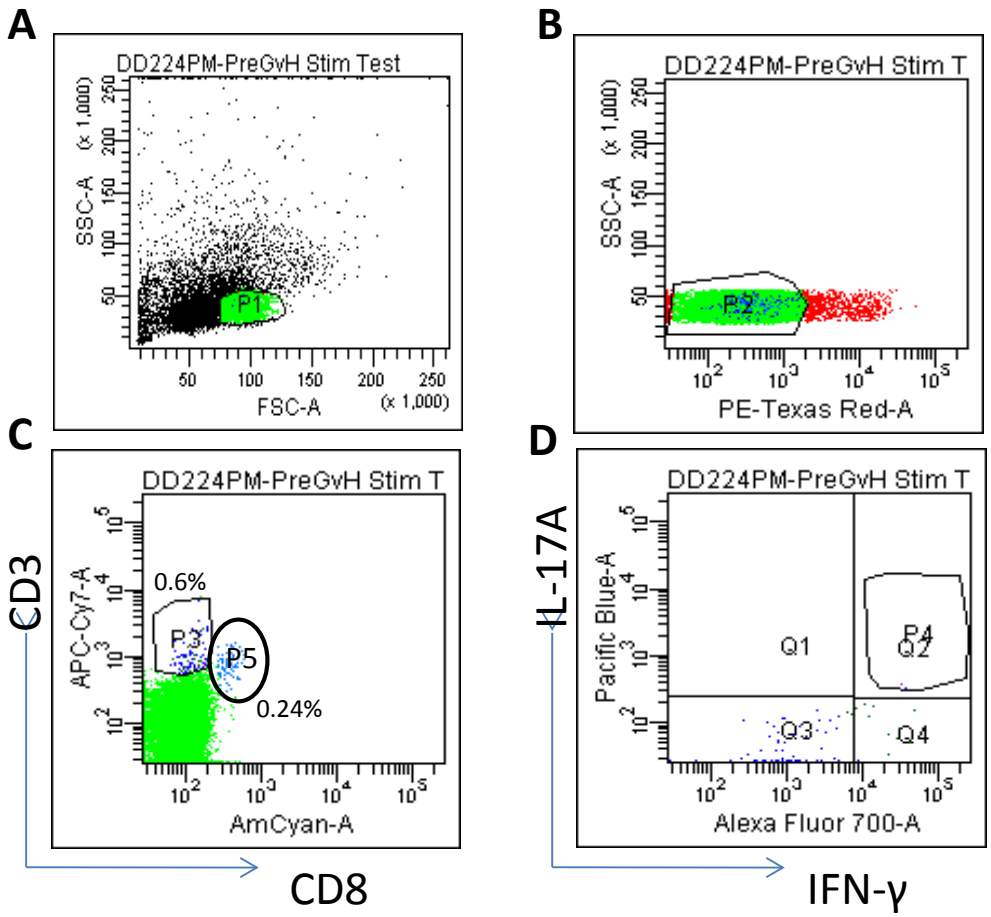


Figure 5.3.4.1 Flow cytometric plots showing gating for Th17/Th1 intracellular cytokine staining in a patient sample with low CD4+ and CD8+ T cell frequencies

The sample was taken from a patient at 8 weeks post-transplantation and was stained according to the Th1/Th17 intracellular cytokine staining protocol. The lymphocyte population was gated in the forward/side scatter plot (Gate P1 (A)) and live cells were selected with gate P2 (B). CD4+ (P3) (identified as CD3+ CD8- cells) and CD8+ (P5) frequencies were 0.6% and 0.24% respectively. From this low-frequency CD4+ gate, IL-17A and IFN-γ secreting cell levels were determined (D).

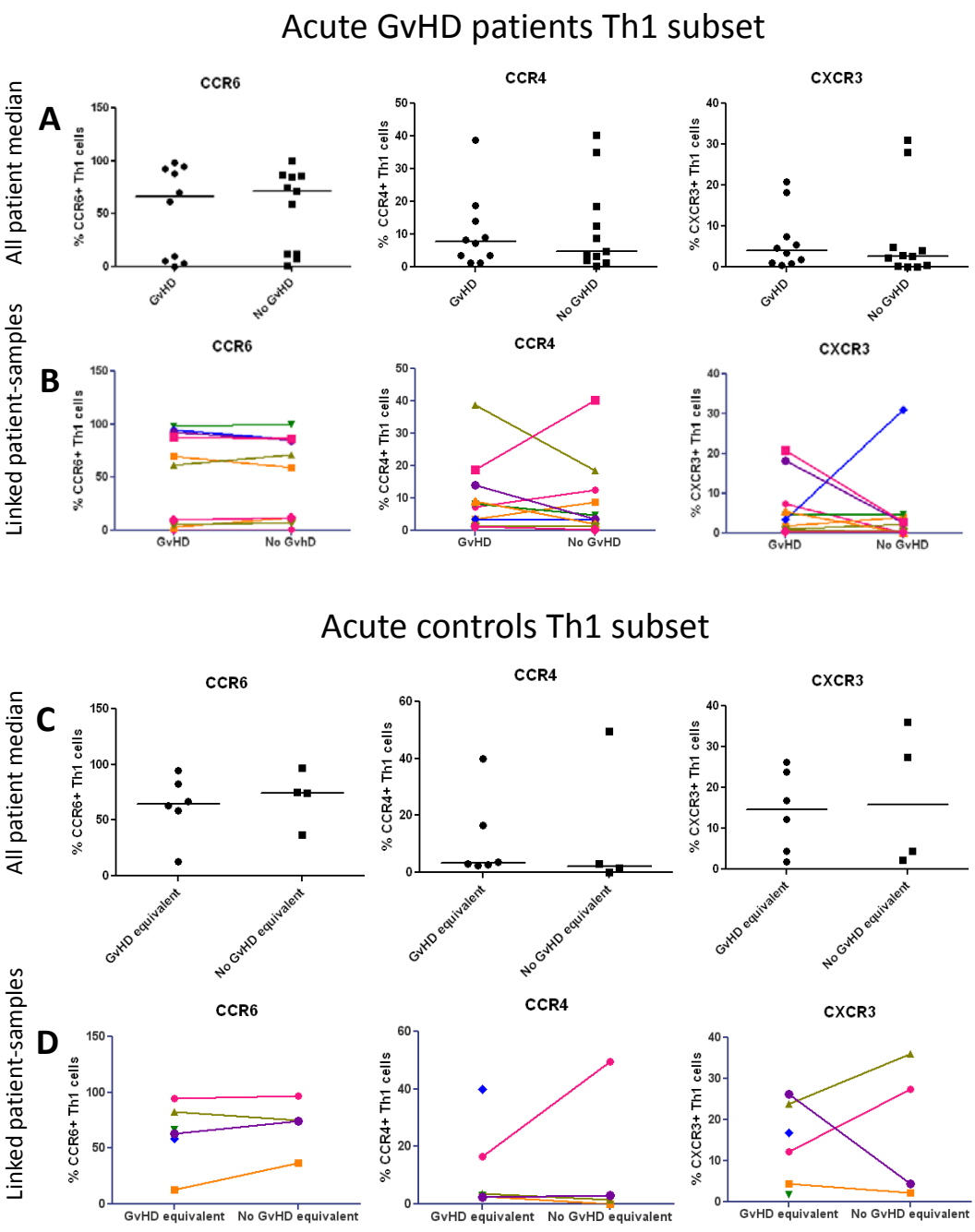


Figure 5.3.4.2 Chemokine receptor expression on Th1 cells in acute GvHD patients and time-matched controls

Levels of Th1 cells were established in peripheral blood samples taken from acute GvHD patients either during, or before/after GvHD, by intracellular cytokine staining to measure the frequencies of IFN-γ secreting cells. Chemokine receptor expression by cells in this subset was measured. The frequencies of cells expressing CCR6, CCR4 and CXCR3 are shown

(y-axis) as medians of all patients in samples taken during GvHD and before/after GvHD (No GvHD) (x-axis) (A). These data are shown again with each individual patient's samples linked (B). Chemokine receptor expression was also measured on Th1 cells in samples taken at similar times in control SCT patients who did not have GvHD, shown as grouped medians of samples taken at equivalent times to the GvHD samples or the before/after GvHD samples (C). Individual patients' samples are shown again as linked points (D).

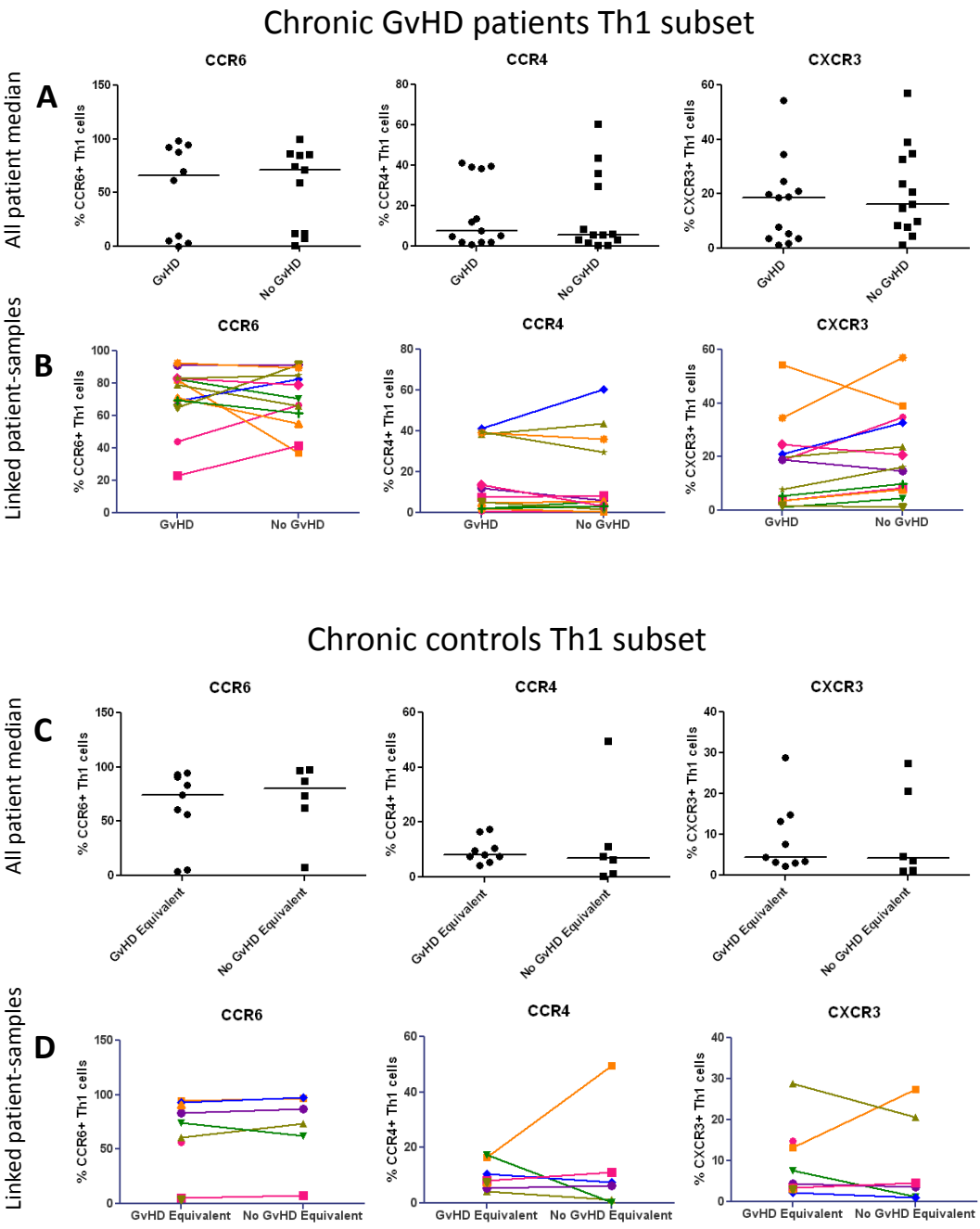


Figure 5.3.4.3 Chemokine receptor expression on Th1 cells in chronic GvHD patients and time-matched controls

Levels of Th1 cells were established in peripheral blood samples taken from chronic GvHD patients either during, or before/after GvHD, by intracellular cytokine staining to measure the frequencies of IFN-γ secreting cells. Chemokine receptor expression by cells in this subset was measured. The frequencies of cells expressing CCR6, CCR4 and CXCR3 are shown

(y-axis) as medians of all patients in samples taken during GvHD and before/after GvHD (No GvHD) (x-axis) (A). These data are shown again with each individual patient's samples linked (B). Chemokine receptor expression was also measured on Th1 cells in samples taken at similar times in control SCT patients who did not have GvHD, shown as grouped medians of samples taken at equivalent times to the GvHD samples or the before/after GvHD samples (C). Individual patients' samples are shown again as linked points (D).

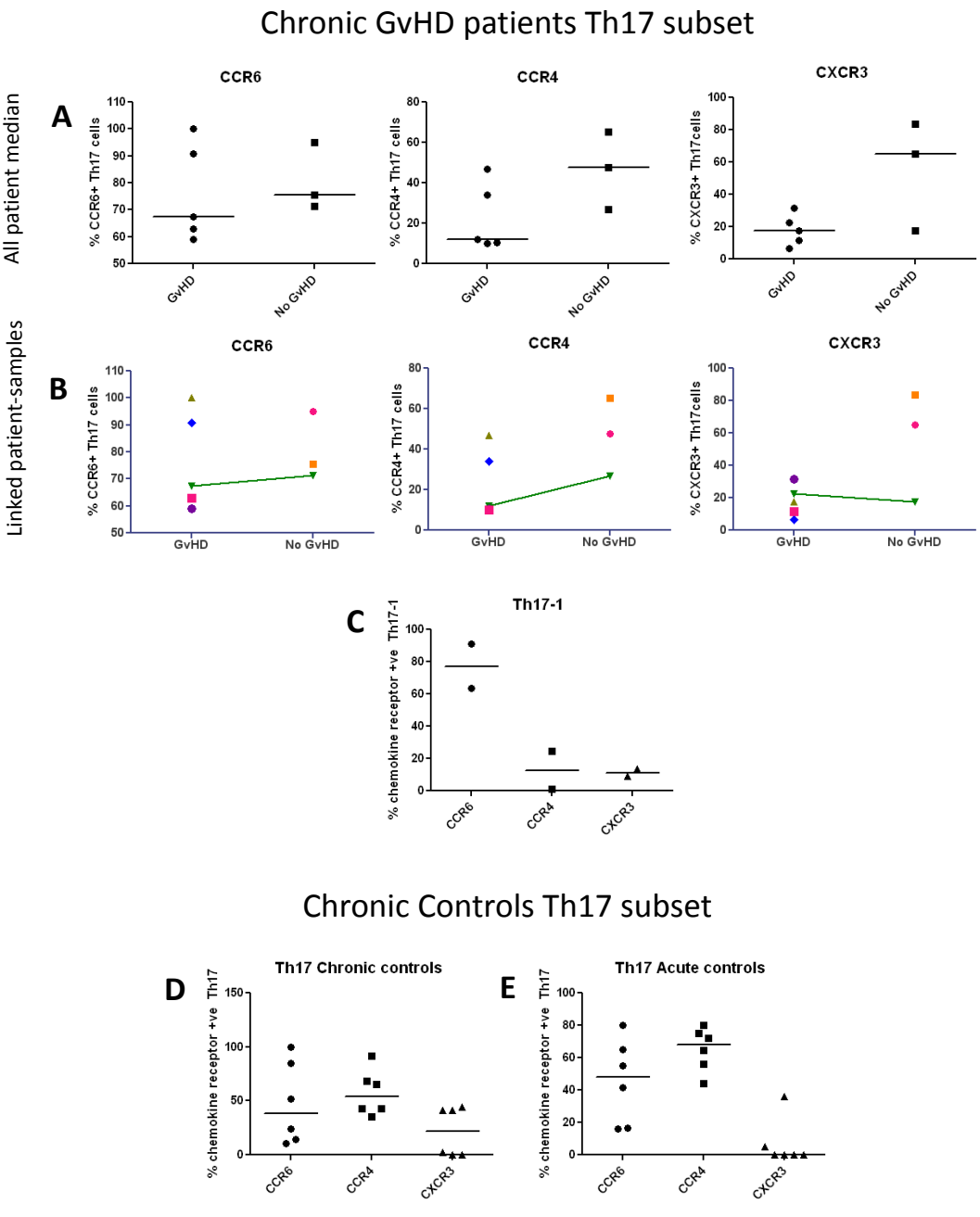


Figure 5.3.4.4 Chemokine expression on Th17 and Th17-1 cells

Levels of Th17 cells were established in peripheral blood samples taken from chronic GvHD patients either during, or before/after GvHD, by intracellular cytokine staining to measure the frequencies of IFN-γ secreting cells. Chemokine receptor expression in this subset was measured. The frequencies of cells expressing CCR6, CCR4 and CXCR3 are shown (y-axis) as medians of all patients in samples taken during GvHD and before/after GvHD (No GvHD) (x-axis) (A). These data are shown again with each individual patient's samples colour coded

and linked where data from both GvHD and non-GvHD was available (B). One patient had data for both GvHD and non-GvHD samples. Chemokine receptor expression data on Th17-1 cells was only available from two patients (C). Frequencies of chemokine receptor expression for chronic (D) and acute (E) controls is shown as means of all samples with sufficient Th17 cells. No control patients had chemokine receptor expression data for more than one sample.

5.4 Detection of cytokines and chemokines in the serum of GvHD patients

Serum was isolated from blood collected in coagulant-free tubes, which were taken at the same time as heparinised blood used for PBMC isolation. Samples were available for most patients that were screened for Th17, Th1 and Treg frequencies. These samples were analysed for the concentrations of 25 cytokines and chemokines by luminex assay. The panel of analytes is detailed in table 5.4.1. The method is explained in Chapter 2 (section 2.5).

Measurements for IL-10 and CCL27 (CTACK) fell below the threshold of detection (2.44 pg/ml) in all samples, so are not included here. Many cytokines or chemokines could not be detected in all samples.

IL-1β, IL-4, IL-5, IL-13, IL-21, IL-22, IL-23, TNFα, CCL2 (MCP-1), CCL4 (MIP-1β), CCL20 (MIP-3α), and CCL5 (RANTES) could be measured in the patients' serum but did not have any pattern between samples, with some patients having increased and some decreased levels in each cohort. Key cytokines (eg IL-17A, IFN-γ), and those with any possible pattern are shown.

IL-17A and IFN-γ were detected at very low concentrations (figure 5.4.1.1) and there was no discernable relationship between serum cytokine concentration and GvHD. Control samples also contained variable levels of cytokine. One patient in each of the acute and chronic cohorts had an increase in IFN-γ levels. Concentrations in the acute patient increased from 6.12 to 55.91 and then 58.03 pg/ml, which corresponded to a cellular Th1 increase from 6.2% to 12.82%, then to 32.17%. In the chronic GvHD patient, the serum concentration increased from 187.81 to 980.51 pg/ml whilst their Th1 frequency increased from 0.7% to 3.08%. Curiously, this Th1 frequency is very low in comparison with other patients who had much larger frequencies and greater changes in cell frequency that were not reflected in the serum concentration measurement.

IL-6, IL-7 and IL-15 were present at low concentrations (figure 5.4.1.2). IL-6 concentrations were elevated from undetectable levels, to concentrations between 45 and 97 pg/ml during chronic GvHD, and decreased following GvHD, except in one patient where IL-6 concentrations rose to around 21 pg/ml after GvHD, having previously been undetectable. No pattern was observed in time matched samples from control patients, although IL-6 was only detectable in a few control samples. This may reflect that IL-6 levels were generally higher in GvHD patients. IL-6 is a proinflammatory cytokine so may well be involved with the pathogenesis of GvHD. IL-7 and IL-15 both promote T cell proliferation and concentrations of these cytokine decreased following acute GvHD in six of seven (IL-7) and five of eight (IL-15), after chronic GvHD in four of four (IL-7) and three of four (IL-15) patients. However, maximum detected IL-7 concentrations were <60pg/ml (acute GvHD patients) and <80pg/ml (chronic GvHD patients), and IL-15 concentrations were <80pg/ml (acute GvHD patients) and <200pg/ml (chronic GvHD patients). Modest decreases at low concentrations may reflect intra-well variability.

The chemokine ligands for CXCR3 are CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC). There was no pattern to the concentrations of these chemokines (figure 5.5.1.3), but the cellular assays did not reveal any pattern of receptor expression either. CCL20 (MIP-3α) is a ligand for CCR6, but was not detected. Ligands for CCR4 (CCL17 (TARC) and CCL22 (MDC)) were not available for analysis by luminex.

There were decreases in the concentrations of CCL7 (MCP-3), CXCL1 (fractalkine) and CCL3 (MIP-1α) following acute GvHD. All three chemokines are inflammatory and CXCL1 has a role in cell recruitment. It is not surprising then, that these may be elevated during GvHD. There was insufficient data to draw conclusions about their role in GvHD, or to show an increase from concentrations prior to acute GvHD.

Cytokines	Chemokines	Alternate chemokine nomenclature	Chemokine ligand
TNF-α	**CXCL9**	MIG	CXCR3
IFN-γ	**CCL3**	MIP-1α	CCR5
IL-1β	**CCL4**	MIP-1β	CCR5
IL-4	**CCL20**	MIP-3α	CCR6
IL-5	**CCL5**	RANTES	CCR1
IL-6	**CXCL10**	IP-10	CXCR3
IL-7	**CXCL11**	ITACK	CXCR3
IL-10	**CCL2**	MCP-1	CCR2
IL-13	**CCL7**	MCP-3	CCR10
IL-15	**CCL27**	CTACK	CCR10
IL-17A	**CX3CL1**	FRAC	CX3CR1
IL-21			
IL-22			
IL-23			

Table 5.4.1 Panel of cytokines and chemokine analytes used for 25-plex luminex assay

Serum concentrations of 25 cytokines and chemokines in samples from stem cell transplant patients were measured by luminex assay. Samples were screened for levels of fourteen cytokines (1st column) and 11 chemokines (2nd column) which are detailed with their alternate nomenclature (3rd column) and the chemokine receptor (4th column).

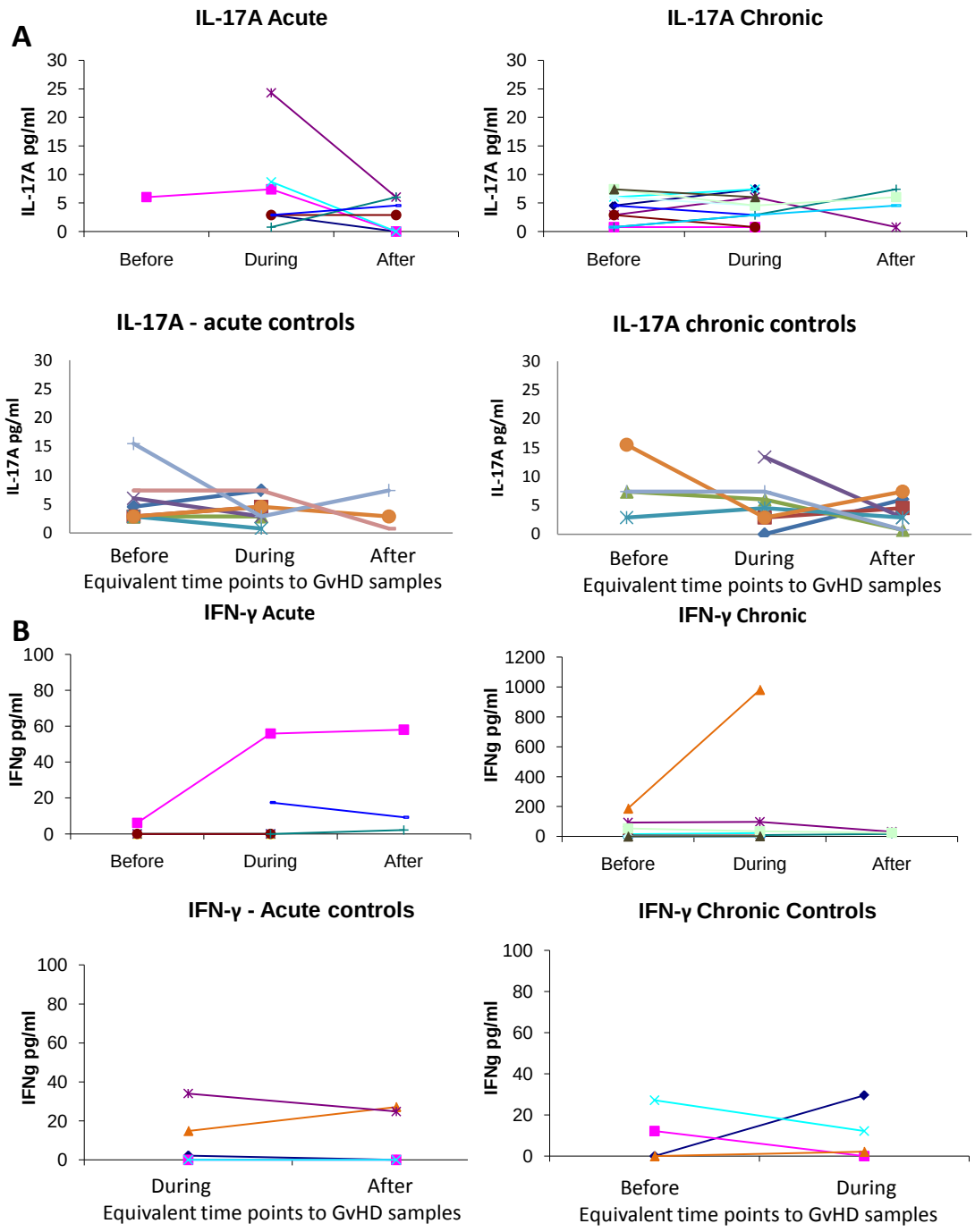


Figure 5.4.1.1 IL-17A and IFN-γ concentrations in serum of patients

Concentrations of IL-17A and IFN-γ (y-axis) were measured in the serum of peripheral blood samples taken during GvHD episodes, and compared with levels in samples taken before or after GvHD, or taken at equivalent times from control patients who had not had GvHD.

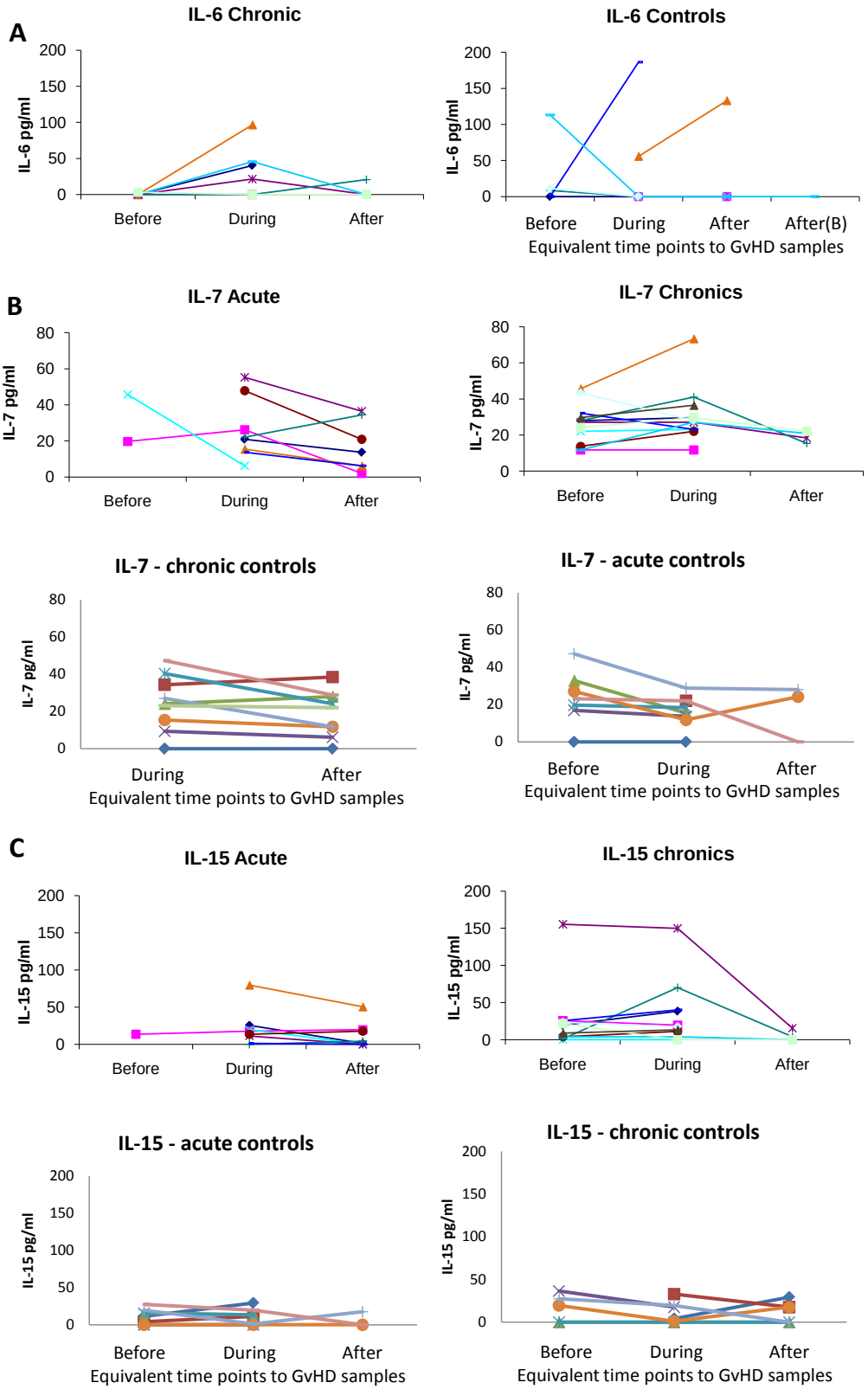

A
IL-6 Chronic
IL-6 pg/ml
200
150
100
50
0
Before
During
After
IL-6 Controls
IL-6 pg/ml
Before
During
After
After(B)
Equivalent time points to GvHD samples
B
IL-7 Acute
IL-7 pg/ml
80
60
40
20
0
Before
During
After
IL-7 Chronics
IL-7 pg/ml
Before
During
After
IL-7 - chronic controls
IL-7 pg/ml
During
After
Equivalent time points to GvHD samples
IL-7 - acute controls
IL-7 pg/ml
Before
During
After
Equivalent time points to GvHD samples
C
IL-15 Acute
IL-15 pg/ml
Before
During
After
IL-15 chronics
IL-15 pg/ml
Before
During
After
IL-15 - acute controls
IL-15 pg/ml
Before
During
After
Equivalent time points to GvHD samples
IL-15 - chronic controls
IL-15 pg/ml
Before
During
After
Equivalent time points to GvHD samples

Figure 5.4.1.2 Serum concentrations of IL-6, IL-7 and IL-15 in samples taken before/after and during GvHD, or from time-matched controls

Concentrations of IL-6, IL-7 and IL-15 (y-axis) were measured in the serum of peripheral blood samples taken during GvHD episodes, and compared with levels in samples taken before or after GvHD, or taken at equivalent times from control patients who had not had GvHD. IL-6 levels were below the level of detection of detection in all acute GvHD patient samples.

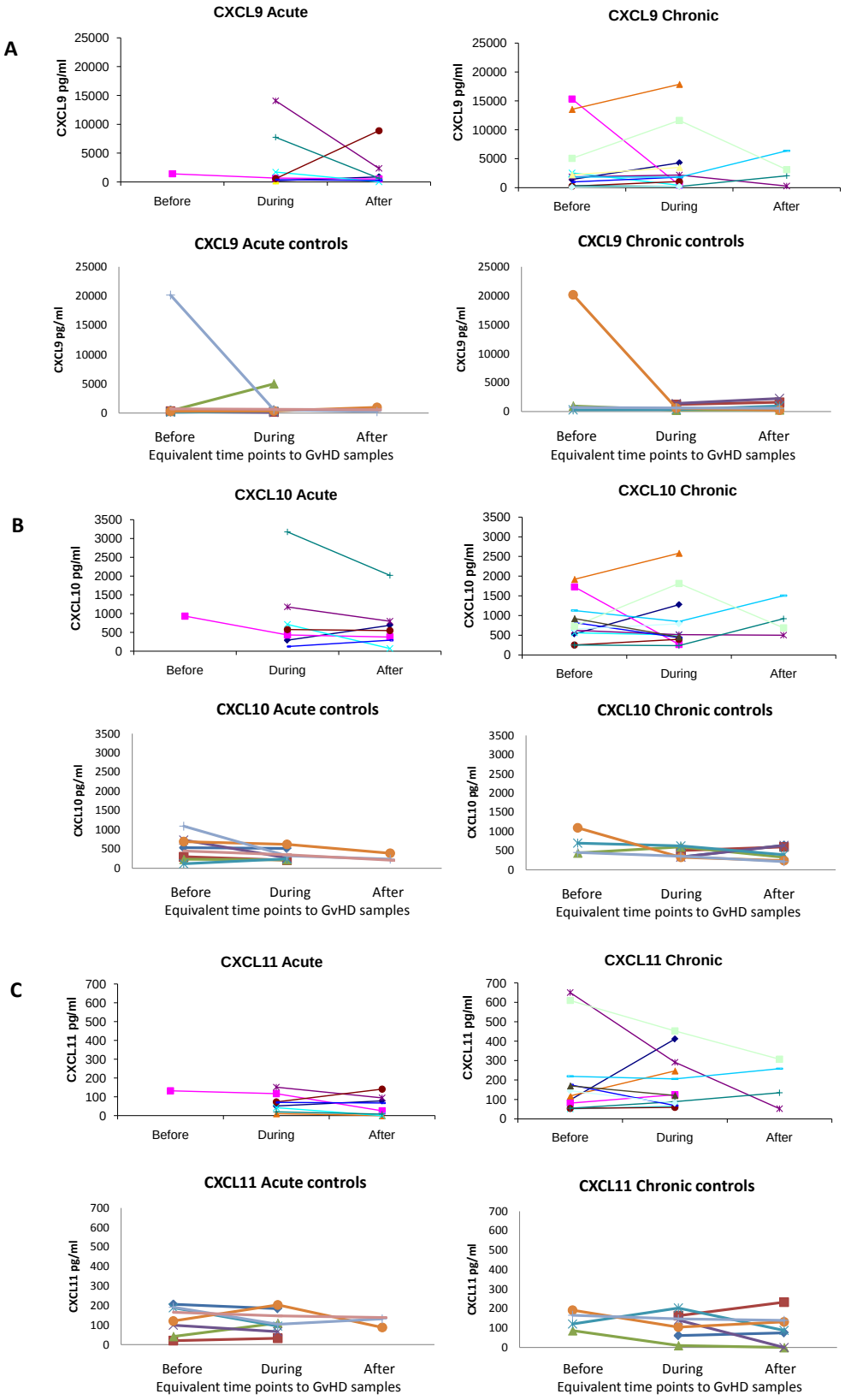

A
CXCL9 Acute
CXCL9 pg/ml
25000
20000
15000
10000
5000
0
Before
During
After
CXCL9 Chronic
CXCL9 pg/ml
25000
20000
15000
10000
5000
0
Before
During
After
CXCL9 Acute controls
CXCL9 pg/ml
25000
20000
15000
10000
5000
0
Before
During
After
Equivalent time points to GvHD samples
CXCL9 Chronic controls
CXCL9 pg/ml
25000
20000
15000
10000
5000
0
Before
During
After
Equivalent time points to GvHD samples
B
CXCL10 Acute
CXCL10 pg/ml
3500
3000
2500
2000
1500
1000
500
0
Before
During
After
CXCL10 Chronic
CXCL10 pg/ml
3500
3000
2500
2000
1500
1000
500
0
Before
During
After
CXCL10 Acute controls
CXCL10 pg/ml
3500
3000
2500
2000
1500
1000
500
0
Before
During
After
Equivalent time points to GvHD samples
CXCL10 Chronic controls
CXCL10 pg/ml
3500
3000
2500
2000
1500
1000
500
0
Before
During
After
Equivalent time points to GvHD samples
C
CXCL11 Acute
CXCL11 pg/ml
700
600
500
400
300
200
100
0
Before
During
After
CXCL11 Chronic
CXCL11 pg/ml
700
600
500
400
300
200
100
0
Before
During
After
CXCL11 Acute controls
CXCL11 pg/ml
700
600
500
400
300
200
100
0
Before
During
After
Equivalent time points to GvHD samples
CXCL11 Chronic controls
CXCL11 pg/ml
700
600
500
400
300
200
100
0
Before
During
After
Equivalent time points to GvHD samples

Figure 5.4.1.3 Serum concentrations of chemokine ligands for CXCR3 in samples taken before/after and during GvHD, or from time-matched controls

Serum concentrations (y-axis) of chemokines CXCL9 (MIG) (A), CXCL10 (IP-10) (B), and CXCL11 (I-TAC) (C) were measured by luminex assay in the serum of acute and chronic GvHD patients from peripheral blood samples taken during and before/after GvHD. Samples from time-matched control patients who did not have GvHD were also screened.

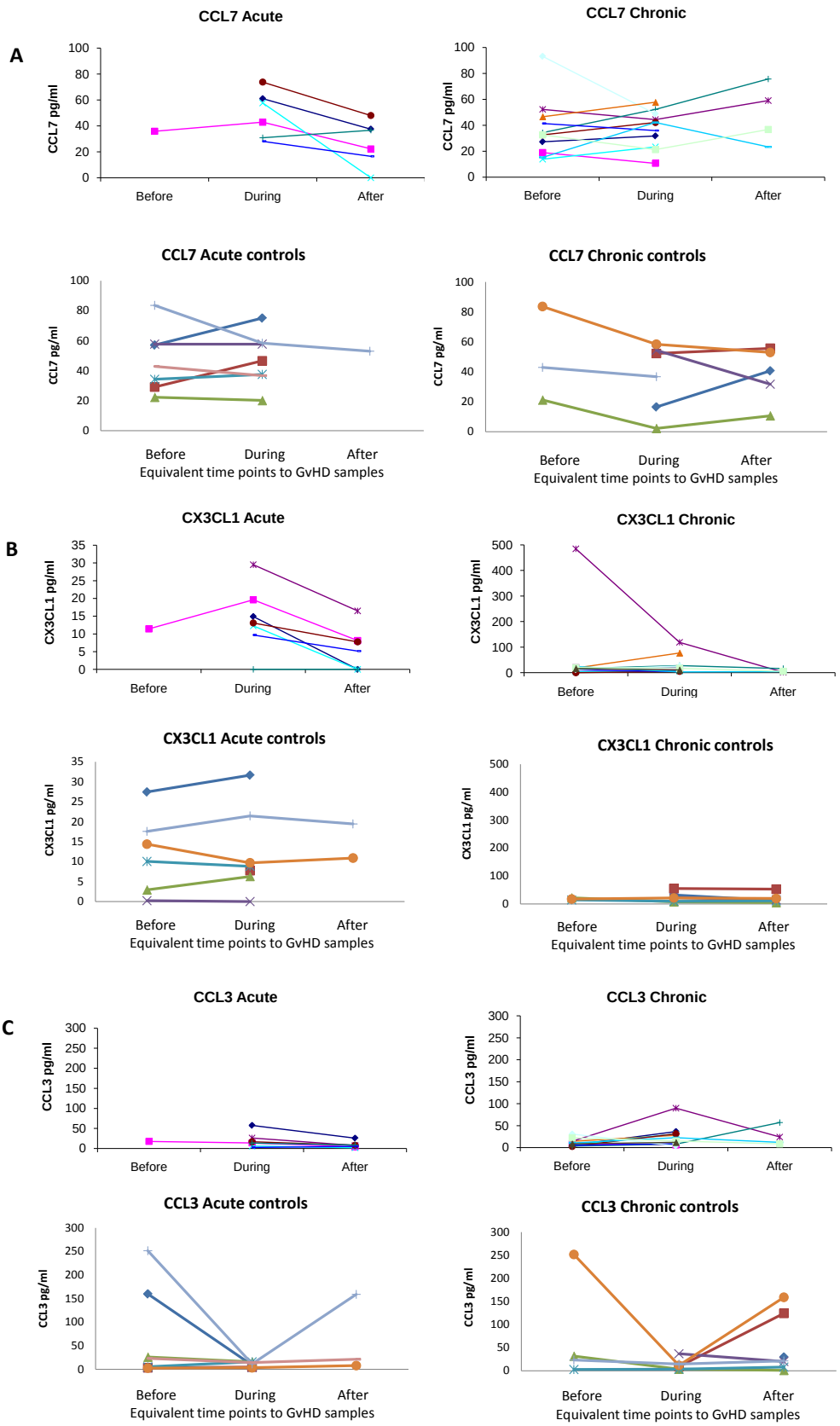

A
CCL7 Acute
CCL7 pg/ml
100
80
60
40
20
0
Before
During
After
CCL7 Chronic
CCL7 pg/ml
Before
During
After
CCL7 Acute controls
CCL7 pg/ml
Before
During
After
Equivalent time points to GvHD samples
CCL7 Chronic controls
CCL7 pg/ml
Before
During
After
Equivalent time points to GvHD samples
B
CX3CL1 Acute
CX3CL1 pg/ml
35
30
25
20
15
10
5
0
Before
During
After
CX3CL1 Chronic
CX3CL1 pg/ml
500
400
300
200
100
0
Before
During
After
CX3CL1 Acute controls
CX3CL1 pg/ml
Before
During
After
Equivalent time points to GvHD samples
CX3CL1 Chronic controls
CX3CL1 pg/ml
Before
During
After
Equivalent time points to GvHD samples
C
CCL3 Acute
CCL3 pg/ml
300
250
200
150
100
50
0
Before
During
After
CCL3 Chronic
CCL3 pg/ml
Before
During
After
CCL3 Acute controls
CCL3 pg/ml
Before
During
After
Equivalent time points to GvHD samples
CCL3 Chronic controls
CCL3 pg/ml
Before
During
After
Equivalent time points to GvHD samples

Figure 5.4.1.4 Serum concentrations changes of chemokines

Serum concentrations (y-axis) of chemokines CCL7 (MCP3) (A), CX3CL1 (Fractalkine) (B) and CCL3 (MIP-1α) (C) were measured by luminex assay in the serum of acute and chronic GvHD patients from peripheral blood samples taken during and before/after GvHD. Samples from time-matched control patients who did not have GvHD were also screened.

5.5 Case studies

The data from the Th1/Th17/Foxp3 staining experiments and the luminex assay presented here do not show strong associations of any particular subsets with GvHD. Few samples were taken at diagnosis when disease would be untreated, and therefore most active. Four patients were screened at the time of diagnosis when they had active, untreated GvHD.

KJ299PM

This patient was diagnosed with chronic gut GvHD at 41 weeks post-transplantation, which was of sufficient severity to warrant hospital admission. A sample was also available from 37 weeks when there was no known GvHD. The patient had previously had acute gut GvHD and a number of CMV disease complications. The patient died 12 weeks later from a combination of GvHD and CMV disease. There was an increase of both Th1 (from 4.18% to 24.98%) and Th17 (from 0.05% to 0.4%) cells in the GvHD sample compared to the pre-GvHD sample and Treg frequencies increased with Th1 and Th17 during GvHD (figure 5.5.1.1A). CCR6 expression on the Th1 subset decreased from 91.46 to 64.92%. The frequencies of chemokine receptor expression could be measured on all effector subsets in the GvHD sample, and CCR6 was around 63% on all subsets. Th17 and Th17-1 cells had similar frequencies of expression of CXCR3 (11.76% and 9.17% respectively) and Th1 subset expression was lower at 1.7%. Th17 cells had the highest frequency expression of CCR4 at 10.22% compared with 5.07% in Th1 cells and 0.85% of Th17-1 cells.

Despite there being very low frequency expression of CXCR3, which did not change in relation to GvHD, there was a marked decrease in serum CXCL10 (from 1727.7 to 267.45 pg/ml) and CXCL9 (from 15335.7 to 430.639 pg/ml) during GvHD, both of which are ligands for that receptor (figure 5.5.1.1C). Serum concentrations of IL17A and IFN-γ were below the level of detection in all samples from this patient.

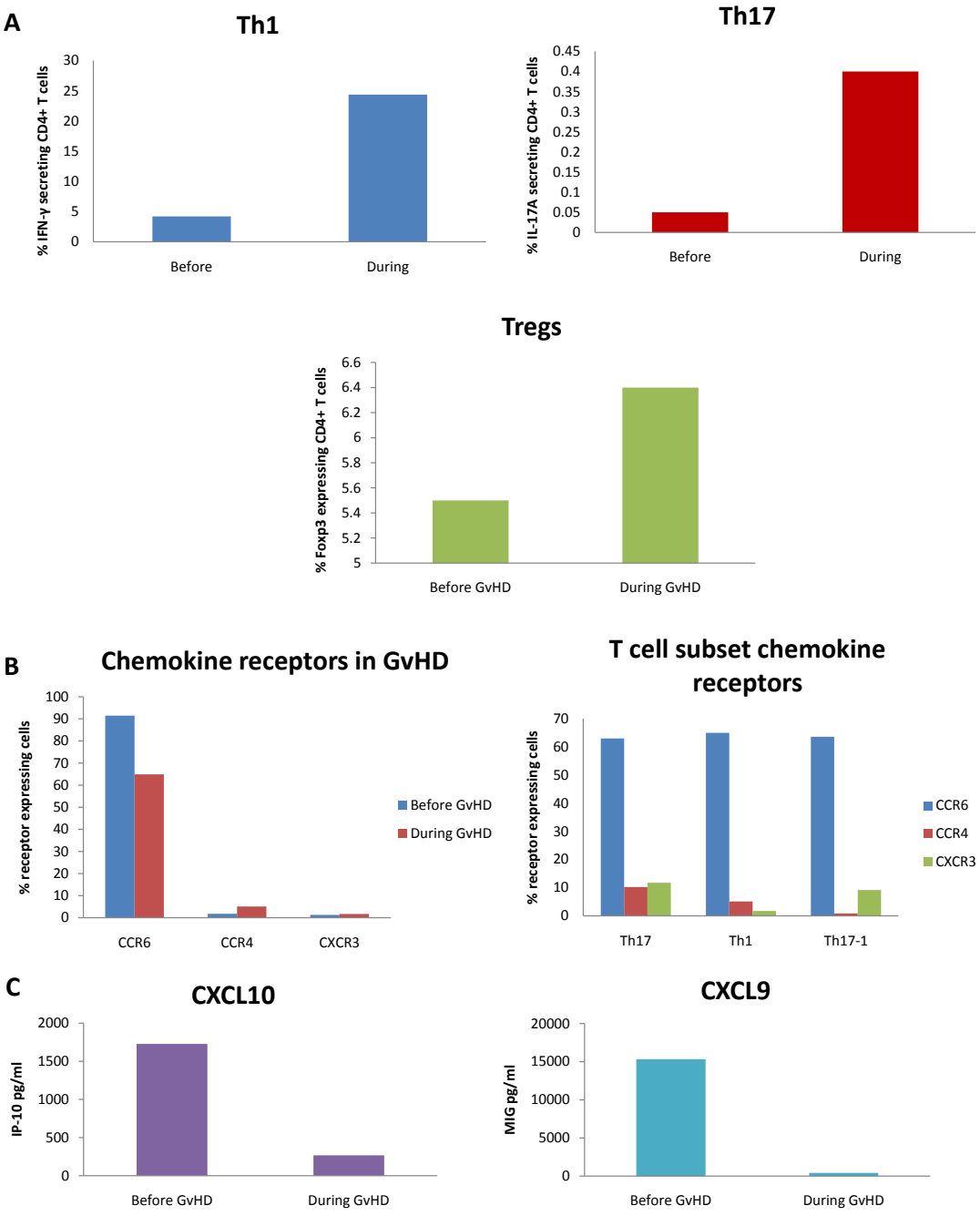


Figure 5.5.1.1 T cell subset frequencies, chemokine receptor expression, serum chemokine levels around the time of chronic gut GvHD in patient KJ299PM

Th1, Th17 and Treg frequencies (y-axis) as determined by intracellular staining for IFN-γ (Th1), IL-17 (Th17) and Foxp3 (Tregs) (A), in peripheral blood samples taken at 37 weeks post-transplantation when there was no known GvHD (before) and at 41 weeks, when the patient was admitted to hospital with chronic gut GvHD (during). Th1 and Th17 subsets

were also stained for chemokine receptors CCR4, CCR6 and CXCR3 (B). Receptor frequencies in the Th1 subset are shown for samples taken before and during GvHD, but there were insufficient cells to determine receptor frequencies for Th17 and Th17-1 subsets in the pre-GvHD sample. Chemokine receptor frequencies for each T cell subset are shown for the sample taken during GvHD. Serum IL-17A and IFN-γ were below the level of detection of the luminex assay, but there were large decreases in CXCL10 (IP-10) and CXCL9 (MIG) (C).

RA296PM

Acute GvHD patient RA296PM was diagnosed with grade II skin GvHD at 5 weeks post-transplantation (figure 5.5.1.2). A post-GvHD sample was taken at 12 weeks post-transplantation. Th1 cells decreased (from 14.78% to 5.62%), as did Th17 cells (from 0.09% to 0.04%) from the GvHD sample to the non-GvHD sample (figure 5.5.1.2A). The serum concentration of IFN-γ reflected this as it decreased from 17.44 to 9.28 pg/ml, but IL-17A concentration increased from 2.88 to 4.54 pg/ml. However, these concentrations were so low and the changes so small, it is difficult to draw conclusions, and may reflect variability between wells in the assay. Chemokine receptor expression was generally low frequency on Th1 cells, with CCR6 around 10-12%, both CCR4 and CXCR3 below 2%. Ligands for the latter receptor, CXCL10 (IP-10) and CXCL9 (MIG) were low compared to other patients. There was an increase in IP-10, but a decrease in MIG during GvHD. A previous study also reported in increase in CXCL10 during acute GvHD, although CXCL9 levels were unchanged (Piper et al., 2007a).

JR302PM

Acute GvHD patient was diagnosed with grade I skin GvHD at 7 weeks post-transplantation, and a post-GvHD sample was available at 16 weeks (figure 5.5.1.3).

No Th17 cells were detected in either sample, but the luminex data shows an increase in IL-17A concentration from below 0.76 to 6.03 pg/ml. Th1 cells increased from 4.02% to 16.43% between the GvHD and post-GvHD samples. Chemokine receptor expression frequency was low, despite this patient having considerably higher serum chemokine concentrations as compared with RA296PM. The frequency of CCR6 expressing cells increased from 5.49% to

7.86% during GvHD. A modest decrease in CXCR10 concentrations from 3174 to 2016 pg/ml was observed after GvHD and CXCL9 decreased from 7755 to 649 pg/ml.

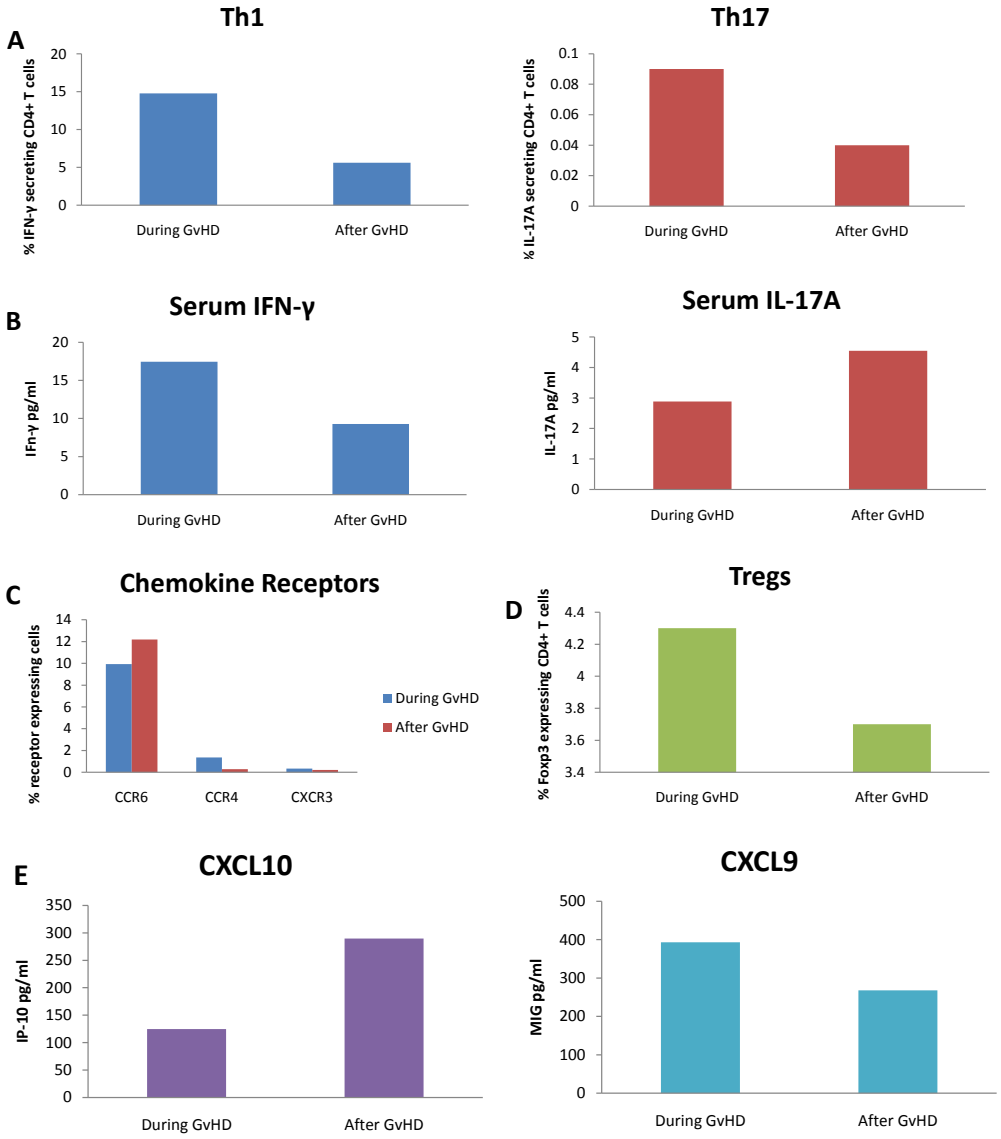


Figure 5.5.1.2 T cell subset frequencies, chemokine receptor expression, and serum cytokine/chemokine levels around the time of acute skin GvHD in patient RA296PM

Th1, Th17 and Treg frequencies (y-axis) as determined by intracellular staining for IFN-γ (Th1), IL-17 (Th17) (A) and Foxp3 (Tregs) (D), in peripheral blood samples taken 5 weeks post-transplantation at diagnosis of acute skin GvHD, and at 12 weeks post-transplantation when GvHD had been resolved. Chemokine receptor frequencies of CXCR3, CCR4 and CCR6 were measured on Th1 cells (C). Serum cytokine and chemokine concentrations were measured by luminex (B, E).

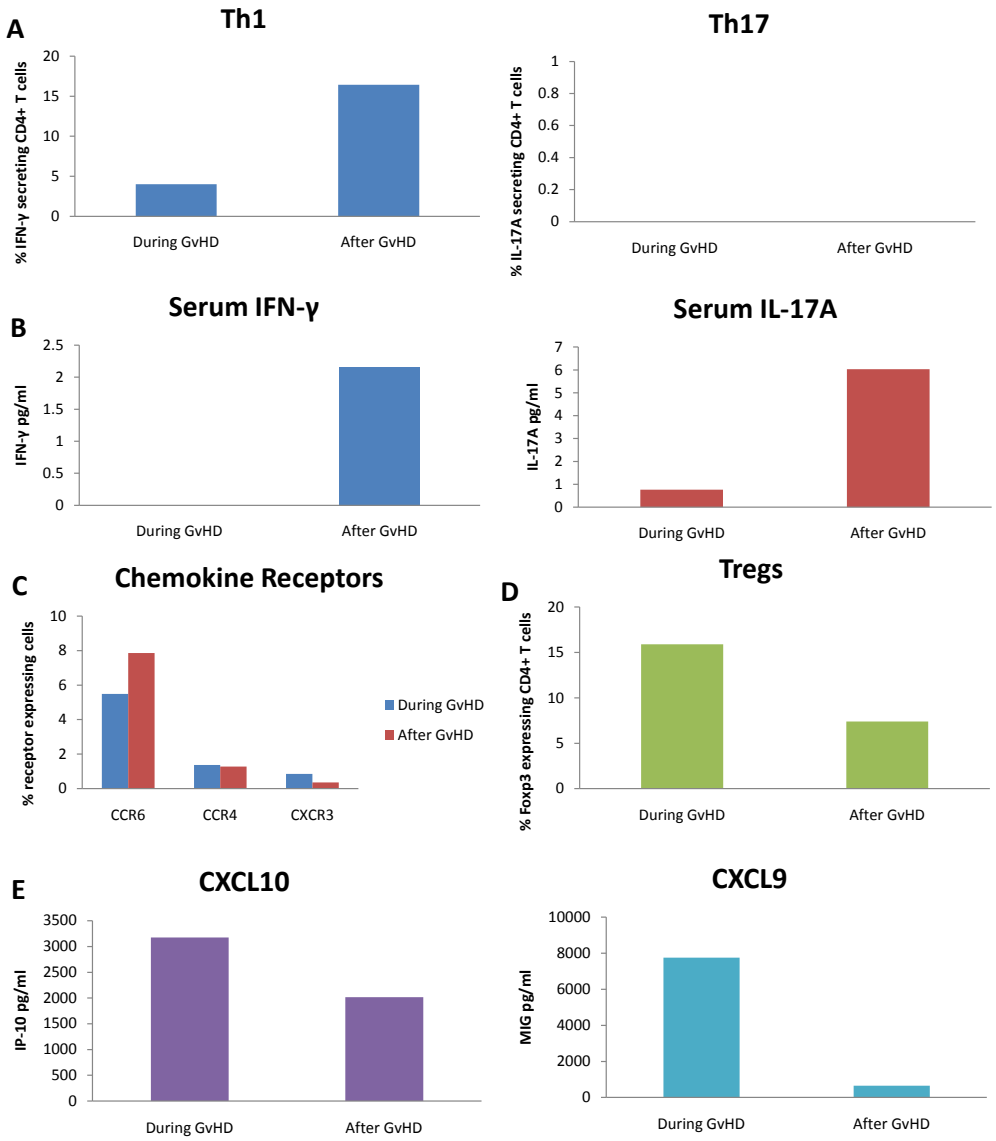


Figure 5.5.1.3 T cell subset frequencies, chemokine receptor expression, and serum cytokine/chemokine levels around the time of acute skin GvHD in patient JR302PM

Th1, Th17 and Treg frequencies (y-axis) as determined by intracellular staining for IFN-γ (Th1), IL-17 (Th17) (A) and Foxp3 (Tregs) (D), in peripheral blood samples taken 7 weeks post-transplantation at diagnosis of acute skin GvHD, and at 16 weeks post-transplantation when GvHD had been resolved. Chemokine receptor frequencies of CXCR3, CCR4 and CCR6 were measured on Th1 cells (C). Serum cytokine and chemokine concentrations were measured by luminex (B, E).

CM308PM

This patient was diagnosed with acute gut GvHD (grade III) at 3 weeks following transplantation and a follow up sample was obtained after resolution of GvHD at 16 weeks post-transplantation. There was a slight decrease in Th1 frequencies, from 4.61% to 4.14% after GvHD and there was a four-fold increase in Th17 cells, from 0.04% to 0.17%. Th1 levels were very low as was chemokine receptor expression on this subset, although CCR6 expression increased following GvHD from 3.45% to 12%. Serum was not available for cytokine analysis (figure 5.5.1.4).

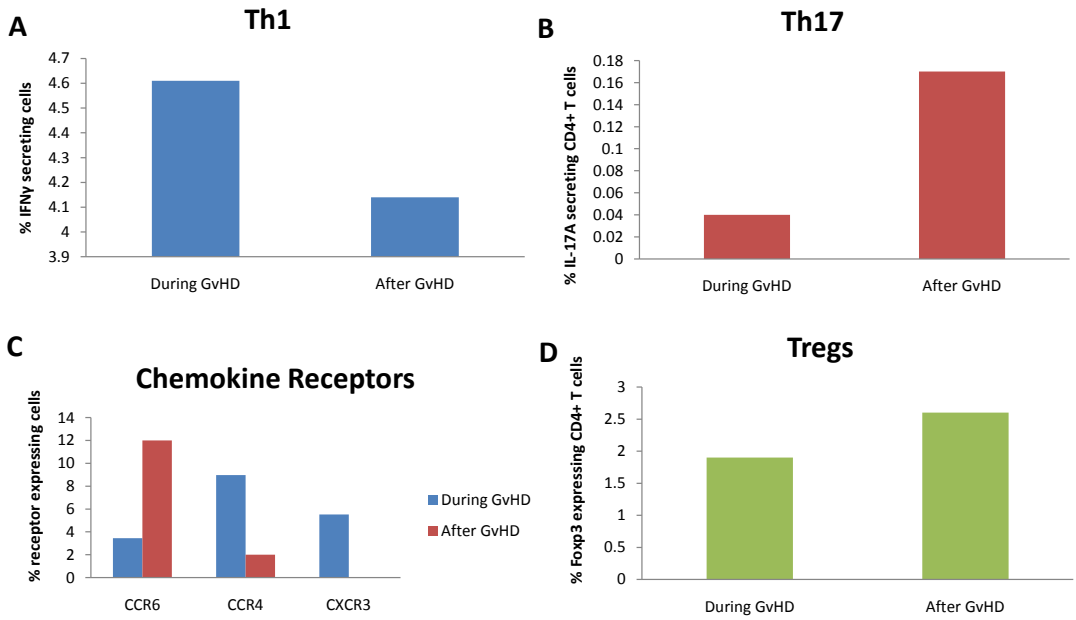


Figure 5.5.1.4 T cell subset frequencies and chemokine receptor expression around the time of acute gut GvHD in patient CM308PM

Th1, Th17 and Treg frequencies (γ-axis) as determined by intracellular staining for IFN-γ (Th1) (A), IL-17 (Th17) (B) and Foxp3 (Tregs) (D), in peripheral blood samples taken 3 weeks post-transplantation at diagnosis of acute gut GvHD, and at 16 weeks post-transplantation when GvHD had been resolved. Chemokine receptor frequencies of CXCR3, CCR4 and CCR6 were measured on Th1 cells (C). Serum samples were not available for the measurement of cytokines and chemokines.

5.6 Discussion

This study sought to establish whether there was any relationship between frequencies of IFN-γ secreting Th1 cells, IL-17A secreting Th17 cells or Foxp3 expressing Tregs and GvHD. Fifty-three allograft patients were studied, of which 18 had acute GvHD, 14 were diagnosed with chronic GvHD and 21 did not suffer any GvHD. Samples had been taken during GvHD episodes and frequencies of these subsets were compared with the levels in samples taken before and/or afterwards when there was no evident GvHD. The frequencies of these T cells expressing chemokine receptors CCR4, CCR6 and CXCR3 were measured and levels of various cytokines and chemokines in the serum of patients were established. Samples from allograft patients who did not have any GvHD were used as controls.

These data did not show any clear associations between T cell subset frequencies, chemokine receptor expression, serum cytokine/chemokine concentrations and GvHD. This was surprising as GvHD is a T cell-mediated inflammatory condition. Therefore, one might expect that Th1 and Th17 cell frequencies would increase whilst Tregs may, certainly initially, be decreased, and that proinflammatory cytokines in the serum of patients would be elevated as compared to samples when no GvHD was evident.

There may be a number of explanations for this counterintuitive result. This was a retrospective study using previously collected, cryopreserved PBMC samples. Clinical data was recorded at the time of the patients' appointments by clinicians at a stem cell transplant patient clinic. Selected information was then made available to the investigator via a database. This study was part of a larger study investigating the immunology of stem cell transplantation for which patient samples were taken according to a collection schedule. The ethical approval allowed for *ad hoc* extra samples to be collected (subject to clinical approval and at not more than two week intervals). Blood samples were collected prior to

consultation with the clinician. There was an inherent complication caused by sample collection. The opportunity for sample collection had already passed when a GvHD diagnosis was made, and a further sample could not be taken for two weeks. Therefore, the majority of samples were taken at some time between diagnosis of GvHD and the time where a clinical assessment was made that there was no longer disease. It was not possible to accurately know the degree of severity of disease at the time of sample collection. It is likely that the patient would have had some form of treatment before the sample had been taken. Of 32 GvHD samples, only four were known to have been taken at the time of diagnosis. The data for all four of these patients has been presented as case studies in section 5.5.

Severity of GvHD was assessed by a clinician at diagnosis. Acute GvHD has a grading system of increasing severity from grade I to grade IV. However, there is no such system for chronic GvHD which made it difficult to retrospectively establish how severe the disease was. As the grading was made at diagnosis, but most samples were collected some time afterwards, the initial grading may not reflect the severity of disease at the time when the sample was taken. Treatment drugs were recorded, but it was not possible to assess how the doses of steroids had affected the immune system of the patients. However, if it was known that a patient had been on intravenous steroids such as prednisolone, then the sample was avoided as this would be likely to have a global immunosuppressive effect.

Another potential confounding factor was that of infections. Fungal infections have been associated with increased levels of Th17 cells (Huang et al., 2004). One patient had relatively high frequencies of Th17 cells regardless of GvHD and had previously had CMV reactivation, although this had been resolved prior to the samples being taken for this study. Another patient, also with higher Th17 levels had had a fungal infection early after transplantation. Although these infections had also long been cleared, there remains the possibility that

earlier infectious events could have skewed reconstitution towards having higher frequencies of Th17 cells. A mouse study has shown that early fungal infections after stem cell transplantation are associated with increased likelihood of developing GvHD (Echtenacher et al, 2009 – Poster abstract, ECI 2009). It would be tempting to speculate, that if Th17 cells were associated with GvHD, then the early infectious event could have pre-empted development of the disease by increasing the overall frequency of Th17 cells. However, the data in this study does not support such an association.

These confounding factors should be considered because the data collected in this study show many conflicting changes in patients. Frequencies of all subsets studied could increase, decrease or remain unchanged in different patients with similar GvHD. Control patients also followed these patterns. Th17 levels changed in the same, or the opposite way to Th1 cells and indeed, Tregs. The lack of patterns may be due to samples being collected at different stages of disease, effects of treatment or indeed natural resolution. It may be that Th1 or Th17 levels increase at different times and one or other may already be in decline when a particular sample was taken. Similarly, if Treg frequencies increase after the inflammatory subsets, then timing of sample collection from the onset of disease is crucial. Even if a sample is taken on the day of diagnosis, it was not known if the symptoms had appeared that day, or several days previously. This study would have benefitted from having prospective sample collection by an allied clinician who could have more fully documented the patients' experiences, symptoms and infectious events. Another drawback to this study is that the non-GvHD samples may have been collected a long time prior to, or following GvHD, and was not consistent between patients.

Fluctuations in cell populations may be normal, particularly for stem cell transplant patients, who have a profoundly disrupted immune environment. It is not clear the extent to which

stability of T cell lineages could affect these populations. There is increasing evidence of plasticity between suppressive or effector lineages (reviewed (Zhou et al., 2009)). This concept may contribute to the apparent inconsistencies between patients in the levels of T cell subsets, and serum cytokines and chemokines. Even in the patients where a sample was definitely taken on the day of GvHD diagnosis there are differences in the way T cell frequencies and serum chemokines changed. These patients did not have any treatment bias.

Two studies have recently claimed to show an association between Th17 cells and GvHD. One study apparently used prospectively collected samples (Dander et al., 2009). However, their control samples used throughout were inconsistent as they claimed that samples from transplant patients who had not suffered GvHD were identical to samples from non-transplanted healthy controls, although limited evidence was produced to support this claim. In my study, the controls behaved in a similar way to the GvHD patients which suggests that changes seen may not be related to GvHD. The Dander study indicated that the association between peripheral blood Th17 frequencies and GvHD was difficult to make, but that there was a localised increase when they studied biopsies. Here, it may be possible to obtain prospective biopsy specimens from a similar cohort to investigate the levels of IL-17A producing T cells at sites of GvHD. Good control samples would be essential as reliable staining by immunohistochemistry is difficult to achieve with currently available antibodies (C. Schmutz - personal communication). If sufficient cells could be obtained from biopsy samples, then Th17 levels at the sites of GvHD could be established by flow cytometry using the antibodies already tested in this study.

Even the chemokines studied in diagnosis samples were inconsistent between patients. Serum concentrations of IFN-γ, CXCL10 and CXCL9 did not change the same way in both

patients, or even in the same patient. In one patient, RA296PM, serum IFN-γ and CXCL9 decreased whilst CXCL10 increased. In JR302PM, IFN-γ increased whilst both CXCL10 and CXCL9 decreased. Secretion of both of these chemokines is induced by IFN-γ, so it would be expected that as IFN-γ increased, then so would CXCL10 and CXCL9. However, IL-10 blocks these chemokines, so it is possible that a suppressive response was underway to counter the IFN-γ effects. IL-10 concentrations were too low to detect in any sample. It should be noted, however, that the concentrations of IFN-γ recorded were so low as to be close to the lower limit of detection of the assay, which could make the data unreliable.

The chronic GvHD case study patient was diagnosed with GvHD in the gut which led to hospital admission. Frequencies of both Th1 and Th17 cells were clearly elevated. Tregs were also elevated. This would suggest that the inflammatory subsets were increased because of the disease, and that Tregs were raised in order to combat the inflammation. However data from one patient is insufficient evidence. It is interesting to note that a considerable decrease in serum concentration of both IP-10 and MIG occurred during GvHD. This was surprising as a previous study showed increase of IP-10 during acute skin GvHD (Piper et al., 2007a). In the two case studies here which had acute skin GvHD, one had increased and one had decreased CXCL10 serum concentrations. The reason for these conflicting changes is unclear but could be related to transplant conditioning as RA296PM had myeloablative conditioning whereas JR302PM had a reduced intensity transplant. Both of these patients had follow-up samples some weeks following GvHD. There may be an unrecorded infectious event, or some other complication affecting reconstitution that affected the patient's CXCL10 levels.

Overall, it was not possible to make any association between frequencies of Th17, Th1 or Tregs with the occurrence of GvHD. Serum IL-6, IL-7, IL-15, CCL7, CX3CL1 and CCL3 were

slightly elevated in a number of cases, although concentrations were low. All of these are proinflammatory cytokines and chemokines. There was no apparent relationship between chemokine receptors CCR4, CCR6 and CXCR3 and GvHD, despite changes in two CXCR3 ligands, CXCL10 and CXCL9.

In order to reduce the influence of treatment being given between diagnosis and the time of sample collection, a more structured prospective approach should be taken with samples collected at very regular intervals so that a sample would be available close to any incidence of GvHD, at diagnosis and immediately upon resolution of the disease.

Chapter 6: Discussion

One of the most challenging issues facing stem cell transplantation research is how to enhance the GvL effect whilst abrogating GvHD. Much of the effort made to elucidate natural GvL targets has been focussed on immunity against overexpressed tumour antigens such as WT1 or PRAME. This study has shown that an immune response can be raised against CTAgs in AML and MM patients following allogeneic SCT. Although the number of patients in whom a response could be detected was not as high as reported in myeloma patients who have not undergone transplantation (Goodyear et al., 2005), it is an important demonstration of tumour-directed immunity in the post-transplant setting. The true frequency of patients with CTAg-specific T cell responses could also have been underestimated because of practical restrictions on the number of peptides that may be used in the screening panels, due to assay limitations and HLA-genotypes of the patients. As the number of defined immunogenic CTAg epitopes increases, larger panels can be used that are tailored to each patient's HLA-genotype. In addition, inclusion of peptides recognised by CD4+ T cells would allow investigation of the wider immune response to CTAgs by including assays that measure IL-10 and IL-17, as well as IFN-γ. Understanding of potential roles of Th1, Th2, Th17 and Treg subsets in a CTAg response may help devise therapeutic strategies for boosting an anti-tumour response.

Whilst it may be possible to develop a vaccine strategy that could be used to augment an anti-tumour response in patients following transplantation, it remains to be seen when the optimum time for such a strategy would be. In the very early period post-transplantation, the disrupted immune environment may prevent the development of a robust T cell response, particularly where conditioning agents lead to delayed T cell reconstitution. An alternative strategy, although controversial, may be to vaccinate the stem cell donors, enabling either tumour-specific cells to be present in the graft, or to be expanded and

adoptively transferred at a suitable time. Such an approach could not be considered with an overexpressed antigen due to the risk of toxicity to the healthy donor. However, as CTAgs should not be expressed in any immunogenic capacity in healthy individuals, this would not be expected to cause tissue damage. One might even speculate that there could be some potential benefits to inducing an anti-tumour immune response in a healthy individual.

Another potential strategy for the immunotherapeutic targeting of tumours is adoptive transfer of tumour antigen-specific T cells to patients. Such an approach has already been used to prevent of combat EBV-related lymphomas (Rooney et al., 1995) and CMV infections (Peggs et al., 2003, Walter et al., 1995) by transferring virus-specific T cells to the patients, and the expansion of these cells has been shown (Heslop et al., 1996). It may be possible to transfer T cells specific for tumour antigens to create, or enhance anti-leukaemic immunity. One method of generating these cells is to transduce T cells with a TCR which will recognise peptides from a tumour antigen. A murine model using a WT-1-specific TCR has shown that this approach is effective in killing leukaemic cells in NOD-SCID mice (Xue et al., 2005, Xue et al., 2010). However, although WT-1 is overexpressed by tumour cells, it is not restricted to malignant cells in the same way as CTAg-specific cells, so carries the potential risk of tissue-directed responses leading to GvHD. If commonly recognised CTAg targets can be identified by building on the work in this study, then suitable TCRs could be cloned for an adoptive transfer approach which would limit GvHD risk. It may be that mixed populations of T cells with TCRs for different CTAgs, both CD4+ and CD8+ T cells, could be generated which were tailored for the patient's HLA-type and transferred to prevent relapse.

Alternatively, there is the possibility of epigenetically upregulating the expression of CTAgs on any residual tumour, in order to increase the opportunity for the transplanted immune response to mount a response against the malignant cells. As this study has already

demonstrated the ability of the transplanted donor immune system to mount a response to CTAgs, then it is conceivable that this component of the GvL response would be of a greater magnitude if antigen concentration was higher.

Expression of CTAg genes is suppressed by hypermethylation. Studies have shown that hypomethylating agents such as 5-aza-2-deoxycytidine can upregulate CTAg expression on solid tumours (Guo et al., 2006, Weber et al., 1994), and also in chronic myeloid leukaemia (Dubovsky et al., 2009). Azacitidine can upregulate the expression of a number of CTAgs in AML and MM cell lines, an effect which is enhanced by the administration of the histone deaceylase inhibitor (HDACi) sodium valproate. Induction of a CTAg-specific CD8+ T cell response was demonstrated in AML patients following combination therapy with azacitidine and sodium valproate (Goodyear et al., 2010). Trials are ongoing to establish whether this effect can be brought about following stem cell transplantation. Use of these agents for anti-cancer therapy is already established and they have been used as a treatment for leukaemia (Soriano et al., 2007). It has been proposed that they have a direct cytotoxic effect on malignant cells by inducing apoptosis through expression of DNA-damage pathway genes (Gomyo et al., 2004). However, given the observations of a CTL response to tumour antigens following treatment, their therapeutic effect may be two-fold – both via direct cellular cytotoxicity and through enhancement of tumour immunogenicity. It remains to be seen if the immunological response generated against these epigenetically manipulated tumours can confer any long term benefit or will be hampered by establishment of tolerance. Nevertheless, current literature does allow the possibility that the GvL effect could be enhanced in some patients using epigenetic manipulation to induce CTAg expression.

One of the main challenges to GvHD therapy is suppression of the immune response directed against patient tissues without compromising general immunity in order that a GvL effect can be established, and to prevent infections. Currently, patients are given prophylactic immunosuppressive therapy in the early transplant period. This is withdrawn at the earliest opportunity in order not to compromise anti-leukaemic immunity. Patients who subsequently present with GvHD like symptoms are generally treated with broad effect immunosuppressant drugs. Prolonged, high-dose steroid treatment carries the risk of infection, viral reactivations, treatment-related toxicity or relapse. Patients are often given antibacterial, antifungal and antiviral drugs along with steroids. An alternative treatment is extracorporeal photopheresis whereby white blood cells are collected from the patient, incubated with a DNA-intercalating agent and exposed to ultraviolet (UV) light. Murine models showed that this had the effect of increasing the number of Tregs which suppressed GvHD (Gatza et al., 2008). Therefore, there is interest in the immune mechanisms underpinning the pathophysiology of the disease to develop treatment strategies.

As described in Chapter 1, previous studies have produced conflicting evidence for the role of T cell subsets in GvHD and whether they inhibit or exacerbate the condition. The data in this study did not show any definite correlations between Treg frequencies, effector T cell subsets, cytokine concentrations and GvHD occurrence. As discussed in Chapter 5, this may be due in part to the timing of sample collection. The primary aim of this arm of the study was to establish whether there was a particular role for IL-17A producing T cells in the pathogenesis of the disease and there was no apparent relationship. A recent publication found that Th17 cells were elevated in peripheral blood of patients with active GvHD, decreased in patients with inactive GvHD and that there was an increased Th17 infiltrate into skin, gut and liver tissues in patient biopsies (Dander et al., 2009). The use of controls in this

paper is curious, as they claim that blood samples from healthy donors are comparable to those from SCT patients without GvHD, and proceed to only show healthy control data in all but one figure. Here, little difference was seen between GvHD and non-GvHD patients, begging the question as to whether use of non-GvHD SCT patient controls would have showed the same significances in that paper. Nevertheless, it is interesting that they show decreased Th17 frequencies in GvHD patients with inactive disease. Indeed, the author did state that having samples taken during active disease was crucial for detection of elevated Th17 (E. Dander - personal communication). This further suggests that the samples available here, which cannot be directly related to disease status at the time of collection, may cause the data to be difficult to interpret.

Iclozan and colleagues, as described in Chapter 1, found that the Th17 subset had a role in GvHD, but was not essential, and that in the absence of IL-17A secreting cells, other T cell subsets would compensate (Iclozan et al., 2010). This suggests that the Th17 subset is part of a normal inflammatory response in GvHD, and therapeutic interventions targeting IL-17A secreting cells would be of little benefit if that led to increased contributions of other subsets.

As the current broad immunosuppressive approach is not optimal, better prediction or targeted therapies are required. Very regular sampling of SCT patients might allow the detection of a predictive marker, such as a population of T cells or perhaps a soluble factor. One study addressed this by screening all SCT patients for Th17 levels and looking for increases prior to symptoms of GvHD (Lange, et al., 2008. Poster, ASH). The author stated that it was difficult to establish a clear pattern (Lange - personal communication), and as yet, the work has not been published. The poster showed that Th17 frequencies were often increased in the week prior to GvHD diagnosis, but had reduced by the time disease was

diagnosed. In some cases, the frequencies were below previous measurements and Treg levels were elevated. This would suggest that transient increases in Th17 populations had already occurred before patients in this study were diagnosed with GvHD. A sample collection strategy based on regular, prospective monitoring could lead to an informative study, particularly if a broad range of investigations could be carried out. For example, luminex assays of patient serum could establish an early increase in cytokines or chemokines. If treatment was started at an early stage, before symptoms had been established, then potentially lower doses of drugs may be required for disease control and resolution.

As Dander and colleagues reported, there may be an accumulation of Th17 cells in the tissues of GvHD patients (Dander et al., 2009). However, further studies on the roles of T cell subsets at the site of GvHD, along with the mechanisms of chemoattraction by chemokines and the dynamics of regulation at these sites, may allow the development of targeted therapies for GvHD to allow swift resolution of disease without a global impairment of immune function. Dander reported considerable technical difficulties with immunohistochemistry (IHC) staining for IL-17A secreting cells (E. Dander - personal communication), and colleagues have also described problems with Th17 IHC staining. If sufficient biopsy samples can be collected locally, and an IL-17A antibody can be satisfactorily validated, then the presence of Th17 cells at GvHD sites could be further investigated by IHC, or preferably using flow cytometry if adequate numbers of T cells can be isolated. However, a recently published, extensive study of biopsy samples from GvHD sites in gut and skin found that was difficult to determine whether Th17 cells were particularly elevated in GvHD as compared to non-GvHD patients, or whether elevated IL-17 secreting cells were causative in GvHD episodes or contributed to tissue damage, although the ratio of

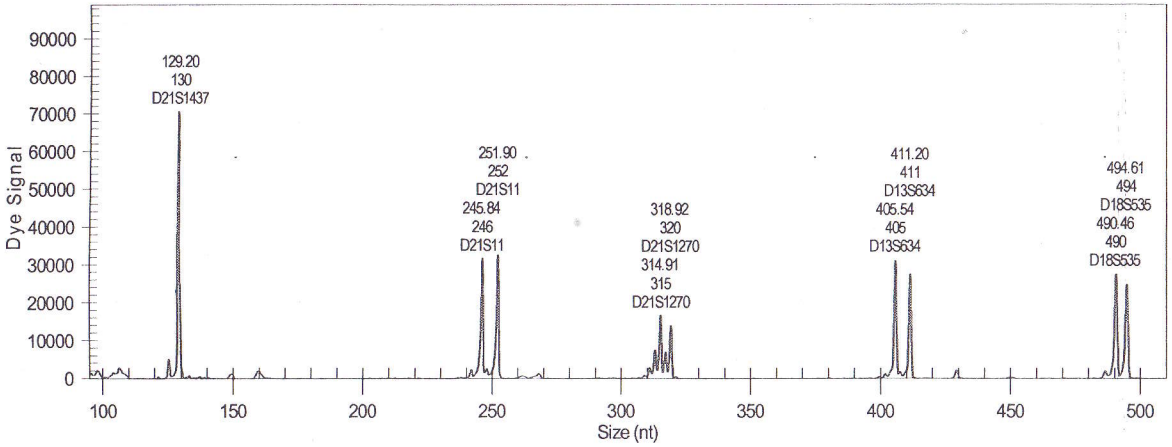
Th17/Tregs at these sites may be indicative of GvHD (Ratajczak et al., 2010). One limitation of their study was that biopsy material from the study cohort was not available from times when there was no evidence of GvHD. Therefore, whether Th17 frequencies increased during GvHD could not be determined. The authors suggest that comparing frequencies between different patients is not necessarily informative, as baseline levels can vary considerably without evidence of pathology. Again, if biopsy samples could be obtained before and after GvHD as well as during disease episodes, a clearer picture of whether Th17 cells do indeed play a role in GvHD may emerge. Such an investigation would depend on obtaining appropriate ethical approval, and the willingness of patients to provide additional skin biopsy samples.

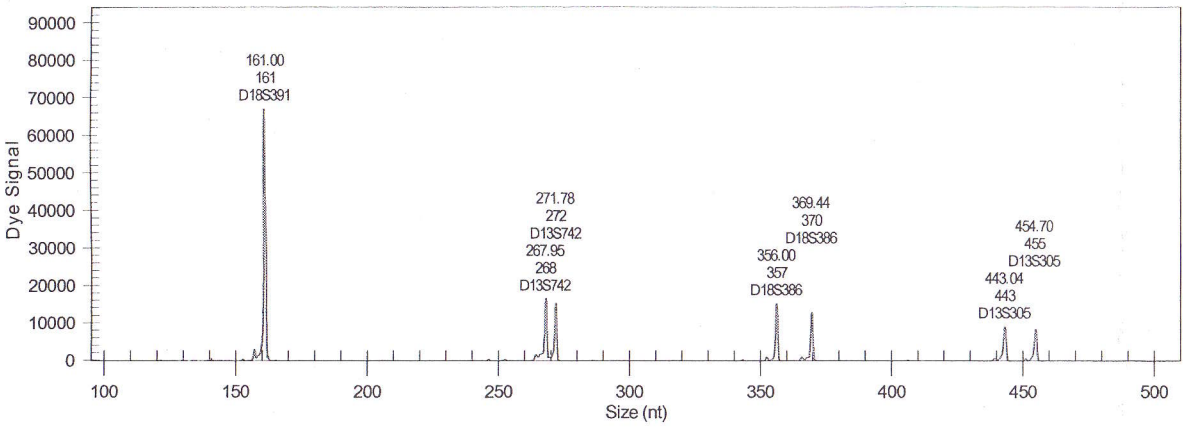
It could be interesting to relate early infectious events to GvHD and the immune repertoire. In this study, it was observed that one patient had comparatively high levels of Th17 cells even before the onset of GvHD. The patient had had a fungal infection early in the post-transplant period, and Th17 responses are thought to have a role in anti-fungal defences. A murine study showed that early *Aspergillus spp*. infections following SCT led to increased incidence of GvHD (Echtenacher et al, 2009 – Poster Abstract, ECI 2009). A human study related *Candida spp* colonisation to occurrence of GvHD and it may be that early infectious events could shape immune reconstitution, and potentially be a predictor of GvHD. Certain patients could potentially be deemed as higher risk for GvHD and could be targeted for prophylactic treatment.

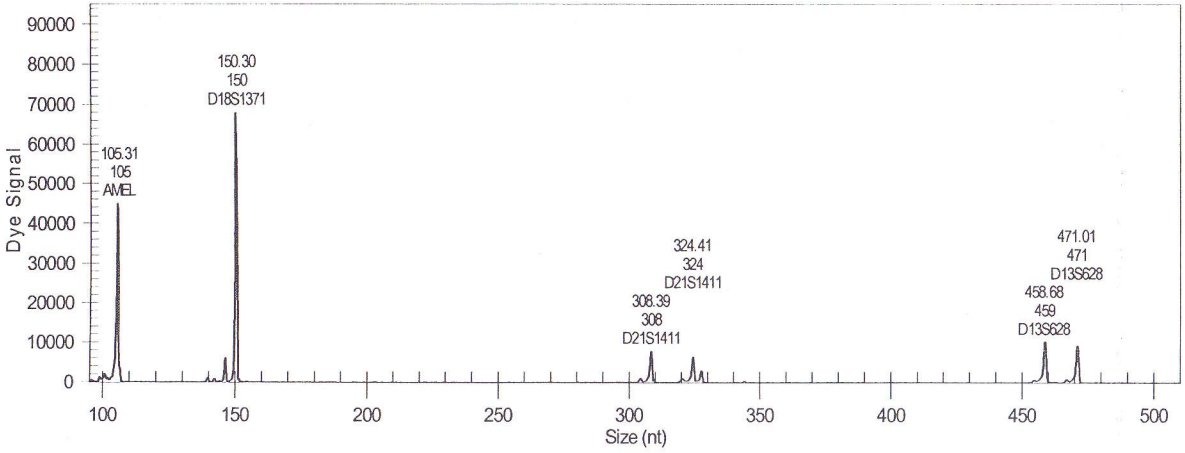
The main conclusion of this project was the demonstration of T cell immunity to CTAgs following stem cell transplant, and this established for the first time that MAGE family proteins are immunogenic in AML patients. These findings lead to the possibility that this

family of tumour-specific genes could be manipulated to enhance the GvL effect in allo-SCT patients.

Appendix A: Chimerism Raw Data







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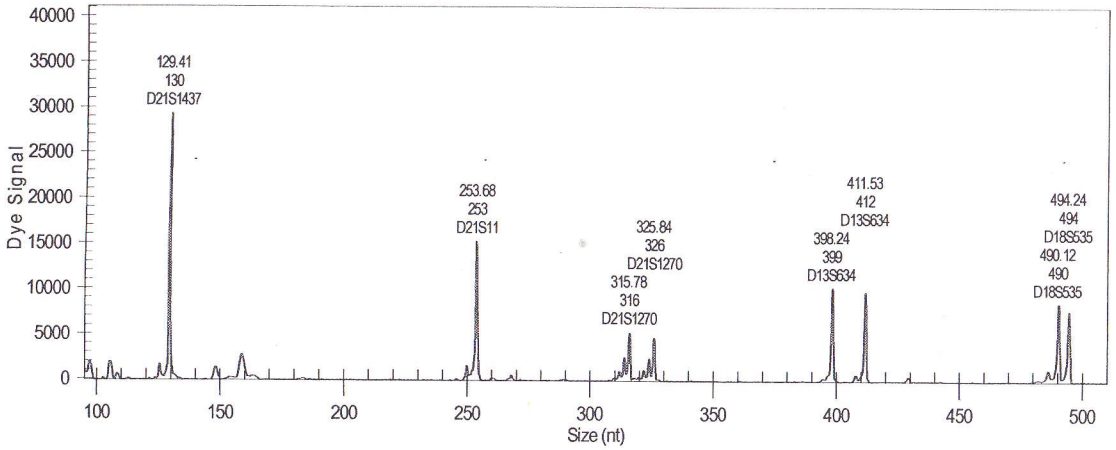

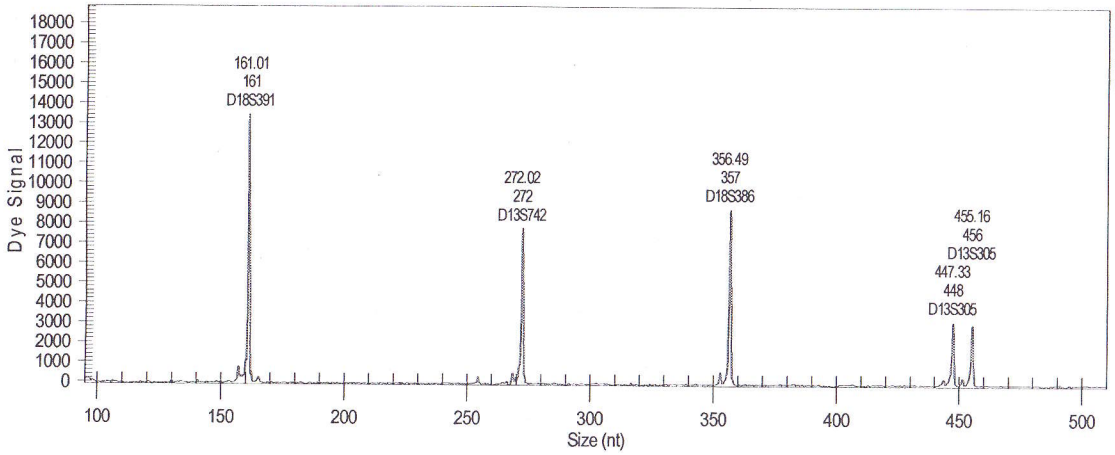

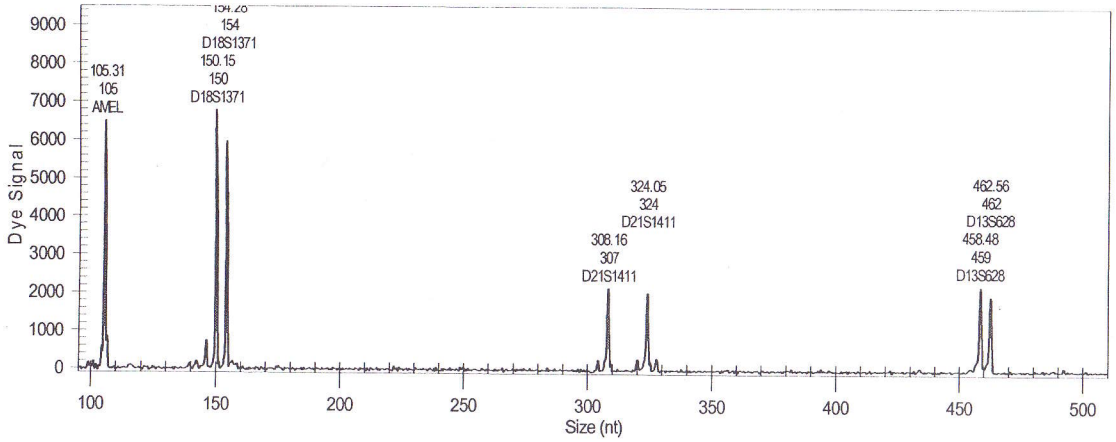

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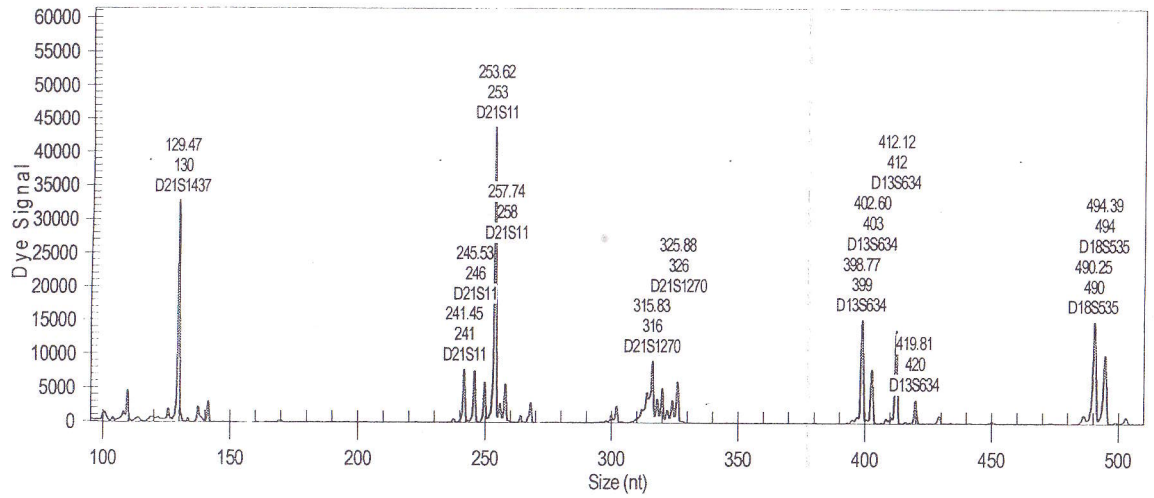
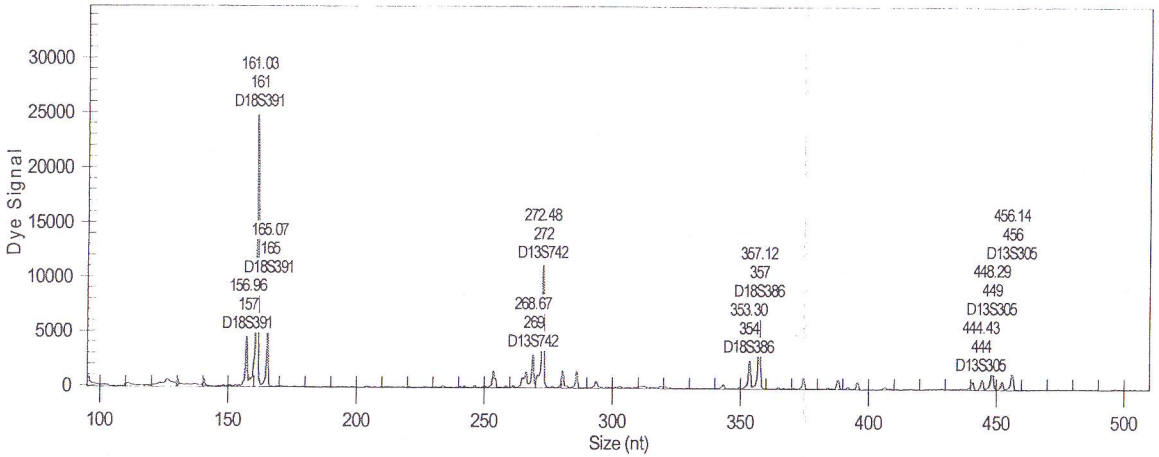
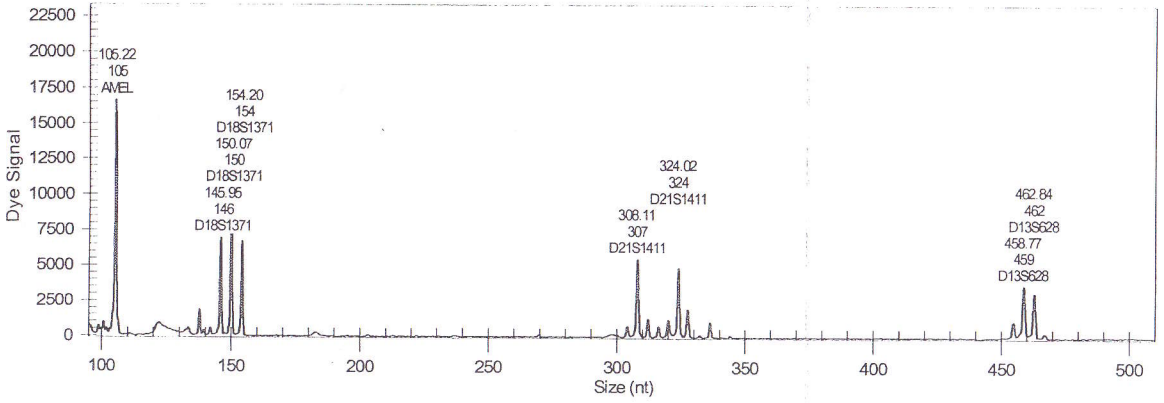
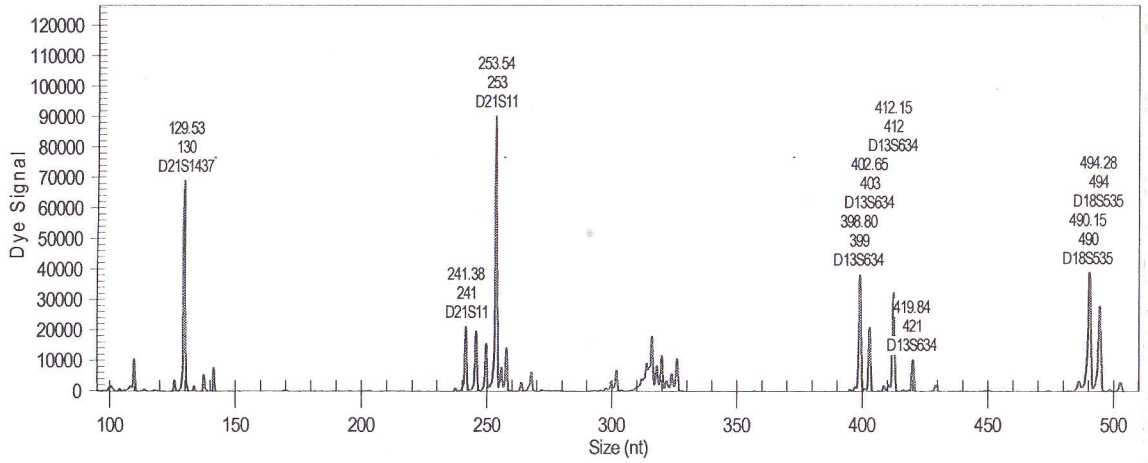
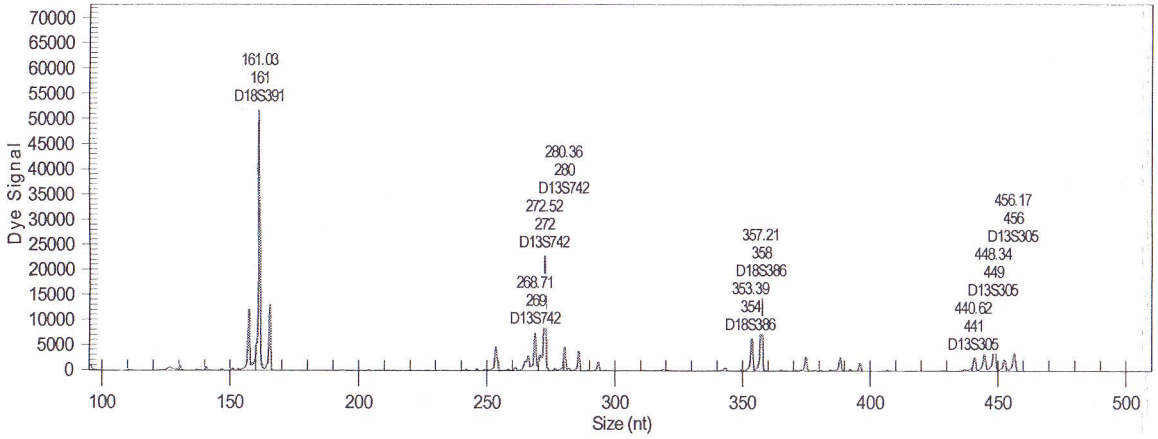


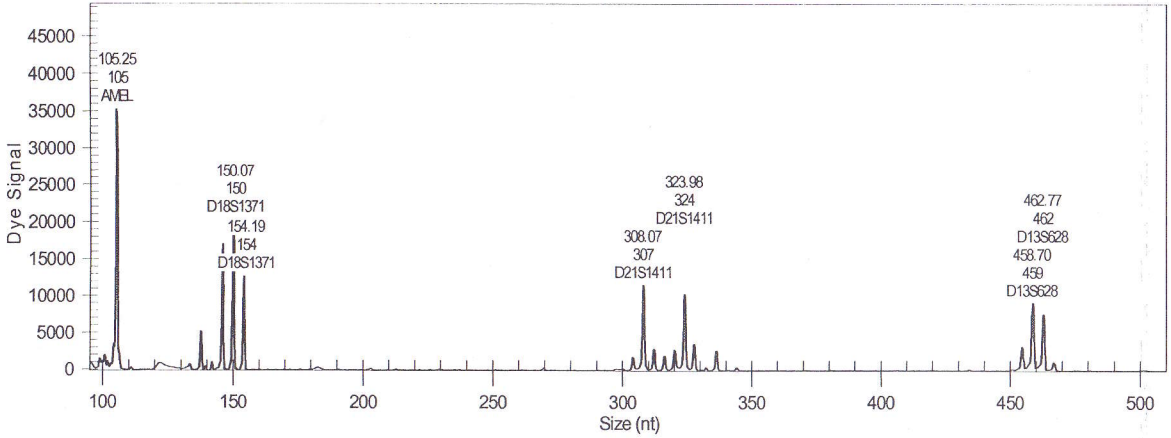




T-cell clone IG9SS







T-cell clone IB8SS

Appendix B: Published Journal Article

CD8+ T-cell immunity against cancer-testis antigens develops following allogeneic stem cell transplantation and reveals a potential mechanism for the graft-*versus*-leukemia effect

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ABSTRACT

Background
Allogeneic stem cell transplantation is associated with a powerful 'graft-*versus*-leukemia' effect that is generally considered to result from an alloreactive T-cell immune response. However, disease remission can also be observed after syngeneic transplantation and we investigated whether a T-cell immune response to cancer-testis antigens can be detected in patients in the post-transplant period.

Design and Methods
The T-cell immune response against cancer-testis antigens was studied in a cohort of 41 patients who underwent allogeneic stem cell transplantation for the management of acute myeloid leukemia or multiple myeloma. The cytokine secretion assay was combined with magnetic selection to allow detection of an interferon-γ-secreting T-cell response to a panel of cancer-testis antigen peptides.

Results
A cancer-testis antigen-specific CD8+ T-cell immune response was observed in the peripheral blood of five patients with an average magnitude of 0.045% of the CD8+ T-cell repertoire. Four of these patients had undergone reduced intensity conditioning transplantation with alemtuzumab for the treatment of acute myeloid leukemia and three remain in long-term remission. T-cell immunity was focused against peptides derived from MAGE proteins and was markedly increased within the bone marrow.

Conclusions
Functional cancer-testis antigen-specific CD8+ T-cell immune responses develop in the early period following reduced intensity allogeneic stem cell transplantation and are preferentially localized to bone marrow. These immune responses are likely to contribute to the cellular basis of the graft-*versus*-leukemia effect.

Key words: allogeneic stem cell transplantation, antigen-specific, CD8+ T cell, graft-*versus*-leukemia effect.

*Citation: McLarnon A, Piper KP, Goodyear OC, Arrazi JM, Mahendra P, Cook M, Clark F, Pratt G, Craddock C, and Moss PAH. CD8+ T-cell immunity against cancer-testis antigens develops following allogeneic stem cell transplantation and reveals a potential mechanism for the graft-*versus*-leukemia effect. Haematologica 2010;95(9):1572-1578. doi:10.3324/haematol.2009.019539*

Funding: this work was funded by a grant from Leukaemia Research (04029)

Manuscript received on November 11, 2009. Revised version arrived on March 29, 2010. Manuscript accepted on March 29, 2010.

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The online version of this article has a Supplementary Appendix.

Introduction

Allogeneic stem cell transplantation is used widely in the management of hematologic malignancies but the frequency of disease relapse remains a major challenge. The 'graft-*versus*-leukemia' (GvL) effect of allogeneic transplantation results from the recognition of host tumor cells by the donor immune system[1-4] and an improved understanding of the cellular targets of this response would facilitate the development of novel immunotherapeutic approaches.

The magnitude of the GvL effect is related to the degree of histoincompatibility between the donor and recipient and this has focused interest on minor histocompatibility antigens as the predominant target for T cells which mediate allogeneic immune responses.[5-7] However, it is now appreciated that GvL responses may also be observed in the setting of syngeneic transplantation in which there is no genetic mismatch between donor and recipient[8] and this has concentrated attention on the possibility that stem cell transplantation may be associated with immune responses against 'tumor-specific' antigens.[9]

Cancer-testis antigens (CTAg) are proteins whose physiological expression is restricted to germline tissue and are, therefore, not exposed to the systemic immune system so that immunological tolerance is not established. CTAg expression is often observed in malignant cells and may reflect induction of a 'germline transcription' profile during cell transformation.[10-13] CTAg-specific humoral and cellular immune responses have been reported in patients with a variety of solid tumors[14-16] and there is increasing interest in their potential role in hematopoietic malignancies.[17-21]

CTAg may also represent an important target for the GvL effect of allogeneic transplantation and antibodies to CTAg proteins have been demonstrated in patients who have undergone stem cell transplantation for the treatment of myeloma.[21] A T-cell response to the CTAg protein NY-ESO was also observed in one patient in this cohort but this was coincident with disease relapse. In this study we screened 41 patients for the presence of a $CD8^+$ T-cell immune response to CTAg peptides in the post-transplant period and related this to clinical outcome. Such immune responses were observed in five patients and, when present in the early period after transplantation, were associated with long-term disease control.

Design and Methods

Patients

Forty-one patients with a primary diagnosis of acute myeloid leukemia (AML) or multiple myeloma (MM) who were undergoing allogeneic stem cell transplantation were studied. Written informed consent was obtained prior to enrolment in the study and appropriate ethical approval was obtained from the South Birmingham Regional Ethics Committee. Thirty-five patients had a diagnosis of AML, while the remaining six were undergoing treatment for MM. Twenty patients received a myeloablative conditioning regimen consisting of cyclophosphamide (60 mg/kg x 2 days) and 14.4 Gy total body irradiation, and 21 received a reduced intensity conditioning regimen incorporating fludarabine (25 mg/m^2 x 5 days), alemtuzumab (140 mg/m^2) and melphalan (10 mg x 5 days) (Table 1).

Patients' samples

Heparinized peripheral blood samples of 50 mL were taken prior to the transplant and then at several time points in the post-transplant period. Bone marrow samples were obtained at the time of routine clinical monitoring and mononuclear cells were isolated from all samples by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). All assays were carried out using freshly isolated peripheral blood or bone marrow samples.

Detection of cancer-testis antigen-specific T cells

A panel of 20 peptides (Alta Biosciences, Birmingham, UK) from ten CTAg gene families was chosen on the basis of having been previously identified as T-cell epitopes (as detailed on the website of the Academy of Cancer Immunity) and also shown to have RNA expression in AML and/or MM.[22,23] The peptides, their HLA restriction and gene derivation are detailed in the *Online Supplementary Table S1*. Peptide-specific T cells were identified from peripheral blood mononuclear cells using detection of interferon-gamma (IFN-γ) cytokine secretion and an enrichment assay kit (CSA, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Freshly isolated peripheral blood mononuclear cells were seeded into wells of tissue culture plates (Iwaki) in RPMI 1640 media (Invitrogen) supplemented with 10% human serum (H+D Supplies) and L-glutamine (Invitrogen) at a cell density of 1×10^7/mL and left overnight without stimulation at 37°C in 5% CO_2. Peptides were then added either individually or in pools of not more than six at a final concentration of 10 μg/mL. An equivalent volume of dimethylsulfoxide (Sigma, UK) was added to a negative control well which was then used to determine gate positioning for FACS analysis, as shown in Figure 1. Staphylococcal enterotoxin B (Sigma, UK) was used as a positive control at a concentration of 1 μg/mL. Following a 3-h stimulation period, the cells were labeled for 5 min with IFN-γ catch reagent and incubated under continuous rotation for 45 min at 37°C. The cells were then labeled with IFN-γ detection reagent conjugated to a phycocerythrin fluorochrome, followed by anti-phycoerythrin magnetic beads. Magnetic selection was carried out either manually using MS columns (Miltenyi Biotech) for double positive selection, or using the equivalent "posseld" selection program on an autoMACs (Miltenyi Biotech). Pre- and post-selection samples were labeled with CD4-fluorescein isothiocyanate, CD8-phycoerythrin cyanin 5.1 monoclonal antibodies (Beckman Coulter, High Wycombe, UK) and propidium iodide (1 μg/mL, Sigma, UK) to exclude dead cells. Flow cytometric analysis was carried out using either a Beckman Coulter XL-2 flow cytometer (Beckman Coulter, High Wycombe, UK) with WinMDI software for analysis (Scripps Institute, La Jolla, USA) or a BD LSRII with FACS DIVA analysis software (BD Biosciences). The percentage of antigen-specific T cells in the $CD4^+$ or $CD8^+$ pool was calculated using the number of cytokine-secreting cells in either the positively selected fraction or pre-selected samples gated on $CD4^+$ or $CD8^+$ cells within the peripheral blood mononuclear cells. Any IFN-γ-secreting cells detected in the unstimulated negative control were considered to be background and subtracted from the frequency of cells producing IFN-γ following peptide stimulation.

Results

Cancer-testis antigen-specific $CD8^+$ T-cell responses can be detected in patients following stem cell transplantation

The IFN-γ cytokine secretion assay was used to identi-

fy CTAg-specific T-cell responses to a panel of 20 immunodominant peptides derived from ten CTAg genes (*Online Supplementary Table S1*). Peptides were selected on the basis that they were derived from proteins for which RNA expression had previously been demonstrated in AML and/or MM, and immunogenicity had been previously documented in patients with solid tumor malignancies.[24] Peripheral blood lymphocytes were isolated from patients at several time points following transplantation and were stimulated with CTAg peptides appropriate to the HLA genotype of the patient.

Overall, CTAg-specific CD8+ T cells were identified in five patients who were screened at a wide variety of time points in the post-transplant period. The frequency of the CTAg-specific CD8+ T-cell response was variable and ranged between 0.0005% and 0.2% of the total CD8+ T-cell pool. The mean frequency of responses was 0.045%, which equates to approximately 1 in 2000 CD8+ T cells and is comparable to the magnitude of many virus-specific immune responses. Of the 20 patients who received reduced intensity conditioning with alemtuzumab, four had detectable CD8 responses to CTAg peptides, whereas of the 21 who underwent myeloablative conditioning only one had CTAg-specific CD8 (Figure 2).

The majority of CTAg-specific immune responses could be detected without the requirement for magnetic enrichment, although selection was used to increase the reliable sensitivity of the IFN-γ cytokine secretion assay down to 0.0001%.[25] If a CTAg-specific response was only apparent after magnetic selection then the precursor frequency was calculated on the basis of the post-selection sample, although this approach tends to underestimate the true value due to cell loss during selection. An example of flow cytometric analysis before and after magnetic enrichment is shown in Figure 1. These responses could be detected serially in some cases and the absolute magnitude of the response varied at different time points in the post-transplant period.

Patients were recruited to the study at a range of different time points in the post–transplant period (Figure 2) and the association between CTAg-specific immunity and time post-transplant is discussed below.

Cancer-testis antigen-specific CD8+ T-cell responses were observed in two patients with long-term disease-free survival

CTAg-specific CD8+ T cells were detected in two patients within the first year following transplantation and both of these had undergone reduced intensity conditioning allografting for AML (Figure 3). AML patient 189A (Figure 3A) had a T-cell response to peptide RAGE-1$_{11\text{-}20}$ and also to the peptide pool comprising MAGE-C2$_{336\text{-}344}$/MAGE-A1$_{289\text{-}298}$. These responses were observed at 9 weeks post-transplant which was the first time point of analysis. Of note, the patient had suffered from grade IV graft-*versus*-host disease (GvHD) of the skin 3 weeks after transplantation and this had been treated with oral prednisolone. The T-cell response to RAGE-1$_{11\text{-}20}$ was present at five separate times of analysis until 51 weeks post-transplant although the magnitude of the response was variable and was undetectable on four occasions. Cyclosporine A treatment had been discontinued at 27 weeks post-transplantation and this coincided with a transient increase in the RAGE-specific T-cell response (Figure 3A).

CTAg-specific T cells were detected in patient 235A at

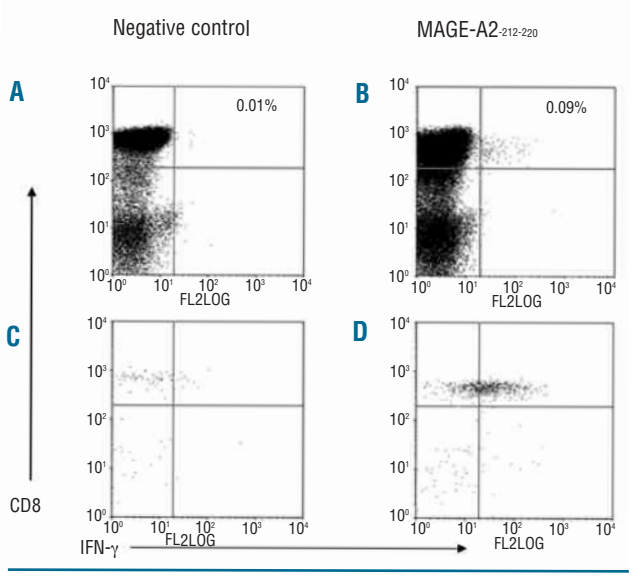

Figure 1. Flow cytometric enumeration of frequency of CTAg responding CD8+ cells. Following the IFN-γ cytokine secretion assay and enrichment, perioheral blood mononuclear cells incubated with dimethylsulfoxide only during the stimulation period are shown, prior to (A) and following (C) magnetic selection, along with IFN-γ responses to MAGE-A2$_{212\text{-}220}$ prior to (B) and following (D) magnetic selection.

Table 1. Clinical characteristics of the patients undergoing stem cell transplantation.

Age (years): median (range)	50 (23-63)
Gender, number (%)	
Male	21 (51)
Female	20 (49)
Underlying disease, number (%)	
Acute myeloid leukemia	35 (85.4)
Multiple myeloma	6 (14.6)
Transplant type, number (%)	
Myeloablative	
Sibling	14 (34.2)
Unrelated donor	6 (14.6)
Non-myeloablative	
Sibling	11 (26.8)
Unrelated donor	10 (24.4)
HLA-types of patients, number (%)	
A1	20 (44.4)
A2	26 (57.7)
A3	11 (24.4)
A24	4 (8.8)
B7	16 (35.5)
B18	2 (4.4)
B44	17 (37.7)
Cw7	30 (66.6)
Cw16	4 (8.8)
Disease state, number (%)	
Relapsed	8 (19.5)
Non-relapsed	33 (80.5)

17, 23 and 29 weeks after transplantation and this also coincided with tapering of cyclosporine A therapy, which was finally discontinued by 25 weeks. However, at 20 weeks, no response was detected (Figure 3B). This patient was one of 13 also screened prior to beginning conditioning for transplantation, although no pre-transplant responses were detected.

Importantly, patients 189A and 235A have remained in remission since transplantation and now remain disease-free at 52 and 37 months post-transplant, respectively. Overall, there were 14 patients who were screened for CTAg-specific immune responses on two or more occasions within the first year post-transplant and who are alive without relapses. As CTAg-specific T cells were observed in two of these, we were able to demonstrate immunity to this relatively limited CTAg peptide panel in 14.3% of this group. Screening began on an additional AML patient (138A) at 92 weeks post-transplant and a T-cell response was observed against peptides MAGE-C2$_{336-344}$ and MAGE-A1$_{161-169}$ but not detected thereafter (*data not shown*). This patient has also remained relapse-free during follow-up.

Cancer-testis antigen-specific T-cell responses can also be detected at the time of disease relapse

Our previous studies of CTAg-specific immunity in patients with myeloma revealed that CD8$^+$ responses are often observed around the time of disease relapse and

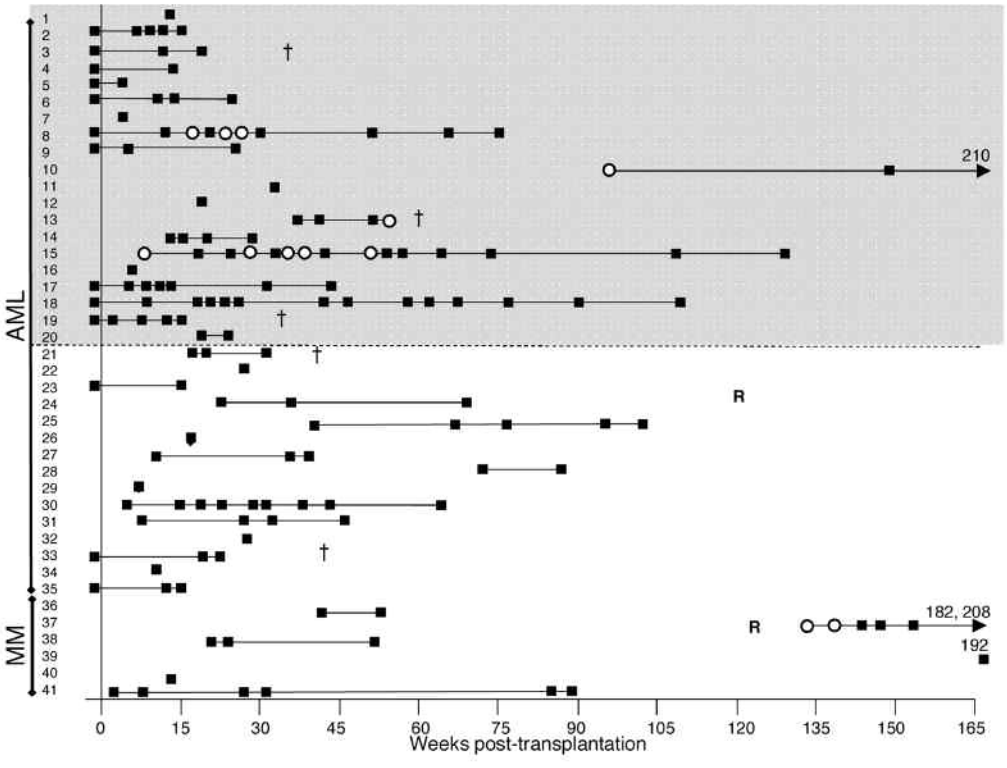

Figure 2. Summary of the screening of all patients' samples. The times of all screens are shown in weeks post-transplantation (y-axis). Assays in which no responses were seen are shown as black squares and positive responses are shown as open circles. Samples screened more than 165 weeks post-transplantation are indicated by a number representing the week at which the sample was screened. A cross (†) denotes time of death of patients who died due to relapse, and a letter R shows when patients suffered relapse but were successfully treated and have survived. The gray area shows those patients who received reduced intensity conditioning. Response frequencies in patients 15 and 8 are shown in Figure 3 (A and B, respectively), and patients 37 and 13 are detailed in Figure 4 (A and B, respectively).

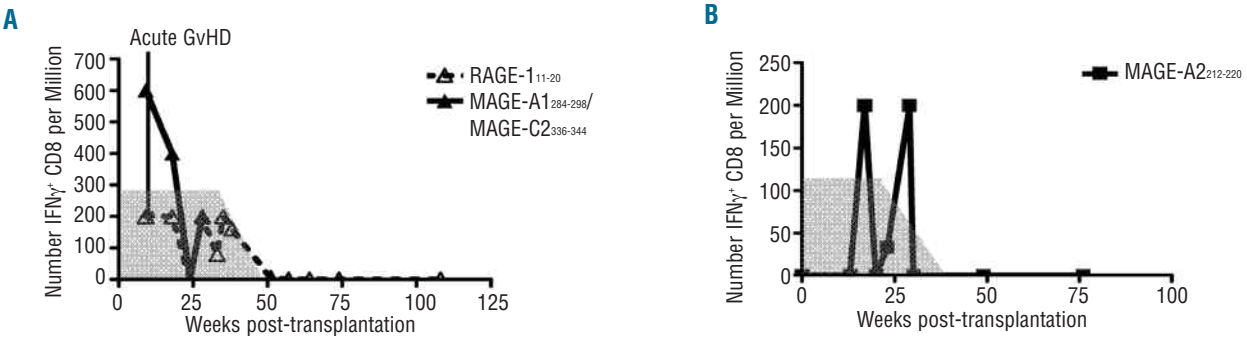

Figure 3. Responses to CTAg peptides were detected at various times post-transplantation in AML patients The frequencies over time of CTAg-specific T cells in two AML patients who had responses are shown. The gray area indicates prophylactic tapered cyclosporine treatment given during the early weeks following transplantation. Patient 189A (A) suffered grade IV acute graft-*versus*-host disease during week 7 post-transplantation which was treated with prednisolone. Patient 235A (B) had fluctuating responses between weeks 17 and 29 which were undetectable at week 20 post-transplantation.

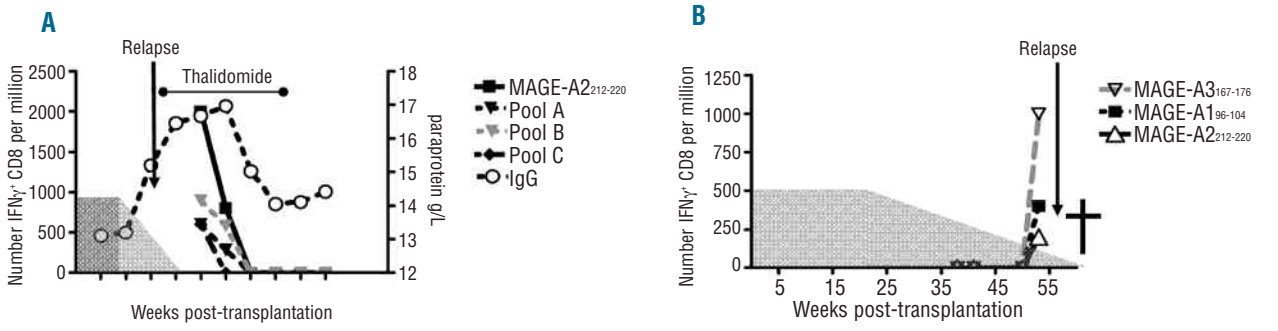

Figure 4. Responses to CTAg peptides were detected post-transplantation in relapsed patients The frequencies over time of CTAg-specific T cells in relapsed myeloma patient 125M are shown (A). The gray area indicates prophylactic tapered cyclosporine treatment given during the early weeks following transplant. Paraprotein levels are plotted on the right-hand axis. CTAg-specific responses were detected in AML patient 172A shortly before relapse (B).

probably reflect immune response to increased tumor load.[19] A similar pattern was observed in patient 125M with a diagnosis of MM who was initially studied more than 2 years after stem cell transplantation with a myeloablative conditioning regimen. The serum paraprotein level had risen at 124 weeks post-transplant and indicated the onset of disease relapse. The first CTAg analysis was undertaken 10 weeks after this point and revealed a broad CTAg peptide-specific immune response with specificity for peptides in three different peptide pools and included reactivity against the peptide MAGE-A2-212-220. The patient was treated with thalidomide and the next sample was taken at 143 weeks at which time the magnitude of the MAGE-A2212-220 -specific response had fallen by 60% and responses to other peptide pools were not observed. Interestingly, the patient responded well to thalidomide with a reduction in the paraprotein level and no CTAg-specific immune response could be detected in subsequent analyses (Figure 4A).

Patient 172A with a pre-transplant diagnosis of AML was screened from 38 weeks post-transplant and responses were seen at 53 weeks to peptides MAGE-A196-104, MAGE-A2212-220 and MAGE-A3167-176. At 55 weeks the patient was diagnosed with a relapse of AML and died shortly afterwards (Figure 4B).

RAGE-1-specific T cells can be detected at higher frequency within bone marrow than within peripheral blood

Paired blood and bone marrow samples from patient 189A were obtained at 35 and 51 weeks post-transplantation. T cells specific for RAGE-111-20 were detected by the IFN-γ cytokine secretion assay and the frequency was found to be up to seven times higher in bone marrow than in peripheral blood mononuclear cells. In peripheral blood, at both 35 and 51 weeks, 0.0006% of CD8+ T cells were specific for RAGE-111-20, whereas in the bone marrow, the frequencies were 0.0021% at 35 weeks and 0.0042% at 51 weeks (*Online Supplementary Figure S1*).

The cancer-testis antigen-specific CD8+ T-cell response post-transplantation is preferentially focused on peptides derived from the MAGE family of proteins

Twenty CTAg peptides were used in this study and T-cell responses within patients undergoing stem cell transplantation were detected in response to six of these. Interestingly, responses were focused on peptides derived from the MAGE family of proteins and four patients showed T-cell immune responses against peptide MAGE-A2212-220 (*Online Supplementary Figure S2*). Overall, CD8+ T-cell responses were observed against peptides derived from the MAGE subfamily proteins A1, A2, A3 and C2, and a strong immune response against RAGE-1 was also observed in one patient.

Discussion

Almost all experimental studies of GvL have focused on minor histocompatibility antigens and the potential role of 'tumor-associated' proteins has been relatively ignored. CTAg are important targets in autologous cancer-specific immune responses and have the capacity to contribute to the allogeneic GvL response. The development of a humoral immune response against CTAg proteins has been reported as a frequent event in patients undergoing stem cell transplantation for MM, and T-cell clones against NY-ESO have been reported in one patient at the time of disease relapse.[21]

We studied CTAg-specific T-cell immunity in a large cohort of patients undergoing allogeneic stem cell transplantation for AML and MM, and observed CTAg-specific CD8+ T cells in five of the 41 patients. While this may seem a relatively low frequency it must be appreciated that the peptide pool only contains a small fraction of the potential immune epitopes from CTAg proteins, and in addition the potential relevance of each peptide is limited by the HLA genotype of the patient. As such the use of a broader range of CTAg peptides would be expected to facilitate the detection of many more CTAg-specific T-cell responses within this group of patients. The magnitude of individual CTAg peptide-specific immune responses averaged 0.045%, or around 1 in 2000 of the CD8+ T-cell repertoire. This is comparable to the level of T-cell response to many viral epitopes[26] and indicates that the immunogenicity of these proteins can be quite considerable in this setting. The range of responses varied between 0.0005% and 0.2% of the CD8+ T-cell pool and suggests that individual peptides are likely to reflect

either dominant or subdominant epitopes within each patient.

Interestingly, the strength of the CTAg-specific immune responses also varied over time within individual patients and seemed to correlate with clinical events. The reduction in prophylactic immune suppression after stem cell transplantation is often associated with increased allogeneic immune responses and in two patients (189A and 235A) we were able to detect CTAg-specific T cells immediately following cessation of cyclosporine A treatment. The magnitude of CTAg-specific immunity that is required for clinical efficacy is unclear but in CTAg vaccine trials for metastatic melanoma, tumor regression has been demonstrated even though it is not always possible to detect tumor-specific T cells. This has led to the belief that very low numbers of T cells are required for an effective anti-tumor response[16,27] and the levels within our cohort of patients are certainly in this range. In the setting of allogeneic transplantation it is well documented that patients can achieve durable remissions on withdrawal of immunosuppression.[28]

The CTAg-specific immune response in patient 235A was interesting in that it fluctuated quite markedly in magnitude. Two strong positive responses were seen at weeks 17 and 29 post-transplantation but on two occasions between these dates the response was much lower, and in one case undetectable. The reason for this is unclear but is possibly related to biological factors such as T-cell homing and tissue distribution which frequently results in some degree of fluctuation in T-cell responses. However, although the IFN-γ cytokine secretion assay has been validated in our laboratory, we cannot rule out the possibility that the lack of a detectable response on that occasion could have been due to a technical problem with the assay. The IFN-γ secretion assay is a powerful technique but the sensitivity of detection that was required for this study is at the limit of current technology.

Interestingly, all of the AML patients who developed CTAg-specific immune responses had undergone reduced intensity conditioning which includes the use of alemtuzumab. This regimen is strongly immunosuppressive and provides valuable prophylaxis against GvHD. However, the clinical outcome for AML patients after reduced intensity conditioning has been very encouraging[29] and it is tempting to speculate that induction of a CTAg-specific immune response could provide one explanation for this.

Although our studies were performed largely on peripheral blood samples, CTAg-specific T cells are only likely to be effective in the control of hematopoietic malignancies if they are able to enter the bone marrow. We found that CTAg-specific $CD8^+$ T cells were selectively recruited to marrow such that their frequency was up to seven-fold higher at this site. We have recently described a unique 'bone marrow homing' chemokine receptor profile on $CD8^+$ T cells[30] and in future studies it will be important to determine the phenotype of CTAg-specific T cells at these sites. Interestingly, a recent study investigated T-cell immunity to the leukemia-associated antigens WT1 and proteinase-3 in patients with myeloid malignancy and also found higher frequencies in the bone marrow than in peripheral blood.[31]

The major clinical question must relate to the potential contribution of the CTAg-specific T-cell response to the GvL effect. It seems clear that such responses were induced as a result of the allogeneic transplant as no immune response was observed in the 13 patients who were screened prior to transplantation, and the magnitude of the CTAg-specific population was increased in association with withdrawal of immune suppression in two patients. CTAg-specific T cells have been shown to have the capacity to kill primary tumor cells *in vitro*[32] and it is, therefore, tempting to speculate that such immune responses do indeed contribute to disease control.

However, CTAg-specific T-cell immune responses were also seen in two patients at the time of disease relapse. We have seen this pattern previously in patients with MM and believe it reflects the response of $CD8^+$ T cells to increased availability of CTAg protein from recurrent tumor.[18] Interestingly, the allogeneic NY-ESO-specific T-cell response reported by Atanackovic *et al.* was also observed at the time of relapse of MM.[21] Future studies will need to address the phenotype and magnitude of T-cell responses in relation to relapse. Interestingly, the AML patient in our study in whom we detected a CTAg-specific $CD8^+$ response at relapse had very low levels of donor chimerism prior to relapse and it will be important to address whether CTAg-specific immunity at relapse reflects expansion of recipient-derived T cells.

Somewhat to our surprise, the majority of T-cell responses were specific for peptides derived from MAGE proteins. Although it was initially felt that MAGE was not expressed in AML,[33] mRNA expression has recently been reported.[34] The fact that MAGE expression is highly restricted to germ cells and malignant tissue is likely to dictate that MAGE-specific immune responses are manifest as a GvL response rather than GvHD.[35,36]

In conclusion we have demonstrated frequent occurrence of CTAg-specific $CD8^+$ T cells in patients following reduced intensity stem cell transplantation. The association between the early detection of such an immune response and prolonged disease remission provides support for the concept that CTAg-specific immunity plays an important role in GvL. Further studies are now indicated to explore the potential for enhancing CTAg-specific immunity in the post-transplant setting.

Authorship and Disclosures

AM carried out the experiments, designed the research, and drafted the paper; KP and OCG designed the research and reviewed the paper; JA, MC, FC, GP, PM and CC consented patients and provided clinical information; PAHM designed the research and drafted the paper.

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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