Alloantigen specific T cell depletion from haematopoietic stem cell grafts for the prevention of graft versus host disease

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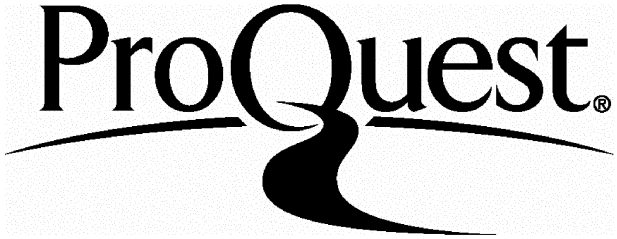

dedicated to Mum and Dad… for their continuing faith in me

and to N. Charnley……for inspiration and everything else

"This was a good year for persimmons. Autumn in the mountains was beautiful."

Palm of the Hand Stories *Y. Kawabata*

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ABSTRACT

Graft versus host disease (GvHD) is a multi-step immune process involving lymphocyte activation and proliferation. We have devised a novel method of selectively depleting alloreactive cells from haematopoietic stem cell grafts while retaining a pool of immunocompetent non alloreactive lymphocytes possessing anti-viral and possibly anti-leukaemic activity and capable of hastening immune reconstitution. This method involved identifying the alloreactive cells thought to initiate GvHD by means of an activation marker, CD69 in an in-vitro system and depleting these cells by paramagnetic bead sorting. The temporal dynamics of CD69 expression as well as other activation markers in an allogeneic setting was first initially determined to determine the optimal marker and time for depletion. Using flow cytometric analysis and cell proliferation data, the engineered graft was shown to retain only 12% of its original alloreactivity but preserving 78% of its 3rd party reactivity. This system has been tested on mismatched and histocompatibility matched donor/patient pairs. A modified mixed lymphocyte culture system using cytokines has also been studied and this has proved to be of clinical significance in predicting GvHD. Cytokines, in particular, γ-interferon was shown to upregulate various cell surface molecules important in antigen presentation and may explain in part the crucial role of cytokines in GvHD. This depletion strategy was then tested in a NOD/SCID murine GvHD model. This involved comparing the intraperitoneal injection of 5-10x10^6 unmanipulated T cells from a CBA (H-2^k) mouse into a non-lethally irradiated (250 cGy) NOD/SCID (H-2^{g7}) recipient (positive control), with that of mice who received allo-depleted cells. This allodepletion strategy protected against death from lethal GvHD in a complete MHC mismatched setting (survival 12.5% in positive control versus 71.4% in allo-depleted group). In parallel, by using tetrameric HLA-peptide

complexes and looking at CMV+, HLA-A2 individuals, it has been demonstrated that the non-alloreactive fraction using this strategy retained 90% of the specific anti-CMV activity, suggesting that these grafts would protect from CMV reactivation. Moreover, the alloreactive cells are easily recoverable in this selective T-cell depletion strategy for cryopreservation and ready for immediate access as therapeutic donor lymphocyte infusions in cases of frank relapse post-transplant.

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ABBREVIATIONS:

γ-IFN	gamma-interferon
2ME	2-Mercapto-ethanol
^{3}H-thymidine	Tritiated thymidine
AIM	activation inducer molecule
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APB	alkaline phosphate buffer
APCs	antigen presenting cells
ATG	anti thymocyte globulin
BMT	bone marrow transplantation
BSA	bovine serum albumin
CD	cluster of differentiation
CD40L	CD40 ligand
CIK	cytokine induced killer
CM	complete medium
CML	chronic myeloid leukaemia
CMV	Cytomegalovirus
CNS	central nervous system
cpm	counts per minute
CR	complete remission
CTLA4-Ig	cytotoxic lymphocyte antigen-4
CTLp	cytotoxic T lymphocyte precursor
CTLs	cytotoxic lymphocytes
DAG	1,2 diacylglycerol
DLIs	donor leukocyte infusions
DMEM	Dulbecco's modified eagles medium
DMSO	dimethylsulphoxide
DSBR	double-strand break repair
DSCA	double strand conformation analysis
EAE	experimental allergic encephalomyelitis
EBMT	European Blood and Marrow Transplantation
EBNA	Epstein Barr Nuclear antigens
EBV	Epstein Barr virus
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot assay
FACS	fluorescent activated cell sorting
fas-L	fas-ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward angle light scatter
GCSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GvHD	graft versus host disease
GVHR	graft versus host reaction
GvL	graft versus leukaemia
GvT	graft versus tumour

HBSS	Hanks buffered saline solution
HHV6	human herpes virus 6
HLA	human leukocyte antigen
HPA-3	human platelet antigen-3
HTLp	helper T-lymphocyte precursor
i/p	intra-peritoneal
i/v	intravenous
IBMTR	International Bone Marrow Transplant Registry
ICAM-1	intracellular adhesion molecule-1
IL	Interleukin
IL-2R	IL-2 receptor
IP_3 inositol	1,4,5-triphosphate
ITAM	immunoreceptor tyrosine based activation motif
KIRs	killer inhibitory receptors
LAK	lymphokine activated killer
LDA	limiting dilution assay
LFA-1	lymphocyte function associated antigen-1
LMP	latent membrane proteins
LPS	lipopolysaccharide
mAb	monoclonal antibodies
MACS	magnetic cell sorting
MFI	median logarithmic fluorescent intensity
mHags	minor histocompatibility antigens
MHC	major histocompatibility complex
MIP-1α	macrophage inhibitory protein-1α
MLC	mixed lymphocyte culture
mMLC	modified MLC
$NaHCO_3$	sodium bicarbonate
NF-AT	nuclear factor of activated T cells
NK	natural killer
NO	nitric oxide
NOD	non obese diabetic
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSCT	peripheral blood progenitor stem cell transplantation
PBST	PBS + 0.1% Tween20
PE	phycoerythrin
PerCp	peridinin chlorophyll protein
PHA	phytohaemagglutinin
PMA	phorbol-12-myristate-13-acetate
PTLD	post transplant lymphoproliferative disease
PWM	pokeweed mitogen
RFLP	restriction fragment length polymorphism sequence
RPMI	Roswell Park Memorial Institute hydrogen-carbonate-buffered medium
RRI	relative response index
s.d.	standard deviation
SBA	soybean lectin agglutination
SCID	severe combined immunodeficiency disease
SI	stimulation index
SSC	side angle light scatter

SSCP	single strand conformation polymorphism
SSOP	sequence specific oligonucleotide probing
SSP	sequence specific primers
TBI	total body irradiation
TBST	TBS + 0.05% Tween20
Tc	cytotoxic T
TCD	T cell depletion
TCR	T cell receptor
Th	T-helper
TNF-α	tumour necrosis factor-alpha
TNFR	tumour necrosis factor receptor
URSTO	universal recombinant site targeting oligonucleotide
VCAM	vascular cell adhesion molecule-1
WT-1	Wilm's tumour gene encoded transcription factor

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1. Chapter 1. Main Introduction:

Initial sporadic attempts to harness the potential benefits of allogeneic bone marrow transplantation (BMT) were followed by rapidly expanding use in the treatment of acute leukaemias. Early efforts were directed towards those refractory to conventional chemotherapy. The prognosis and survival of acute leukaemias then in the 1950s was dismal and a search for better survival with other forms of therapy was required. Vast improvements have occurred since then and allogeneic BMT is now used to treat a wide range of diseases. The main indications are still haematological malignancies (chronic and acute myeloid leukaemia in particular, acute lymphoblastic leukaemia, lymphomas, multiple myeloma); others include myelodysplastic syndromes, aplastic anaemia and benign disorders such as immune deficiencies and inherited metabolic disorders. Data collated by the International Bone Marrow Transplant Registry (IBMTR) shows a sharp increase in the number of allogeneic transplants since the 1980s with an estimated 17000 allotransplants performed in 1997.

Allogeneic transplantation however is still associated with a substantial array of potentially life threatening complications. The major risks include: 1) the acute and late toxicity attributable to the intense "myeloablative" chemotherapy and radiotherapy containing conditioning regimens, 2) graft rejection, 3) graft versus host disease (GvHD), 4) life threatening infective complications resulting from poor immune reconstitution and 5) disease relapse. Improvements in the conditioning regimens and supportive care have considerably reduced morbidity and mortality and the issue of gentler, "non-myeloablative" conditioning regimens is being actively investigated.

Graft rejection is uncommon in a matched related unmanipulated transplants (Anasetti et al 1989). GvHD, infection and relapse however remain major obstacles to a successful transplant outcome.

A further issue is donor availability. It has been estimated that only 30% of patients undergoing allogeneic stem cell transplantation have a genotypically matched sibling (Madrigal et al. 1997). Even with the substantial volunteer registries, the probability of finding a phenotypically matched donor remains less than 70% in the Caucasian population. One solution to this problem of limited availability of matched donors is the used of mismatched donors. At present, mismatched donor transplants however carry a higher risk of morbidity and mortality largely due to a higher incidence of graft versus host disease and delayed immune reconstitution.

Clearly, for allogeneic transplantation to be made safer and applicable to a broader range of diseases by the use of a wider pool of donors to be made available, the twin issues of GvHD and immune reconstitution need to be addressed since these account for the most common causes of peri-transplant mortality. This is especially salient when considering allogeneic transplantation for other less immediately "life-threatening" diseases such as sickle cell disease. The benefits and safety of transplantation must clearly outweigh the risks involved.

Clinically severe GvHD still occurs despite post transplant immunosuppression. Pan T cell depletion is extremely effective in preventing GvHD but leads to an increased incidence of graft rejection, delayed immune recovery and leukaemic relapse in malignant disease, implying a crucial role of T cells in these processes (Horowitz et al 1990). Besides being involved in the graft versus host (GvH) reaction, they are both

the initiators of the beneficial graft versus leukaemia (GvL) effect and the principal mediators in providing immunity from infection. A successful strategy for GvHD prevention will need to dissect the complex role T cells play in these processes which can be beneficial or deleterious to the outcome of transplantation.

1.1 The scientific basis for allogeneic BMT:

1.1.1 Chemotherapy vs transplantation:

The field of clinical allogeneic bone marrow transplantation has expanded exponentially since the first reports of its success in the treatment of acute leukaemia. (Thomas et al. 1959). The mainstay of treatment of the acute leukaemias has been with chemotherapy consists of a combination of different cytotoxic agents (combination chemotherapy) often given in cycles to achieve the maximum log kill of leukaemia cells. Initially, it was thought that the purpose of an allogeneic BMT was to allow for more intensive and "myeloablative" doses of chemotherapy/radiotherapy to be given, with the transplant procedure providing the stem cell rescue for haematopoeitic recovery (Thomas et al. 1975a). This seemed consistent with the fact that patients with leukaemia, which proved refractory to standard doses of chemotherapy achieved remission following "myeloablative" conditioning that included total body irradiation (TBI) (Thomas et al. 1975b)

1.1.2 Immune basis for cure by alloBMT

Allogeneic BMT as a form of immunotherapy was first perceived by the Harwell group of scientists (Barnes & Loutit 1957). However, it was only when excessive relapse

rates were encountered following T cell depleted allografts that it was fully appreciated how much the anti-leukaemic effect of the transplant procedure was due to an immunologically mediated GvL effect (Apperley et al. 1986). When a retrospective analysis of allogeneic BMT was performed (Horowitz et al. 1990), it was found that for a comparable cohort of patients with the same disease, the probability of relapse was lower for patients receiving an allogeneic marrow compared to a syngeneic marrow. This was despite the fact, that in both situations, the "myeloablative" conditioning regimens used were similar. The only difference between these comparable cohorts were the difference in the origins of the donor marrow: matched sibling vs identical twin. This implied that something in an allogeneic graft in contrast to a syngeneic graft was responsible for the significant decrease in relapse risk.

Weiden had also pointed out the anti-leukaemic effect of chronic GvHD in contributing to an improved survival after allogeneic transplantation (Weiden et al. 1981), (Weiden et al. 1979). This data was corroborated by Sullivan who demonstrated that GvHD, both acute and chronic, improved the probability of disease free survival (Sullivan et al. 1989). There have also been anecdotal reports of leukaemia remission during episodes of acute GvHD (Higano et al. 1990). Conversely, the absence of GvHD predicted a higher chance of relapse, especially in patients with chronic myeloid leukaemia (CML) patients. The fact that T cell depletion was associated with an increased chance of relapse indicated that T cells responsible for GvHD were also capable of mediating the anti-leukaemia effect (Horowitz et al 1990). This anti-leukaemic effect correlates best with the presence of chronic GvHD but the lowest rate of relapse is seen in patients with both acute and chronic GvHD. As for the donor, syngeneic transplants are rarely complicated wuth GvHD but have a significantly higher risk of relapse than allogeneic transplant recipients. (Gale et al 1984). Conversely, patients transplanted from

unrelated donors have a significantly higher rate of GvHD and a lower risk of relapse compared to matched sibling transplants (Gajewski et al. 1990). In other words, the greater the degree of antigen mismatching and potential for alloreactivity, the greater the likelihood for more severe GvHD, but also for a more potent GvL effect. Any immunosuppresive treatment designed to prevent or treat GvHD such as cyclosporin or methotrexate also diminishes the GvL effect (Barrett et al 1997), (Bacigalupo et al. 1991). The advent of donor leukocyte infusions (DLIs) has provided the strongest evidence so far of the importance of a GvL effect (Kolb et al. 1995).

The GvL response has been best characterised and described for CML and extended to the acute leukaemias. Since then, the more embracing term: "graft versus tumour (GvT) effect has been coined to include the range of applicable diseases like multiple myeloma (Verdonck et al. 1996), myelodysplasia (Porter et al. 1996) and lymphoma (Jones et al. 1991).

1.2 Graft versus host disease:

1.2.1 Incidence and characteristics:

GvHD is a pathophysiological process caused by alloreactive donor T cells which culminates in multi-organ system dysfunction and destruction. This is a reflection of exaggerated but normal physiological inflammatory mechanisms occurring in a setting where they are undesirable. RE Billingham's prerequisites from 1966 regarding the key characteristics of the process which define GvHD still holds true (Billingham 1966) and remain crucial to the development of our graft engineering strategy:

"the graft must contain immunologically competent cells", meaning it is immune mediated and initiated by mature functional donor cells in the graft.

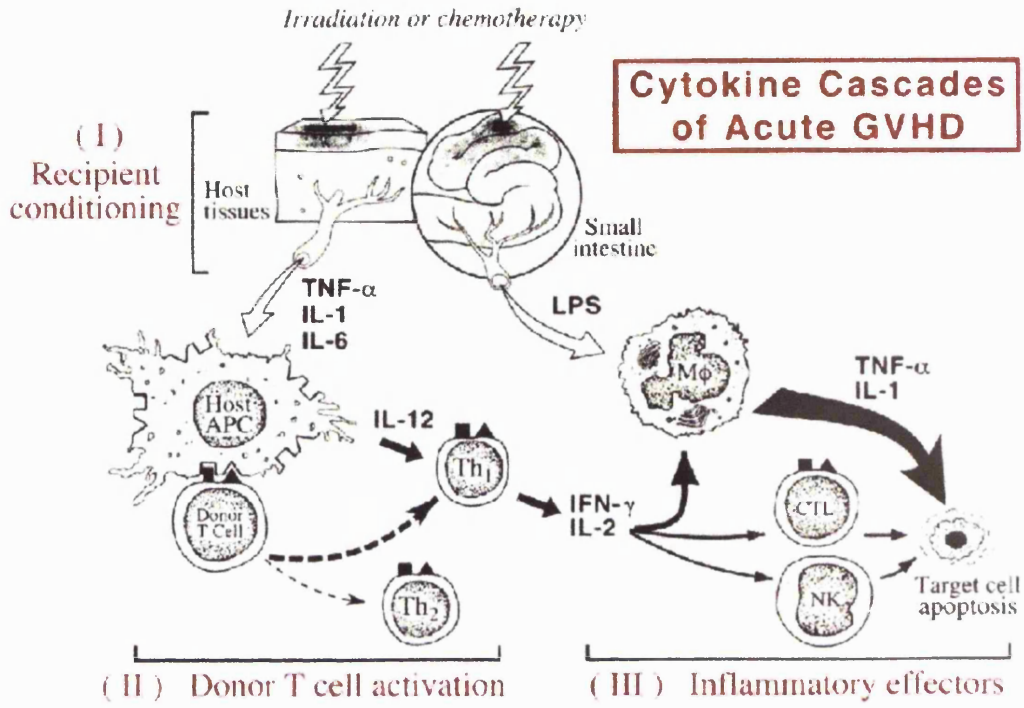
"the host must possess important transplantation alloantigens that are lacking in the donor graft, so that the host appears foreign to the graft" implying an alloreactive process involving recognition of alloantigen disparity and its sequelae.

"the host itself must be incapable of mounting an effective immunological reaction.

GvHD is a major cause of morbidity and mortality following allogeneic BMT (Gale et al. 1987). Despite post transplant immunosuppresive therapy with either cyclosporin or methotrexate as single agents, moderate to severe acute GvHD develops in about 45% of unmanipulated HLA-identical sibling transplants (Gale et al 1987). If both agents are combined, the incidence is reduced to 35% (Storb et al. 1989) but remains considerable. Human leukocyte antigen (HLA) mismatching is the single most important factor for the development of GvHD (Madrigal et al 1997). Other factors include age and sex of both donor and recipient, previous exposure to cytomegalovirus (CMV) infection, the type and stage of disease at time of transplant, details of the transplant regimen including TBI and choice of GvHD prophylaxis including T cell depletion (Atkinson et al. 1990), (Gale et al. 1987).

Ferrara has proposed that dysregulation of complex cytokine networks occurring in **three sequential phases** forms the framework for the pathophysiology of acute GvHD (Antin et al 1992), (Krenger et al. 1996). Figure 1.1 provides an overall view of the multi-step process involved in the pathogenesis of GvHD.

Figure 1.1 Cytokine cascades and the 3 phases of acute GvHD:



taken from "Graft versus host disease" edited by JLM Ferrara, HJ Deeg, SJ Burakoff (Marcel Dekker publishers)

1.2.2 Phase 1 "cytokine storm" and recipient conditioning:

The earliest phase of GvHD begins prior to the infusion of donor cells. The transplant conditioning regimen damages and activates host tissues and leads to the secretion of inflammatory cytokines including tumour necrosis factor-alpha (TNF-α), interleukin 1 (IL-1) and granulocyte-macrophage colony stimulating factor GM-CSF) (Xun et al. 1994). These may upregulate adhesion molecules and major histocompatibility complex (MHC) antigens thereby enhancing the recognition of host MHC or minor

histocompatibility antigens by infused mature donor T cells (Norton et al 1991a), (Chang et al. 1986), (Leeuwenberg et al. 1988). This process is in accordance with the observation that an enhanced risk of GvHD after BMT in both a murine model and a clinical situation is associated with more intensive conditioning regimens and the release of inflammatory cytokines (Clift et al. 1990), (Hill et al. 1997a). Lipopolysaccharide (LPS) also leaks through the intestinal mucosa damaged by the conditioning regimen. The massive outpouring of cytokines during this transplant conditioning has been aptly termed the "cytokine storm" (Ferrara 1993). The importance of this "cytokine storm" is illustrated by the fact that despite injection of 10-fold more donor lymphocytes, graft versus host reaction (GVHR) in unirradiated F1 hosts results in far less mortality and histopathological damage to target organs than does allogeneic BMT involving the same strain combinations (Hakim et al 1997) subjected to a radiation containing conditioning regimen. In an elegant study, a progressive increase in the intensity of conditioning was associated with an increasing severity of GvHD (Hill et al 1997a).

1.2.3 Phase 2 "donor T cell activation":

This phase consists of donor T cell activation leading to the proliferation and differentiation of activated T cells. The degree of donor-recipient histoincompatibility determines the extent of allorecognition and alloreactivity but this involves mature donor T cells recognising recipient peptide-HLA complexes (alloantigens): MHC differences in mismatched and minor histocompatibility antigen disparity in matched grafts. Analysis of T cells in target organs affected by GvHD has also shown specific Vβ usage, supporting the idea of local expansion of T cell clones to host alloantigens (Liu et al. 1996).

The T cell subsets involved depend in part on the histoincompatibility differences between the donor and host. In murine models, the situation may be less complex where the genetic differences can be carefully controlled, but this may not invariably reflect the human situation. CD4+ and CD8+ T cell subsets are each capable of causing GvHD (Korngold et al. 1991), (Korngold 1992). GvHD directed at MHC class I and minor histocompatibility antigen differences is crucially dependent on CD8+ cells (Sprent et al 1990). By contrast, GvHD in fully mismatched and class II disparate transplants is largely dependent on CD4+ T cells which also play an essential role in regulating CD8+ alloreactive T cells (Via 1991). In the majority of clinical HLA-identical transplants, both CD4+ and CD8+ T cells can initiate this afferent arm of GvHD and are likely to do this simultaneously (Herrera et al. 1999). In certain diseases such as CML, CD8+ T cells may be more crucial in the pathophysiology of GvHD and clinical CD8 depletion strategies have demonstrated a decrease though not complete abrogation in its incidence (Nimer et al. 1994). Donor T cell activation is followed by clonal expansion and differentiation. This results in the production of T-helper 1 (Th1) type cytokines like interleukin-2 (IL2) and gamma-interferon (γ-IFN), which in turn further augments T cell activation, proliferation, migration and trafficking. It also leads to the induction of cytotoxic lymphocytes (CTLs) and natural killer (NK) cells, as well as the priming of macrophages to produce interleukin-1 (IL-1), interleukin-12 (IL12) and tumour necrosis factor-alpha (TNF-α), thus setting the scene for the next phase (Ferrara 1993).

1.2.4 Phase 3: "Inflammatory effectors":

Various T cell subsets including CD4+, CD8+ T cells and other novel T cell populations (Sakamoto et al. 1991) have been found in affected tissues. Whatever subset of T cells are predominantly involved, the initial hypothesis that the cytolytic function of CTLs cause the majority of damage is too limited. The other 2 key inflammatory effectors are NK cells and mononuclear phagocytes. NK cells appear to be prominent in several animal models (Ferrara et al. 1989) and they have been found in target tissues in GvHD (Guillen et al. 1986). Varying results have been obtained with NK cell depletion studies (Blazar et al. 1988), (MacDonald et al 1992) but this may reflect the diversity of NK subpopulations. NK cells participate both in target cell killing as well as the secretion of important cytokines. Mononuclear phagocytes which have been primed by donor T cell activation and cytokines released in phase 2 secrete more cytokines including TNF-α and IL-1.

Th1 cytokines are therefore crucial in recruiting NK cells and macrophages and amplifying the GvH reaction. Although Th1 cytokines implies a predominant secretion from CD4+ cells, CD8+ T cells can also be correspondingly subdivided into Tc1 and Tc2 subsets and the Tc1 subset would also contribute to the cytokine storm. IL12 in particular enhances T cell production of Th1 cytokines, NK cell secretion of γ-IFN, and the cytolytic functions of lymphocytes. An amplified loop then occurs as the secreted γ-IFN in turn further stimulates the pro-inflammatory macrophages to secrete more cytokines, resulting in tissue damage and systemic toxicity. In response to LPS, γ-IFN also acts to prime mononuclear cells to secrete TNF-α (Gifford et al 1987). LPS is secreted by intestinal mucosa damaged during the conditioning regimen and can directly stimulate gut macrophages as well as keratinocytes, fibroblasts and dermal

macrophages if it reaches the skin. TNF-α increases MHC expression, facilitates cell-mediated cytotoxicity, (Cavet et al. 1999) mediates tissue destruction and causes systemic morbidity and mortality during this effector phase of GvHD by inducing cell death (Nestel et al. 1992).

The other major pro-inflammatory cytokine is IL-1, principally produced by activated mononuclear phagocytes. The importance of IL1 was confirmed in 2 trials which studied the specific inhibition of IL-1 in steroid resistant GvHD and noted objective responses in patients (Antin et al. 1994), (McCarthy, Jr. et al. 1996) and in a murine model (Hill et al. 1999). In addition, excess nitric oxide (NO), produced by macrophages may contribute to the deleterious effects of GvHD, especially immunosuppression. IL6 and IL8 are 2 other cytokines that have also generated interest recently with regards to their involvement in GvHD (Symington et al. 1992), (Uguccioni et al. 1993).

The crucial role of Th1 cytokines was confirmed when Fowler demonstrated that cells of the Th2 cytokine phenotype prevented LPS-induced lethality during murine GvHD (Fowler et al. 1994). When mature donor T cells were polarised, ex-vivo towards a type 2 phenotype with rIL4, GvHD was abrogated in a murine transplantation model. These polarised cells were thought to function in vivo by inhibiting type 1 T cell responses including production of γ-IFN and thereby attenuating the systemic morbidity of GvHD (Krenger et al. 1995). Cytokine involvement and GvHD was approached from a different angle by examining recipient TNFα and IL10 gene polymorphisms and an association was seen with more severe GvHD and early mortality (Cavet et al 1999).

At the cellular level, the TNFα receptor system (Piguet et al. 1987), (Speiser et al. 1997), Fas/FasL pathway (Baker et al. 1997), (Via et al. 1996) and perforin/granzyme pathway (Blazar et al. 1997a), (Graubert et al. 1997) have all been variously and differentially implicated in the pathogenesis of GvHD. FasL defective donor T cells were shown not to induce cutaneous or hepatic GvHD (Baker et al. 1996) although GI disease was still present while TNF-α appeared to be crucial in the pathogenesis of gut lesions and less so for hepatic lesions (Hattori et al. 1998). The role of perforin/granzyme is more controversial with investigators disputing its relative importance (Blazar et al 1997a), (Tsukada et al. 1999), (Baker et al 1997). This is further complicated by the fact that in murine models, MHC class I mismatches are more susceptible to granzyme B damage compared to class II mismatches (Graubert et al 1996). The importance of cytokines was again highlighted by Martin who showed that even in the absence of Fas or perforin mediated mechanisms, cytokine mediated pathways could induce GvHD (Martin et al. 1998).

In addition, investigators have begun to look at chemokines such as macrophage inhibitory protein-1α (MIP-1α) and demonstrated its possible importance in murine GvHD across a Class I MHC barrier (Serody et al. 1999). Chemokines are a class of predominantly small molecular weight proteins that are involved in the recruitment of multiple effector cells including mononuclear leukocytes and neutrophils (Rollins 1997). It is thought that T cell production of MIP-1α may be important in the recruitment of alloreactive T lymphocytes (Serody et al 1999).

1.2.5 GvHD and infection:

It has been shown in defined murine models (Miconnet, de, V et al 1998) that stimulation of CD4 lymphocytes specific for the endogenous mouse mammary tumour viruses *Mtv-7* and *Mtv-6* play a crucial role in the pathogenesis of GvHD partly through the release of cytokines. Extrapolation to human transplantation is less straightforward as endogenous superantigens have not yet been evidenced. However bacteria (Kappler et al. 1989), Epstein Barr virus (EBV) (Sutkowski et al. 1996) and CMV (Dobrescu et al. 1995) have all been linked to superantigenic responses and the presence of these infectious agents are known to increase the risk of GvHD. Any infection would also stimulate tissue damage, production of cytokines and release of LPS predisposing the target organs to GvHD damage. Compared with conventionally housed irradiated mice, enteric GvHD was significantly reduced in germ free mice given incompatible marrow (van Bekkum et al 1977). Similar findings have been reported using prophylactic antibiotics for gut decontamination in humans. Viral antigens could also possibly function as minor histocompatibility antigens and invoke a T cell response. There may also be a bystander effect involved with concurrent GvHD and viral infections stimulating cross-reactive NK and T cells (Matzinger 1994).

It is well recognised that CMV infection can ~~exercebate~~ exacerbate the GvH reaction and contribute to the profound immunosuppression seen in such instances (Miller et al. 1986), (Grundy et al 1985). This relationship stems from a two way process: the GvH reaction may reactivate latent CMV infection via cytokine interactions and the latter could augment GvHD by altering expression of cell surface molecules important in immune recognition (Grundy et al 1985). In addition to the poorer immune reconstitution seen in allotransplants, this could also explain why CMV disease is more

common in allogeneic compared to autologous transplants. Clearly, any strategy of preventing GvHD that concomitantly also preserves anti-CMV activity would be of potential clinical benefit.

1.2.6 Organ distribution of GvHD:

Despite the ubiquitous expression of Class I on all tissues, GvHD seems to typically involve specific target organs. This implies that although the systemic "cytokine storm" is crucial in the pathogenesis of GvHD, it is not sufficient and other local factors must also play a role. The main organs affected are skin, liver and gastrointestinal system and there is accumulating evidence that the lungs may be another target (Cooke et al. 1998). This may in part be explained by the tissue distribution of minor MHC antigens or it may be related to the differences in peptide presentation by MHC molecules and presence of different populations of professional antigen presenting cells (APCs) in these tissue sites. The patterns of recruitment and migration of alloreactive lymphocytes into specific tissue sites could also be an important factor. In addition, tissue specific neo-antigens may be expressed at these sites with the ongoing inflammation. It has been pointed out that the target organ sites seem to be areas which share an intensive exposure to endotoxin and bacterial products that can trigger and amplify local inflammation in contrast to other organs like the heart and kidney (Ferrara et al 1999).

1.2.7 Major histocompatibility matching:

Histocompatibility antigens most important in transplantation are encoded by a series of genes that reside in a discrete chromosomal region on human chromosome 6 termed

the major histocompatibility complex. The human leukocyte antigen (HLA) system is the MHC equivalent in humans. The importance of the HLA system can be appreciated by the fact that during an immune response, T cells recognise foreign antigens presented as peptide fragments only in association with MHC molecules. This essential role of the MHC in antigen presentation explains in part the extensive polymorphism of the MHC in allowing a vast array of foreign peptides that can be presented to the immune system. In tissue transplantation, their role is central to T cell allorecognition and hence the initiation of the GvH reaction.

The HLA complex on chromosome 6 contains genes encoding for Class I and II molecules. Class I loci consist of 3 allele families termed A, B and C. Class II loci can be divided into DR, DP and DQ alleles. Each family consists of many distinct alleles thus leading to the extreme heterogeneity of individual HLA types. The loci traditionally examined in tissue typing to determine a suitably matched bone marrow donor are A, B, (Class I) and DR (Class II). This implies that there are 6 alleles to be typed and donor-recipient pairs could therefore range from complete mismatched (0/6) to fully matched (6/6). Some transplant centres are now also performing HLA-C typing, important for NK cell alloreactivity, as well as matching for HLA-DRB1 and DQB1 alleles (Petersdorf et al. 1996), (Petersdorf et al. 1995).

Historically, tissue typing was done by serological methods which are increasingly being replaced by molecular typing methods (Van Rood et al. 1975). These include restriction fragment length polymorphism (RFLP) analysis, sequence specific oligonucleotide probe hybridisation (SSOP), sequence specific primers (SSP), heteroduplex analysis, single strand conformation polymorphism (SSCP) and direct nucleotide sequencing (Bidwell 1992), (Krausa et al 1993), (Allen et al 1994), (Clay et

al. 1991),(Orita et al. 1989), (Petersdorf et al 1995). Two novel high resolution techniques have recently been developed: universal recombinant site targeting oligonucleotide (URSTO) and double strand conformation analysis (DSCA) (Arguello et al. 1996), (Arguello et al. 1998). The advent of high resolution typing has meant an explosion of newly defined alleles and consequently previously serologically "matched" pairs may now be molecularly mismatched. In a retrospective analysis of 440 patient-donor pairs who were serologically identical, molecular typing revealed that only 55% were still completely matched (Sasazuki et al. 1998) and that molecular mismatching at class I was clearly associated with an increased risk of GvHD. Ideally, every unrelated donor should be identical with the patient for alleles at all HLA loci. As this becomes increasingly difficult, the challenge is for typing laboratories to formulate a rational donor selection scheme that may allow for certain HLA incompatibilities. This has led to the concept of "permissive matching" (Madrigal et al 1997). In phenotypically matched unrelated transplants, GvHD still occurs with increased frequency compared to genotypically matched related transplants because other than minor histoincompatibility differences, it may be that the current resolution of typing is still insufficient to detect other allelic differences. Also, other HLA molecules other than A, B or DR may be important for matching.

1.2.8 Minor histocompatibility antigens:

Minor histocompatibility antigens (mHags) are immunogenic peptides derived from the expression of polymorphic genes which may differ from host and donor (den Haan et al. 1995). These peptides bind to HLA antigens and, like foreign antigens, are recognised by allogeneic T cells in a HLA-restricted fashion. Within HLA-matched siblings the degree of mHag disparity determines the GvHD response (Gale et al.

1987), (Goulmy et al 1997). The list of mHags is rapidly expanding and includes HA-1 to 7 and 2 HY mHags, of which the peptide sequences of HA-1, HA-2 and both H-Ys have been identified. The distribution of HA-1 and 2 which are HLA-A2 restricted, is confined only to haematopoietic tissues while the others are expressed on all cells. A lower relapse rate is seen in male recipients of female marrow transplants, compared to gender-matched transplants, suggesting that donor recognition of the exclusively male protein H-Y mHag on host leukaemic blasts may result in a GvL effect (Voogt et al. 1988). On the other hand, it may also contribute to GvHD (Gale et al. 1987). Goulmy has demonstrated a significant correlation between HA-1 mismatching and acute GvHD in 50 informative (HLA-A2) donor-recipient pairs (Goulmy et al. 1996). With the use of tetrameric HLA class I-peptide complexes, a significant increase in HA-1 and HY specific CTLs was demonstrated during acute and chronic GvHD which decreased correspondingly with successful treatment (Mutis et al. 1999a).

A large number of CTL clones specific for human mHags have also been isolated from the blood of HLA-matched BMT recipients (Warren, Greenberg, & Riddell 1998), (Goulmy 1996). Other examples of mHags important in transplantation are rapidly being discovered. Polymorphism of the adhesion molecule CD31 has been implicated in GvHD (Behar et al. 1996) and incompatibility for human platelet 3 antigen (HPA-3) was recently described as a risk factor for aGvHD, but only in HLA-A2 patients, strongly suggesting that the mismatch behaved like a mHag and was recognised in a HLA-A2 restricted fashion. The importance of mHags was further supported by observations of low rates of GvHD in populations that feature genetic homogeneity and presumably a lower diversity of mHag disparity (Morishima et al. 1995).

1.2.9 Peripheral blood stem cell transplants and the risk of GvHD:

It is now over 7 years since the first use of peripheral blood progenitor cells for allogeneic transplantation (PBSCT) (Dreger et al. 1993) and the number of transplants using PBSCT continues to rise, constituting about 26% of allografts reported to the European Blood and Marrow Transplantation (EBMT) registry in 1996 (Gratwohl et al. 1998). Compared to marrow, PBSCT recipients receive 4-5 times as many CD34+ cells with a median dose in the range of 4-10x10^6/kg and more with repeated apheresis procedures and if CD34 selection is performed (Russell et al 1998). Despite a higher T cell load, retrospective studies to date have shown no difference in the incidence of acute GvHD between PBSC and marrow (Schmitz et al. 1998), (Bensinger et al. 1996). This may be related to the use of granulocyte colony stimulating factor (GCSF) in mobilising the stem cells. GCSF was found to reduce the proportion of L-selectin+ T cells which has been associated with a greater propensity to induce GvHD by perhaps altering their migration and trafficking pathways (Sugimori et al. 1999). G-CSF also reduces T cell alloreactive capacity and induces a shift to a Th2 pathway (Pan et al. 1995). The incidence of chronic GvHD may however be higher (Storek et al. 1997). It has also been reported that PBSCs possess superior engraftment properties resulting in more rapid immune reconstitution, especially in mismatched transplants and greater GvL activity (Russell et al 1998). The alloreactive potential of various stem cell grafts were compared in a murine assay and confirmed that PBSC had a lower immunoreactive potential compared to marrow (Leung et al. 1999) with deceased IL2 and γ-IFN production in response to alloantigen stimulation (Zeng et al 1997).

1.2.10 Predictive tools for GvHD:

Although matching donors and recipients for HLA reduces the incidence and severity of GvHD, some recipients receiving HLA-mismatched grafts do not develop acute GvHD whereas other recipients of genotypically matched allografts may develop severe, lethal GvHD. Techniques have therefore been developed to assess the likelihood of GvHD and the clinical significance of 1) known HLA mismatched, b) cryptic HLA mismatches and 3) mHag differences. The mixed lymphocyte culture (MLC) assay has been widely used in the past to confirm HLA class II matching (Baxter-Lowe et al. 1992). This technique is however too insensitive to distinguish many mismatches and to accurately predict the incidence or severity of GvHD (Mickelson et al. 1993). The advent of molecular typing has rendered it redundant (Baxter-Lowe et al 1992).

Limiting dilution analysis has proved to be a sensitive tool for the detection and investigation of T lymphocytes of defined specificity. The cytotoxic T lymphocyte precursor (CTLp) and helper T-lymphocyte precursor (HTLp) assays use limiting dilution analysis to quantify the frequency of donor anti-host cytotoxic T and helper T cell precursors capable of responding to mismatched alloHLA antigens presented on the patient cells (Kaminski et al. 1991), (Schwarer et al. 1994), (Schwarer et al. 1993). High CTLp frequencies correlate with class I mismatches usually undetectable by conventional typing and whereas high HTLp frequencies appear capable of detecting class II differences (Kaminski et al. 1989), (Kaminski et al 1991), (Schwarer et al 1994). High CTLp values may occur with no detectable mismatch in unrelated donor-recipient pairs and this is likely due to a high level of undefined class I incompatibilities beyond the resolution of current tissue typing (Madrigal et al 1997).

The CTLp assay is not good in detecting mHag mismatches and hence only useful in unrelated transplants but not in matched sibling transplants (Kaminski et al 1989), (de Bueger et al. 1993). While some studies have suggested a correlation between high CTLp and increased risk of severe GvHD (Kaminski et al 1989), (Roosnek et al. 1993), (Spencer et al. 1995), others find no such correlation (Fussell et al. 1994). Reproducibility appears therefore to be a problem. The same can also be said of the HTLp assay which has been used as a predictive tool in both unrelated (Schwarer et al 1994) and matched related transplants (Theobald et al. 1992) with variable success.

Newer techniques have been developed. One such method is the human skin explant model (Dickinson et al. 1998), (Sviland et al 1999). Donor lymphocytes sensitised against irradiated recipient lymphocytes are subsequently co-cultured with recipient's skin biopsy sections and histological changes assessed and graded. This has been shown to successfully predict acute GvHD in HLA-identical sibling BMT. When directly compared against the CTLp and HTLp assays, only the skin explant model was an accurate indicator of acute GvHD, able to detect presumed mHag disparities whereas the CTLp and HTLp assays were not predictive. An in-vivo GvHD model transplanting human epidermal cells and human lymphocytes into severe combined immunodeficient mice has also been developed (Takakura et al. 1999).

1.3 Graft versus leukaemia:

1.3.1 GvH and GvL as separable phenomena:

Both the immune basis of cure in alloBMT and the association of GvL with GvHD are well recognised. The question remains as to whether all of the GvL effect is part of the general alloreactive process occurring in GvHD. A distinct GvL phenomenon separable from GvHD has been reported in some animal models (Bortin et al. 1973), (LeFever et al 1985), (Truitt et al. 1983). However, some of these animal systems involve experimental virally induced leukaemias and the observations may, therefore not be applicable to humans. Direct evidence for a distinct GvL effect in humans has been more difficult. Retrospective analyses have indirectly shown that GvL occurs in the absence of GvHD because relapse rates are lower in allograft recipients not developing severe acute GvHD than in transplants between identical twins (Horowitz et al 1990), (Gale et al. 1994). Patients relapsing post BMT have attained remission after DLI without developing acute GvHD (van Rhee et al. 1994), (Kolb et al 1995). Moreover, by altering either the number of T cells or the timing of infusion, a GvL effect may be seen independent of GvHD (Truitt et al 1991a), (Johnson et al 1993). The latter though could argue for common rather than distinct effectors but perhaps exhibiting different thresholds of reactivity against normal and leukaemic blasts.

The relative importance of the fas-fas ligand (fasL), perforin/granzyme and TNF-α cytotoxic pathways in mediating target cell death may be different in GvHD and in GvL. Dissection of these pathways is another way of differentiating GvHD from the GvL effect. A murine leukaemia model using either perforin defective or FasL defective animals has demonstrated that the Fas/FasL pathway is crucial for the

induction of GvHD but not for the GvL effect. Conversely, the perforin pathway was important for the GvL effect (Tsukada et al 1999). Disruption of the cytokine network so crucial to the pathogenesis of GvHD is an alternative mechanism of differentiating the GvH reaction from GvL. Both these mechanisms were highlighted when Pan showed that while the alloreactive and cytokine functions of PBSC grafts were reduced, the perforin dependent cytotoxic pathway important in GvL was maintained indicating a functional separation of GvH and GvL activity (Pan et al. 1999). Systemic levels of LPS and TNF-α were also reduced.

1.3.2 Leukaemia target antigens in GvL:

If a specific GvL effect were to be exploited, then central to defining that specificity would be the identification of antigens present on leukaemia cells that are recognised by donor effector cells. Table 1.1 lists some of the potential candidate target antigens.

HB-1 is a novel mHag specific for B cell acute lymphoblastic leukaemia which elicited a donor-derived HLA-B44 restricted CTL response in a patient treated by HLA-matched BMT (Dolstra et al. 1999). The leukaemia restricted distribution of this polymorphic antigen and the ability to generate HB-1 specific CTLs in vitro using peptide loaded dendritic cells offers a novel opportunity for leukaemia specific therapy without invoking the risk of GvHD. Wilm's tumour gene encoded transcription factor (WT1) has recently been described to be associated with malignant transformation of CD34+ progenitor cells. HLA-A0201 restricted CTLs specific for WT1 have been shown to kill leukaemia cell lines and transformed CD34+ progenitor cells isolated from patients with CML while sparing normal CD34+ progenitor cell (Gao et al. 2000).

Table 1.1: candidate target antigens involved in GvL

adapted: (Barrett et al 1996)

Ubiquitous and alleleic:
-- minor histocompatibility antigens H-Y, HA-3, HA-4, HA-5
-- other mHags like CD31, HPA-3 and yet to be defined minor antigens
Lineage restricted:
-- haematopoietic tissue restricted: HA-1, HA-2
-- myeloid specific proteins: proteinase 3, CD33
-- lymphocyte specific proteins: CD19, T cell receptor, B cell idiotype
Leukaemia specific:
-- translocation products BCR-ABL, PML-RARA
-- products of gene mutations: eg ras
-- products of viral transformation eg EBV proteins
-- over-expressed normal proteins: eg proteinase 3, WT1
-- unique T cell receptor for T cell malignancies and idiotype for B cell malignancies
-- mHag specific to leukaemic lineage: HB-1

1.3.3 GvL cellular effectors:

Murine models have variously implicated CD4+, CD8+ and NK cells as effectors in GvL activity without causing GvHD (Truitt et al 1991b),(Jiang et al 1995),(Jiang et al. 1997), (Giralt et al. 1995), (Hsieh et al 2000), (de, V et al. 1999), (Mutis et al. 1999b). CD4+ cells alone have been shown to confer GvL (Jiang et al 1995),(Barrett et al 1996)

in CML and this is supported by good results achieved with CD8 depleted DLIs (Giralt et al 1995).

It is also clear that CD4+ cells can be cytotoxic (Grogg et al 1992) and several groups have raised leukaemia specific T cell clones from matched sibling donors (Faber et al. 1992), (Jiang et al 1995) with lineage specificity to CML cells and normal myeloid cells from the recipient but not T or B cells from the same individual. However, as they did variably react against host-derived haematopoietic cells, it does suggest that a class II restricted haematopoietic lineage related minor histocompatibility antigen may be the target rather than a truly leukaemia specific antigen. The CD4 story may also not be directly applicable to other leukaemias besides CML although they may still be important through the indirect secretion of cytokines. This may be because CML cells express MHC class II molecules which could allow for the direct recognition of tumour cells by CD4+ T cells (Johnson et al 1999).

CD8+ T cells clones reactive with leukaemia cells have been described (Sosman et al. 1990), (Falkenburg et al. 1993) and seen in a clinical situation, involving both CD4+ and CD8+ GvL effectors (Faber et al 1992). Attempts are currently being made to expand these leukaemia-specific T cells.

Further attempts to characterise GvL T cell effectors have defined them in terms of their cytokine profile secretions, being either Th1/Tc1 if they are γ-IFN and IL2 secreting and Th2/Tc2 if IL4 and IL10 are the main cytokines produced (Mosmann et al 1989) It has been reported that the GvL effect is associated with a Th1 phenotype in a murine leukaemia model (Fowler et al. 1996a), (Fowler et al. 1996b). On the other hand GvHD is also thought to be mediated by alloreactive T cells with a type 1

cytokine phenotype and hence the specificity of the GvL effect may not be harnessed (Krenger et al 1995).

Another population of T cells termed γδ T cells has been implicated in pure GvL activity. They are so called because they possess the γδ heterodimer T cell receptor rather than αβ common to most other T cells and recognise targets in a non MHC restricted fashion (Fisch et al. 1990). They often respond to intact polypeptides rather than processed antigen and also to non-peptide based antigens. Although activation may occur via the the T cell receptor (TCR) (Lanier et al. 1987), recognition is not based on this and may instead involve lymphocyte function associated antigen-1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1) interactions (Ensslin et al 1991). Their normal range in peripheral blood is 5-10%. An association has been demonstrated between increased levels of these cells and improved leukaemia free survival without additional risk of GvHD in a cohort of patients and demonstrated leukaemia specific killing with in-vitro assays (Lamb, Jr. et al. 1996). They have been shown to facilitate engraftment in mice (Blazar et al. 1996).

Lymphokine activated killer (LAK) and NK cells have also been implicated (Lowdell et al. 1997), (Delmon et al. 1986), (Murphy et al 1997), (Zeis et al. 1998). Attempts have been made to expand these NK cells or to generate LAK cells using IL2, IL12 (Uharek et al. 1996). Toxicity with in-vivo administration of IL2 remains a problem and ex-vivo expansion of NK cells has proved difficult although recent reports are more encouraging (Miller et al 1998). The relative feasibility of haploidentical transplants has illuminated new aspects of NK cells function. It has been observed that despite extreme T cell depletion, a low incidence of relapse was observed, especially with acute myeloid leukaemia (AML). This correlated with the appearance of

significant numbers of NK and T cells expressing NK markers including killer inhibitory receptors (KIRs) appearing early after engraftment (Albi et al. 1996). This phenomenon is also seen to a lesser extent, in allogeneic transplants. In a haploidentical transplant, the possibility of a KIR mismatch between donor and recipient is high, creating the possibility of alloreactive NK cells recognising recipient haematopoietic cells including leukaemic blasts based upon a lack of inhibition from the appropriate KIR molecule (Aversa et al. 1998), (Russell et al 1998). These alloreactive NK cells seem to only recognise recipient haematopoietic cells and not other host tisue, thereby not inducing GvHD (Ruggeri et al. 1999). The reasons for this have yet to be fully elucidated but would contribute to a GvL effect independent of GvHD. KIR epitope-mismatching in the GvH direction may thus confer unique potential for GvL effect and engraftment. It has also been suggested that allogeneic NK cells contribute to the GvL effect early post BMT whereas T cells are more critical at later time points (Johnson et al 1992).

Another population implicated in GvL activity are termed NK-T cells which share characteristics with both NK and T cells although they are derived from CD3+ T cells (Lu et al 1994), (Schmidt-Wolf et al. 1991), (Schmidt-Wolf et al. 1993), (Hoyle et al. 1998). They have been shown to effectively eradicate human tumour cell lines as well as autologous tumour targets in SCID murine model systems (Lu et al 1994), (Hoyle et al 1998). This cytotoxicity appears to be non-MHC restricted and may be regulated by KIRs which are present on their cell surface (Hoyle et al 1998). In addition, these NK-T cells also produce pro-inflammatory cytokines such as GM-CSF, γ-IFN and TNF-α. Unlike NK cells, there are readily expandable with the use of cytokines (γ-IFN, IL2) and do not require exogenous IL2 for in-vivo activity. They have thus also been called

cytokine induced killer (CIK) cells. Clinical trials are underway. Table 1.2 summarises the properties of these effector cell populations.

Table 1.2: Properties of GvL effector cell populations
Taken from Forman,S, American Society of Haematology education book 1999)

Property	NK	CTL	NK-T cells
Phenotype	CD3-CD56+CD16+	CD3+CD56-CD8+	CD3+CD56+CD16-
Precursor cell	NK	T	T
Antigen specificity	No	Yes	No
Expandable	Limited	Yes	Yes
IL2 in vivo	Yes	No	No

1.3.4 GvL effector cytolytic pathways:

Two pathways of cytolysis have been described. The first involves the exocytosis of perforin and granzymes. Perforin induces pore formation in the target cell membrane which allows the granzymes to enter and induce apoptosis. The second mechanism involves the cell surface expression of fas-ligand (fas-L) on the effector cells which binds to its ubiquitously expressed counter receptor fas on the target cell and induces apoptosis. The importance of these two mechanisms has been demonstrated with the use of elegant murine knock-out models (Kagi et al. 1994a), (Kagi et al. 1994b), (Heusel et al. 1994), (Henkart 1994), (Oshimi et al. 1996), (Rouvier et al 1993) and in-vitro systems (Jiang et al. 1996). CD4+ clones alone may also exert GvL by cellular contact and cytokine release of TNFα and γ-IFN ((Jiang et al 1995), (Barrett et al 1997).

1.3.5 Factors influencing GvL reactivity:

-Type of malignancy (AML, CML, ALL etc)

-Stage of disease (remission or relapse)

-Type of transplant (autologous, syngeneic, allogeneic)

-extent of histocompatibility (sibling, unrelated, partial match)

-treatment of donor marrow (T cell depletion, cell addback)

-environment (microflora, infections, CMV status)

-Post transplant immunosuppression (Truitt et al 1995)

1.4 T cell depletion:

1.4.1 pros and cons:

It was firmly established as early as 1968 that GvHD was mediated by lymphocytes by using discontinuous albumin gradients to selectively remove lymphocytes (Dicke et al 1969), (Dicke at al 1968). This was further corroborated when GvHD in primates was prevented using antithymocyte globulin which is in essence a form of in-vivo T cell depletion (Bekkum et al. 1972). Pan T cell depletion (TCD) strategies have generally been successful in reducing the incidence and severity of acute and chronic GvHD in both matched and mismatched transplants (Waldmann et al. 1984), (Prentice et al. 1984), (Champlin 1990), (Rohatiner et al. 1986), (de Witte et al. 1986), (Filipovich et al. 1984) but unfortunately, they have also been associated with a concomitant rise in the incidence of graft rejection, leukaemia relapse and delayed immune reconstitution (Apperley et al 1986), (Martin et al. 1985), (Marmont et al. 1991), (Hale et al 1988). This has meant that overall survival has not improved in controlled studies involving

HLA-identical transplants. (Maraninchi et al. 1987), (Mitsuyasu et al. 1986). Graft rejection has been reported to increase by tenfold (Marmont et al 1991).

The increase in leukaemia relapse is most striking for patients with CML. Some centres have reported that while 12% of patients in chronic phase relapse after unmanipulated transplants, as high as 50% have relapsed with TCD grafts (Goldman et al. 1988). This was corroborated in a large retrospective analysis which demonstrated that CML patients in 1st chronic phase who received TCD transplants from their HLA-matched siblings were 4.45 times (if GvHD was present) and 6.91 times (if GvHD was absent) compared to recipients of non TCD grafts (Horowitz et al 1990). Three important points can be highlighted: 1) T cell depletion leads to an increased probability of relapse 2) GvHD is associated with a GvL effect and 3) this marked increase in relapse rate is present even when TCD transplant recipients with GvHD are compared with unmanipulated allograft recipients without signs of GvHD. This argues cogently for an anti-leukaemic effect of T cells, independent of GvHD (Horowitz et al 1990).

However, the association of T cell depletion with leukaemia relapse has been challenged by some investigators (Prentice et al 1989), (Young et al. 1992) who have found the risk of disease recurrence to be similar to that after conventional BMT. This however may highlight the multifactorial nature of GvHD which includes other factors like varying intensities of pretransplant chemotherapy, conditioning regimens and patient cohorts used by individual centres.

T cell depletion is the single most effective method of reducing the incidence and severity of GvHD. Studies have also demonstrated that GvHD following T cell

depletion is directly related to the number of residual donor T cells in the allograft (Atkinson et al. 1987). For recipients of TCD HLA-identical sibling grafts, the incidence of acute GvHD is reduced to 5-20%, with only mild to moderate cutaneous involvement and mortality largely eliminated (Champlin 1992). The same applies to mismatched or unrelated donors: a 75% incidence with unmanipulated versus 30% with T cell depletion (Ash et al. 1990).

1.4.2 Techniques of T cell depletion:

A number of techniques have been used in achieving T cell depletion. They can be achieved either by destroying the cells or physically removing them from the graft. In both cases, the procedure may or may not involve the use of T cell directed monoclonal antibodies. The most common approaches include:

1) **soybean lectin agglutination (SBA) and sheep E rosette formation** (Reisner et al. 1981). SBA binds and agglutinates cells bearing N-acetyl-D-galactosamine which is expressed on all mature blood cells, including T cells but not by pluripotent haematopoietic progenitor cells. A 1.26 ± 0.34 log depletion of T cells is achieved with this step alone (Kernan et al. 1986). Another 1 log T cell depletion is achieved if combined with E-rosetting in which the CD2 antigen present on T cells is bound to sheep red blood cells and the complex removed by density gradient. Besides E rosetting, SBA has also been used in conjunction with panning on a plastic surface coated with antibodies to CD5 and CD8 (Gajewski et al. 2000) or with immunomagnetic techniques.

2) **counterflow elutriation** (Almici et al 1992), (de Witte et al 1986). This method separates cells on the basis on their size and density with the use of a spinning chamber under centrifugal force.

3) **Monoclonal antibodies (mAb) used alone or with complement.** This rests on the ability of cell bound mAb to fix and activate complement.

i) **OKT3** (Prentice et al. 1982). This is an anti-CD3 mAb

ii) **cocktail of monoclonal anti-T-cell antibodies** including anti-CD6, anti-CD7 and anti-CD8 (Prentice et al 1984), (Mitsuyasu et al 1986).

iii) **Campath antibodies** (Hale et al. 1990). This is a series of rat mAb directed against a glycoprotein CDw52 highly expressed on lymphocytes and activates the human complement cascade. It is reactive to virtually all T and B cells, macrophages, monocytes, lymphoid leukaemic cells and some NK cells but not to myeloid leukaemias or stem cells (Hale et al. 1985). Campath-1M (IgM monoclonal) has been used in combination with donor serum complement to purge T cells from HLA-matched grafts and was found to be effective in reducing GvHD but graft failure remained significant (Waldmann et al. 1984). This led to the use of Campath-1G (IgG_{2b}) administered both in vivo to patients and in-vitro in the graft (Hale et al. 1994), (Hale et al 1998). Recently, a humanised form, Campath-1H was introduced (Hale et al. 2000)

4) **mAb bound to immunotoxins such as ricin conjugated anti-CD5** (Filipovich et al. 1990)

5) **T cell subset depletion: T10B9.** This is an IgM mAb which targets the $\alpha\beta$ heterodimer of the TCR and spares the $\gamma\delta$ T cells which may be involved in a GvL effect (Lamb, Jr. et al 1996)

6) **Other specific T cell antibodies such as CD6 depletion** (Soiffer et al. 1997). The anti-CD6 mAb recognises an antigen that is present on mature T cells but not B, NK or myeloid precursors. NK cell precursors are therefore spared.

7) **Immunomagnetic T cell depletion.** This is currently widely used in CD34 selection technology. This method physically separates T cells from the graft, thereby avoiding the addition of potentially toxic agents and release of cellular contents from dying cells (de Wynter et al. 1999), (Urbano-Ispizua et al. 1998). Commonly used immunomagnetic separation techniques include the Dynal beads which are superparamagnetic, 2.6*u*m diameter polystyrene beads and the magnetic cell sorting (MACS) nanoparticles (50nm diameter). When a biotinylated anti CD34/avidin column and an anti-CD34 immunomagnetic bead method was compared with Campath and complement, the latter achieved a depletion of 2.16 log and a 56% recovery of CD34+ cells while positive selection resulted in 3.12 log depletion with the avidin column and 4.04 log depletion for the bead method and recovery of CD34+ cells at 27% and 36% respectively (Dreger et al. 1995).

The SBA-erythrocyte rosetting procedure is long and cumbersome and the use of animal serum and cells may be an issue with regulatory agencies. Counterflow elutriation requires experienced operators and may be associated with the loss of small CD34+ stem cells (Noga et al. 1994). A review of results with Campath revealed low incidence of GvHD but graft failure remains a problem (Hale et al 1994) although this has been resolved by the additional use of in vivo Campath (Hale et al 1998).

In pan T cell depletion, it has been estimated that the final graft should contain a T cell dose of $<1X10^5$ T cells/kg recipient body weight to prevent GvHD in matched sibling transplants (Kernan et al 1986). Only grade I-II skin GvHD was seen in a series of 70

recipients (Verdonck et al. 1994). It however results in a higher incidence of mixed donor-recipient chimerism after BMT. So far, successful use of T cell depletion has required the establishment of an appropriate balance between the residual immune response of the recipient, which can be achieved by enhanced conditioning, and the adjustment of the T cell content of the donor bone marrow (Lowdell et al. 1998), (Patterson et al. 1986).

Retrospective analyses of T cell depletion have suggested that certain techniques were associated with relapse more than others. In particular, physical methods of T cell depletion and use of narrow spectrum antibodies to deplete the marrow of T cells reduce the risk of relapse both in matched sibling and unrelated transplants (Marmont et al 1991), (Wagner et al. 1990). In a small series using Campath-1M for T cell depleted matched sibling allografts for AML in 1^{st} remission combined with single fraction TBI, no severe acute GvHD was seen and the relapse rate was only 14% (Lowdell and Craston 1997). Thus, TCD BMT after appropriate conditioning may obviate the issue of graft rejection and prevent GvHD without complete loss of GvL but the problem of poor immune reconstitution remains.

Specific T cell subset depletion such as CD8+ depletion has been performed on matched sibling transplants (Nimer et al 1994) and demonstrated a reduction in GvHD without an increase in leukaemia relapse. This was corroborated by another group where the incidence of grade II GvHD was reduced to 22% from 58% using unmanipulated marrow with a low in cidence of leukaemia relapse although these were mainly in CML patients (Champlin et al. 1991), (Champlin et al. 1990). CD8 depletion may also lead to improved CD4 immune reconstitution with concomitant reduction in post transplant infective complications.

1.4.3 Immune reconstitution post BMT:

Factors determining the speed and extent of immune reconstitution include the source of the graft, recipient conditioning, type of ex-vivo graft manipulation, post transplant immunosuppresive therapy, incidence and severity of GvHD and post transplant infections. The use of a TCD graft generally leads to delayed T cell reconstitution and depressed T cell function (Keever et al. 1989), (Daley et al. 1987) although the reduction in GvHD seen may encourage immune recovery (Noel et al. 1978). The general pattern in allografts is the early appearance of NK cells 2 –3 weeks post transplant (Rooney et al. 1986). Initial reconstitution of immunity post transplant appears almost entirely to be due to the peripheral expansion of memory donor T cells present in the graft (Roux et al. 1996) although it has been reported that naïve T cells also appear early on either via a thymic or extrathymic route (Lowdell et al. 1998). This also holds true for T depleted transplants (Roux et al. 1996). T cell function remains impaired for many months after BMT and the repertoire restricted, especially in unrelated donor TCD grafts (Roux et al. 1996). In unmanipulated matched siblings transplants, Lowdell et al (Lowdell et al. 1998) found that CD4 cells reached the lower end of the normal adult range after 6 months while CD8 recovery was more rapid with normal levels achieved at 3 months. T cell depletion led to a much slower recovery of CD4 cells with numbers below normal range even at one year post transplant. CD8+ recovery was also delayed with TCD but numbers did normalise by 6 months while NK cell recovery was rapid even in the TCD recipients (Lowdell et al. 1998).

The situation with adult recipients of unrelated TCD grafts is worse. TCD is associated with prolonged T cell lymphopaenia and extreme CD4 cytopaenia of less than 200 cells/ul persisting for 12-18 months post transplant (Small et al 1999). This delay in

recovery included CD45RA+ cells (Small et al. 1999). Children having TCD transplants also exhibit poor immune reconstitution but demonstrated no difference between the two types of donors (Small et al 1999). B cell numbers recover to normal levels within 3 months (Small et al. 1990) although plasma cell activity can also be delayed for up to a year after T-depleted transplants affecting humoral immunity (Kelsey et al. 1990). The frequency of both cytotoxic and helper T lymphocyte precursors with anti-leukaemic reactivity can however rise after alloBMT (Jiang et al. 1991) although other groups report lower frequencies and functional responses delayed for 6months (Keever et al.1989), (Daley et al. 1987). Mixed chimerism is also more common in TCD grafts.

These more profound and prolonged immunological deficiencies observed with unrelated donors are a result of various probable factors. It is likely that the greater disparity between donor and host may alter thymic processing and immune reconstitution, and stimulate GvHD leading to immune suppression. The more intensive and prophylatic immunosuppressive regimens used to prevent graft rejection and GvHD including T cell depletion are also likely to contribute (Small et al. 1999).

1.4.4 Infective complications post allografting:

Life threatening infections remain one of the major complications of allogeneic bone marrow transplantation. Potentially fatal infections can be bacterial, viral, fungal or parasitic. The immune deficiency and risk of infections post transplant is a consequence of the following factors:

i. disease process itself as in acute leukaemias or aplastic anaemias
ii. prolonged immunosuppression prior to transplantation

iii. intensive myeloablative conditioning regimen including TBI

iv. T cell depletion determining the numbers of T cells infused in grafts

v. time to engraftment and immune reconstitution

vi. post transplant immunosuppression

vii. graft versus host disease

If T cell depletion and GvHD are critical factors in determining the level of post-transplant immunity, a strategy of depleting only part of the T cell population to ameliorate GvHD while retaining a pool of lymphocytes in the graft to provide immunity should help to reduce the incidence of life threatening infections post allografting. Fatal opportunistic infections after successful engraftment occurred in 12-28% of unrelated transplant recipients compared to 4-15% after HLA-matched sibling allografts (Small et al. 1999). In an analysis of 462 recipients from unrelated allografts, infection and interstitial pneumonia were the primary and secondary cause of death in greater than 30% of patients compared with 14% with disease recurrence (Kernan et al. 1993). T cell depletion increases that risk and in matched sibling allografts, the probability of pneumonia increases from 10 to 21% if anti thymocyte globulin (ATG) and steroids were administered (Small et al. 1997).

The immune system has evolved many mechanisms to protect the host against a wide range of organisms. This immune response can be divided into 2 broad categories: innate and acquired immunity. Innate immunity refers to defence systems already in place which can be activated rapidly following exposure to a pathogen. It is characterised by a broadly specific response and mediated by complement, interferon, phagocytic cells (neutrophils, macrophages) and NK cells. Acquired immunity in contrast is much more specific and largely mediated by T and B lymphocytes. The

hallmarks of these humoral and cellular mechanisms are antigen-specificity and memory leading to a more rapid and sustained secondary response. The innate and acquired immune responses are not mutually exclusive but its cellular effectors are intimately interconnected to each other and by cytokine networks.

Potential bacterial infections are myriad and range from gram-negative septicaemia to Hickman line related staphylococcal infections. The advent of stringent protective barrier nursing techniques, antibiotic prophylaxis as well as the development of and prompt treatment with powerful broad spectrum antibiotics have dramatically reduced the incidence of deaths from bacterial infections. Fungal infections are increasing especially with the use of corticosteroids for the treatment of acute lymphoblastic leukaemia (ALL) and GvHD. Antifungal prophylaxis (fluconazole or itraconazole and amphotericin B by mouth) and prompt commencement of amphoterin or AmBisome remain the treatment of choice. Parasitic infestations are rarer, the commonest being toxoplasmosis.

1.4.5 CMV disease post transplant:

Viral complications include CMV infection and EBV associated post transplant lymphoproliferative disease (PTLD). The CTL response is thought to play an important role in defence against both viruses. CMV has been classified as a member of the β subfamily of the herpesvirus and disease can be due to either de novo infection, from reactivation in a CMV-positive recipient or by transmission from a CMV positive donor to a previously uninfected patient. Prophylaxis includes aciclovir/ganciclovir and ensuring the administration of CMV negative (or leukodepleted) blood products in a CMV-negative recipient as CMV transmission occurs via the leukocytes. The CMV

status of the donor should also be checked and if the option exists, to select a CMV-negative donor over a CMV positive one (Prentice et al. 1997). It has been shown that a transplant from a positive donor to a positive recipient and the transfer of donor-derived antigen specific memory T cell immunity may offer greater protection than that of a negative donor to positive recipient (Grob et al. 1987). CMV can manifest as an asypmtomatic viraemia and quickly progress to pneumonitis, eosophagitis, hepatitis, vasculitis, marrow suppression and central nervous system (CNS) disease. CMV pneumonitis, in particular, is a major cause of death in allotransplants (Forman et al. 1994). The mainstays of treatment for CMV infection are gancivlovir and foscarnet but despite this, mortality remains high (Prentice et al. 1997), (Riddell et al. 1995). It has been estimated that as many as 70% of patients who are CMV positive before transplantation develop CMV infection post transplant and this is the principal cause of death in these patients (Meyers, Flournoy, & Thomas 1986). Moreover ganciclovir can be associated with substantial myelotoxicity and foscarnet with renal toxicity

Four clinical strategies for the control of CMV disease in BMT patients have been proposed (Prentice et al. 1997): 1) prophylaxis; in which anti-viral drugs are given to every patient at risk of developing CMV disease (seropositivity in either donor or recipient). 2) suppression; in which drugs are given when CMV is detected from peripheral sites such as urine and throat washings before development of clinical symptoms. 3) pre-emptive therapy when CMV is detected systemically, either in blood or bronchoalveolar lavage before onset of symptoms. 4) treatment when established disease is present. The results of clinical trials indicate that preventive and pre-emptive alternatives are the most efficient ways of reducing CMV disease with significant reductions in mortality while treatment has been relatively unsuccessful (Prentice et al. 1997).

Immune responses to CMV illustrate the importance of cell mediated immunity. Less specific mechanisms such as α and β interferons, NK cells and macrophages contribute to host resistance to determine the early course of viral infection while T cell mediated immunity dominates the later course (Fletcher et al 1998). This explains why delayed T cell recovery in transplantation or profound CD4 cytopaenia in HIV infection predisposes an individual to CMV infection. Conversely, CMV infection can also delay immune reconstitution (Paulin et al 1985). The humoral response in the form of neutralising antibodies is not thought not to prevent disease but only the severity . Although the CD8+ CTL is thought to be largely responsible for control of disease (Reusser et al. 1991), the CD4+ lymphocyte augments that response through Class II restricted lysis and the direct anti-viral effects of its secreted Th1 cytokines. (Davignon et al. 1996).

1.4.6 EBV infection post transplant:

EBV associated PTLD is a life threatening complication of BMT. The reported incidence has varied from 0.6 to as high as 24% in recipients of mismatched TCD grafts (Zutter et al. 1988), (Shapiro et al. 1988), (Lucas et al. 1998). In particular, it has been associated with TCD grafts and intensive post transplant immunosuppression for GvHD prophylaxis while PTLD complicating unmodified transplants are rare (<1%) (Zutter et al. 1988). Other associations are mismatched unrelated donors and the occurrence of GvHD (Shapiro et a), (Orazi et al. 1997). That T cell depletion remains the most important risk factor is highlighted by the fact that it is the only factor associated with PTLD in autologous transplantation (Chao et al. 1993). However, it is important to note that not all methods of T cell depletion increase this risk: Campath and elutriation are two methods which have not been associated with an increased

incidence (Hale et al. 1988), (O'Reilly et al. 1997). Hale et al reports no lymphomas in a review of 400 recipients treated with Campath-1. This implies that the infusion of EBV infected B cells is another important determinant as Campath removes both T and B cells.

80% of patients with PTLD present with fever, 50% with palpable adenopathy and a proportion with pulmonary, nasopharyngeal, hepatic, gastrointestinal or CNS involvement (O'Reilly et al 1997). The disease can be rapidly progressive and prognosis is often dismal. PTLD in BMT recipients usually arises in EBV infected donor B lymphocytes (Shapiro et al. 1988) and the incidence is highest in the first 6 months after transplantation. PTLD represents a wide spectrum from EBV-driven polyclonal B cell proliferation to monoclonal high grade lymphoma (Knowles et al. 1995). Treatment options include the withdrawal of immunosuppression, combination chemotherapy and donor lymphocyte infusions. CD8+ CTL responses to EBV are crucial in controlling the disease and this is highlighted both by the correlation between the peak incidence of PTLD and levels of EBV-specific CTLs between day 60-90 post transplant and the extent of TCD (Lucas et al. 1996). Nonetheless, it is likely that CD4 helper cells are also crucial in mounting an effective response (O'Reilly et al. 1997).

EBV is a gamma herpes virus that can establish both latent and lytic infections within cells of its hosts and it is estimated that >90% of the population are EBV sero-positive. Latent proteins expressed by infected B cells include 6 Epstein Barr Nuclear antigens (EBNA 1, 2, 3A, 3B 3C and leader) and 3 latent membrane proteins (LMP 1, 2A, 2B) although in the carrier state, most if not all are downregulated (Haque et al. 1999). The key role in maintaining a stable host-virus balance is provided by EBV-specific CD8+ CTLs (Moss et al. 1988). All nine latent proteins and a few of the immediate early and

early lytic gene products have been identified as targets for the CTL (Moss et al. 1988). The role of the CTL response to lytic antigens has only been elucidated recently with especially strong reactivity of an HLA-B8 restricted response to the immediate early VZLF1 protein demonstrated (Bogedain et al. 1995), (Tan et al. 1999).

1.4.7 Graft rejection:

Despite the "myeloablative" conditioning, residual viable host immunocompetent cells can be readily detectable (Butturini et al. 1986) and these may recognise and react against the donor bone marrow leading to graft rejection. Cytotoxic CD8+ T cells and NK cells are primarily responsible for this process (Martin 1993), (Kernan et al. 1989), (Voogt et al. 1990), (Nakamura et al. 1990). Graft rejection is uncommon following an unmanipulated HLA-identical sibling transplant but the incidence rises with HLA-nonidentical donors and with pan T cell depletion. This points to the important role that donor lymphocytes have in preventing graft rejection. Graft rejection is significantly increased from less than 3% with unmanipulated matched sibling transplants to 8-20% with T cell depletion (Maraninchi et al. 1987), (Mitsuyasu et al. 1986), (Ash et al. 1990) and rises to 20-50% with mismatched grafts (Martin et al. 1985). This can be overcome by increasing the conditioning regimen such as switching from multiple to single fraction TBI (Patterson et al. 1986).

The pivotal role of donor CD8+ cells in graft rejection was confirmed in a murine model which demonstrated that this was due to the generation of cytotoxic effectors needed to eliminate residual recipient T cells remaining after pretransplant conditioning (Martin et al. 1998). This was dependent on perforin but minimally on fas/fasL. It was also possible in mice to adjust the numbers of CD8+ cells infused to prevent graft

rejection but not induce GvHD (Martin 1993). This however was unsuccessful when translated to humans (Martin et al. 1999).

The advent of PBSCTs has allowed much larger doses of CD34+ progenitor cells to be transplanted which may compensate for the increase in rejection rates seen with T cell depletion. This was confirmed in a recent paper (Link et al. 1996). Animal data have also suggested that the main cause of graft failure after T depleted transplants was the reduced number of progenitor cells left (Uharek et al. 1992) and Reisner and colleagues have translated this elegantly to show that by increasing the number of transplanted progenitors to "megadose" levels, engraftment was possible even with extreme T cell depletion and across major HLA barriers (Reisner et al. 1995).

1.4.8 Leukaemic relapse:

Post alloBMT, other than fatal infections, relapse of the primary haematological malignancy remains the most frequent cause of treatment failure (Sullivan et al. 1989). The 3 year probability of relapse in the early stages of leukaemia, that is, first complete remission (CR) of acute leukaemia and chronic phase of chronic myeloid leukaemia (CML-CP) is 10-30% (Horowitz et al. 1990). In more advanced disease, this probability rises to between 20-70% (Barrett et al. 1989), (Horowitz et al. 1997). The key to any successful TCD strategy is to ensure that this probability of relapse does not rise any further.

1.4.9 T cell addback and adoptive immunotherapy:

The use of donor leukocyte infusions (DLIs) to treat patients who have suffered a relapse following a transplant provides pivotal proof of the existence and role of a GvL reaction in controlling disease. The concept of DLIs was explored most thoroughly by Kolb in elegant canine experiments. He found that transplantation of T cell depleted marrow could induce chimerism in DLA-identical dogs without further post transplant immunosuppression. In these chimeras, infusion of donor leukocytes converted them into complete donor type without producing any signs of GvHD, if the DLI was delayed for at least 2 months post transplantation. (Kolb et al. 1997).

This principle was transferred successfully into patients and the data analysed in 2 large retrospective studies (Collins, Jr. et al. 1997), (Kolb et al. 1995). The EBMT registry reported results obtained from 135 patients treated at 27 transplant centres. 73% of patients with relapsed CML (88% if cytogenetic relapse, 22% in blast phase) were re-induced into CR with DLI as compared with 29% for AML and 0% for ALL. Many of these remissions were sustained and long lasting although toxicity included clinically significant GvHD (41%) and myelosuppression (34%). Cell doses given ranged from 0.1-15x10^8/kg body weight. Comparable results were obtained from North America: CML (60% CR), AML (15.4% CR), ALL (18.2% CR) though a considerable percentage developed GvHD (60%) and myelosuppression (18.6%). An earlier trial also showed similar results (Kolb et al. 1990). It is now clear that CML responds best to DLIs, ALL least, with AML and myeloma falling in between (Verdonck et al. 1994). And within CML, molecular relapses invariably respond better than cytogenetic and haematological relapses. The use of DLIs in patients with relapsed acute leukaemia has been less successful possibly due to the more rapid proliferative capacity of the

malignant blasts. Some have therefore advocated the use of chemotherapy to debulk the disease prior to DLI.

The mechanism of action of DLI is not known with certainty but in a study analysing the TCR repertoire before and after DLI, it was found that the oligoclonal and clonal patterns present during relapse gradually normalised over 1 year (Storb et al. 1997). It has therefore been postulated that one possible mechanism of response following DLI is the provision of T cell help, which allows for the normalisation of the TCR repertoire and expansion of anti-leukaemic cell populations. The median time to complete response in the various studies has been 4.5 months with some as late as 12 months (Kolb et al. 1990). In murine models, establishment of complete donor chimerism was associated with the presence of alloreactivity and subsequent GvL reactivity while mixed T cell chimerism was associated with little or no GvL reactivity (Truitt et al. 1991a).

GvHD, especially in its chronic form is a common and major complication and a correlation exists between the response in CML to DLI and the severity of DLI. However, it was not an invariable accompaniment in the studies quoted and points to a pure GvL component of DLI therapy which can potentially be harnessed without the incurring the complication of GvHD.

The other major complication of DLI observed has been myelosuppression leading in severe cases to aplasia, particularly in patients with no evidence of donor haematopoiesis at the time of DLI (Keil et al. 1997). The mechanism of myelosuppression is most likely similar to that of transfusion related GvHD.

Adoptive immunotherapy is also another way of restoring the severely impaired immune recovery seen with TCD transplants. Donor lymphocyte infusions with a median dose of $0.5x10^6$ CD3+ T cells/kg can result in the rapid restoration of CD3+, CD4+ and CD8+ T cell numbers as well as antigen-specific T cell responses (Small et al. 1999).

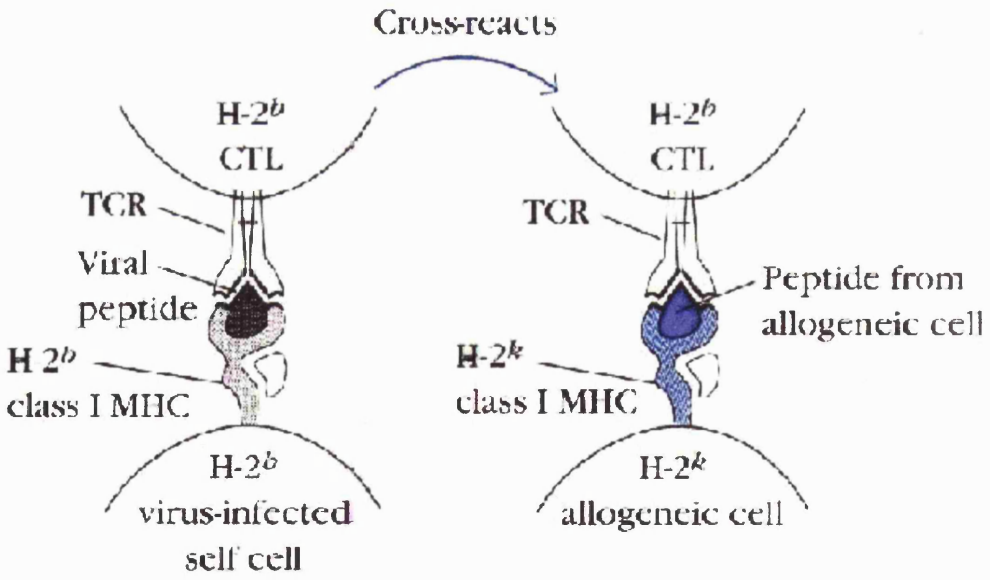
1.5 The T cell response: Alloreactivity, activation, signal transduction:

1.5.1 Allorecognition leading to alloreactivity:

T cell recognition of MHC antigens occurs via 2 distinct mechanisms. Some donor alloreactive T cells recognise peptides complexed to host MHC molecules displayed on the surface of recipient cells (allorestricted or direct pathway) while other T cells interact with processed host derived peptides bound to syngeneic MHC molecules on donor APCs (self-restricted or indirect pathway) (Benichou et al. 1992), (Fangmann et al. 1992), (Liu et al. 1992), (Sayegh et al. 1994). The direct pathway may be a molecular cross-reaction: donor T cells that bear TCRs which recognise a complex formed by a particular peptide (derived from a non-self protein) bound to a self allelic MHC molecule also recognise a different complex formed by other peptides (self and non-self) associated with an alloMHC molecule. This is illustrated in Figure 1.2. Allorestricted T cells reactive with a particular non-self MHC molecule may actually recognise different peptide-MHC complexes. The consequences of multiple cross-reactivity is that unprimed donor lymphocytes may have as many as 1 in 500 T cells capable of reacting with host cells bearing allogeneic MHC molecules in contrast to an unimmunised host only possessing 1:100000 to 1000000 T cells for a particular foreign protein (Pober et al. 1996), (Lindahl et al. 1977). Higher frequencies of at least 1% or

more have also been quoted (Detours et al. 1999), (Sherman et al. 1993). Therefore, allospecific recognition of alloantigens results in a strong primary immune reaction even in an unimmunised host. It may also resemble a memory response as these cross-reactive T cells may have been activated previously by particular contact with their specific foreign peptide. The relative importance of each pathway was examined in a murine model which showed that the direct pathway involving host APCs played a more crucial role in the pathogenesis of GvHD (Shlomchik et al. 1999) .

Figure 1.2 Direct pathway of allorecognition
(taken from J. Kuby: Immunology 3rd edition; W.H. Freeman publishers)



In this direct mode of allorecognition, the "allo-cross-reactive" may recognise two variable determinants. On the one hand, the alloMHC molecule may be the conformational structure that alloreactive T cells respond to independent of the bound peptide (Sherman et al. 1993). M. Bevan hypothesised that the strength of the

alloreactive response was due to numerous non-self MHC molecules on the cell surface that trigger large numbers of high and low avidity T cells directed against polymorphic structures (Bevan et al. 1994). This theory was supported by the finding of an alloreactive CTL line that could be stimulated by immobilised alloMHC molecules stripped of bound peptide (Elliott et al. 1990). On the other hand, alloreactive T cells may recognise alloMHC molecules in a peptide dependent fashion (Sherman et al. 1993), (Rotzschke et al. 1991), (Matzinger et al. 1977). Large numbers of T cells could be stimulated by a diverse array of self-peptides on the non-self MHC molecule. This theory was supported by work using a mouse CTL clone 2C which recognises H-2K^b presenting a synthetic SIYRYYGL peptide as well as non-self H-2L^d bound to a self peptide QLSPFPFDL (Udaka, Tsomides, & Eisen 1992). Different T cell clones therefore recognise different sets of peptides on an alloMHC molecule and contributing to the strength of the alloreactive response. This implies too, the possibility of tumour specific peptide recognition of an alloantigen (Sadovnikova et al. 1996), (Munz et al. 1999).

1.5.2 "NK cell alloreactivity":

NK cell alloreactivity is a new but burgeoning concept. It was traditionally thought that NK cells mediated killing via a non-MHC restricted fashion. Although it is true that the same degree of specificity is not seen as in T cells, NK cells recognition is neither random nor non specific. NK cells possess a set of inhibitory (KIR) and stimulatory receptors, some of which interact broadly with HLA molecules. It is the balance of these opposing receptor engagements that determines whether an "alloreactive" NK cell kills its target. Engagement of the KIR receptors with its appropriate HLA allele on the target cell is thought to generate a dominant inhibitory

signal and to spare the target cell from NK cytolytic function. An individual will therefore kill cells from individuals lacking his/her KIR epitopes. The signals generated by the stimulatory receptors are less well understood (Yokoyama 1998).

1.5.3 T cell activation and signalling pathways:

The activation of T lymphocytes by antigens or mitogens initiates a co-ordinate up- and downregulation of the expression of a wide variety of genes. These various gene products which characterise the activated cell can be divided into immediate, early and late. Immediate genes expressed within half an hour of antigen recognition include the genes for c-fos, c-myc, c-jun, nuclear factor of activated T cells (NF-AT) and NF-*k*B. Early genes expressed within 1-2h include IL2, IL2-R, CD69, IL3, IL6 and γ-IFN and late genes (>2days) are HLA-DR, VLA-4 (Crabtree 1989). Transcription of immediate genes does not require protein synthesis while early and late gene transcription does. Besides the transcriptional activation of a variety of genes, T cell activation is also characterised by the expression of various cell surface molecules. Many of these "activation antigens" have been described.

The general sequence of T cell activation involves the activation of protein kinases leading to the synthesis or phosphorylation of nuclear factors. This results in the functional activation of nuclear factors and the stimulation of gene transcription. Following antigen recognition, a key step in the events following T cell activation is the phosphorylation of proteins that regulate transcription. The first biochemical change known to occur following TCR engagement involves the activation of Fyn and Lck of the Src family (Germain et al. 1999). CD45, a protein phosphatase present in all lymphocytes can either positively or negatively influence this process (Germain et al.

1999). These protein kinases then phosphorylate tyrosine residues in the ε and ζ chains of CD3 in a region termed immunoreceptor tyrosine based activation motif (ITAM). The ITAMs play a primary role in downstream signalling, through the recruitment of the syk-family kinase, Zap-70. Zap-70 and Fyn then phosphorylate phospholipase C ($PLC\gamma_1$), triggering several downstream signalling pathways as illustrated in Figure 1.3:

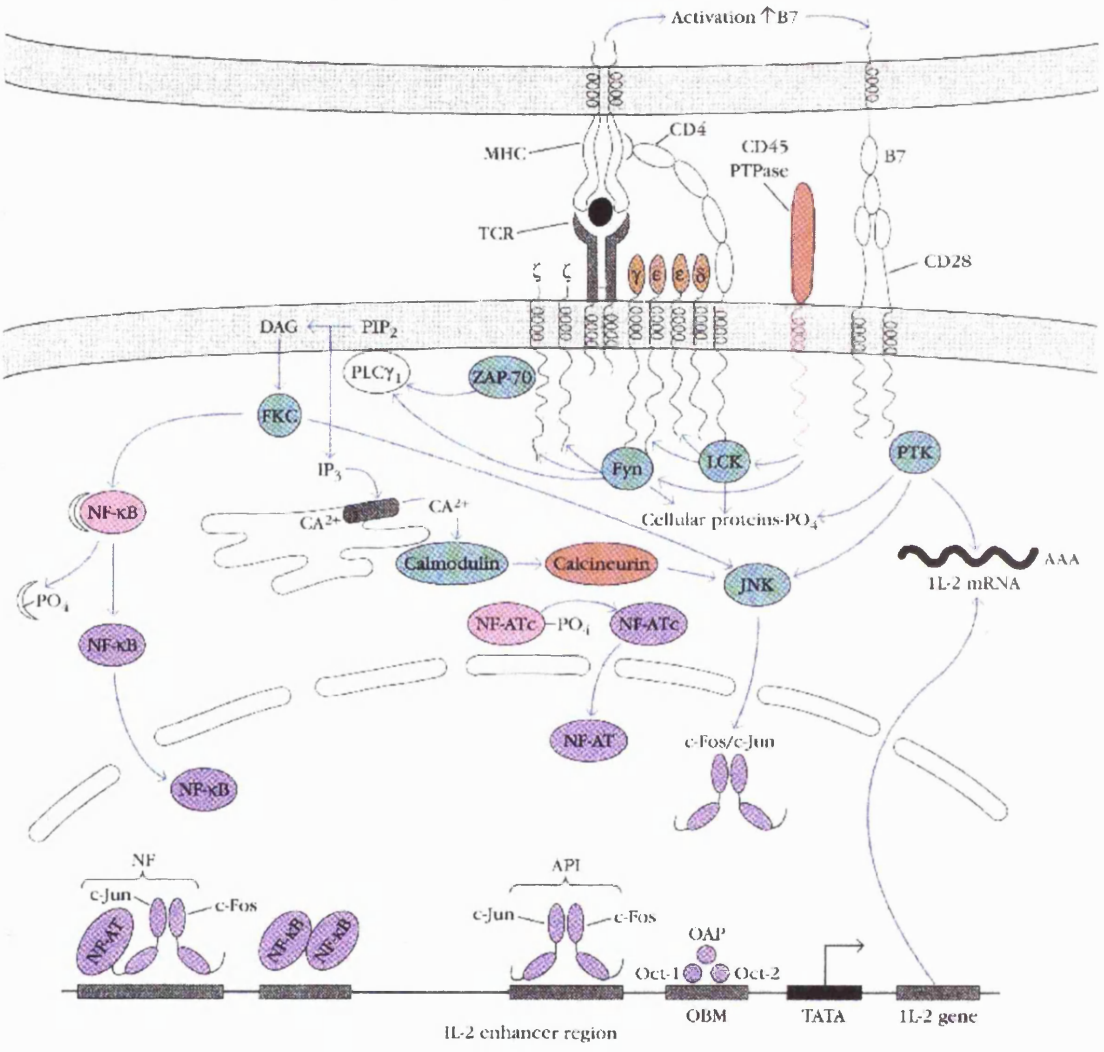
i) 2^{nd} messenger 1,2 diacylglycerol (DAG) leading to activation of protein kinase C (PKC) and Raf

ii) 2^{nd} messenger inositol 1,4,5-triphosphate (IP_3) leading to calcineurin and a rise in intracellular calcium.

Eventually 2 nuclear factors are produced: NF-AT (IP_3 pathway) and NF-κB (DAG pathway) via 2 separate signalling pathways. Binding of these nuclear factors to response elements in the IL2 enhancer region stimulates transcription of the IL2 gene and increased secretion of IL2. Transcription of the IL2 gene begins within 1 hour following TCR-mediated stimulation. A transcription-activating protein complex called AP-1 which is largely composed of the protein products of the c-*jun* and c-*fos* oncogenes binds to the IL2 gene enhancer region. The interaction of IL2 with its high affinity receptor initiates signalling pathways that cause the T cell to transit from the G1 to the S phase.

Figure 1.3 Signalling pathways following T cell activation

(taken from J. Kuby: Immunology 3rd edition; W.H. Freeman publishers)



1.5.4 Activation marker CD69:

CD69 is one of the earliest cell surface molecules to be expressed upon T cell activation, appearing as early as one hour post activation with a potent stimulus like PHA (Testi et al. 1989a). The expression of CD69 results from the *de novo* synthesis

and transcription of CD69 mRNA and protein (Hara et al. 1986). Using Northern blot analysis, CD69 transcripts can be detected in T cells within 30 min of phorbol-12-myristate-13-acetate (PMA) stimulation (Lopez-Cabrera et al. 1993) and peaks in activated T and NK cells after 12 h (Testi et al. 1989a). Thereafter, it remains stable with expression persisting for at least 3 days. It was previously also designated as activation inducer molecule (AIM) (Cebrian et al. 1988), (Santis et al. 1992), Leu 23 (Lanier et al. 1988), (Testi et al. 1989b) and EA-1 (Hara et al. 1986).

CD69 is expressed constitutively on platelets, thymocytes and at very low levels on resting monocytes but markedly upregulated upon activation (Ziegler et al. 1994a). In all other cell types including T cells, B cells, NK cells, neutrophils and eosinophils , it is absent at resting levels and inducible only upon cellular activation. Its association as an activation induced molecule is borne out by the fact that all gut intraepithelial and lamina propria T cells, as well as T cells in the germinal centre and paracortical zone of lymph nodes express high levels of CD69 as they are continually exposed to antigen challenge (De Maria et al. 1993), (Sanchez-Mateos et al. 1989). In the thymus, its expression is mostly restricted to $CD3^{high}$ thymocytes and may represent a marker for positively selected thymocytes (Swat et al. 1993). Table 1.3 summarises the main inducers of CD69 expression in the various cell types (Ziegler et al. 1994a).

It has been reported that CD69 is present on bone marrow derived CD33+ cells and a subpopulation of CD13+ myeloid precursors (Gavioli et al. 1992). Using a novel high affinity anti-CD69 monoclonal antibody, Tassone P et al (Tassone et al. 1996) found expression of CD69 on long term repopulating CD34+Thy-1+ haematopoietic progenitors and also, a subset of myeloid leukaemias but not on lymphoid malignancies.

Table 1.3: Stimuli for induction of CD69 in different cell types

CELL TYPE	STIMULUS
T cell	Anti-CD3, anti-CD2, anti-CD28, PMA, PHA
B cell	SAC (Staphylococcal aureus Cowan I), PMA, anti-IgM
NK cell	IL2, IL12, IFN-α, IL7, PMA, anti-CD16, target cell like K562
Monocyte	GM-CSF, IFN-γ, IL3, LPS
Neutrophil	GM-CSF, PMA
Eosinophil	GM-CSF, IL3, IL5, IFN-γ

PMA: phorbol-12-myristate-13-acetate
PHA: phytohaemagglutinin
GM-CSF: granulocyte-monocyte colony stimulating factor
IL: Interleukin

1.5.5 Structure of CD69:

The CD69 cDNA has been cloned (Ziegler et al. 1993) and nucleotide sequence analysis predicted a polypeptide of 199 amino acids. This indicated that CD69 was a type II integral membrane protein with a single transmembrane domain. The extracellular portion of the molecule (138 amino acids) contains a C-type lectin binding domain which places CD69 as a member of the C-type lectin family, characterised by the ability to bind to sugars in a Ca^{2+}-dependent manner. Other members of the C-type lectin family include CD72, CD94, CD161(homodimer), CD94NKG2 (heterodimer), CD23 (homotrimer), Ly49, CD62L, mannose receptor and macrophage lectin (Yokoyama 1993). This binding to sugars however is not a necessary feature of the C-type lectin family because other members like CD23 and CD72 bind to protein structures instead. CD69 is expressed on the cell surface as a homodimer of differentially glycosylated subunits (Sanchez-Mateos et al. 1991). Murine CD69 has extensive homology to its human counterpart (58% identity) except that the mouse

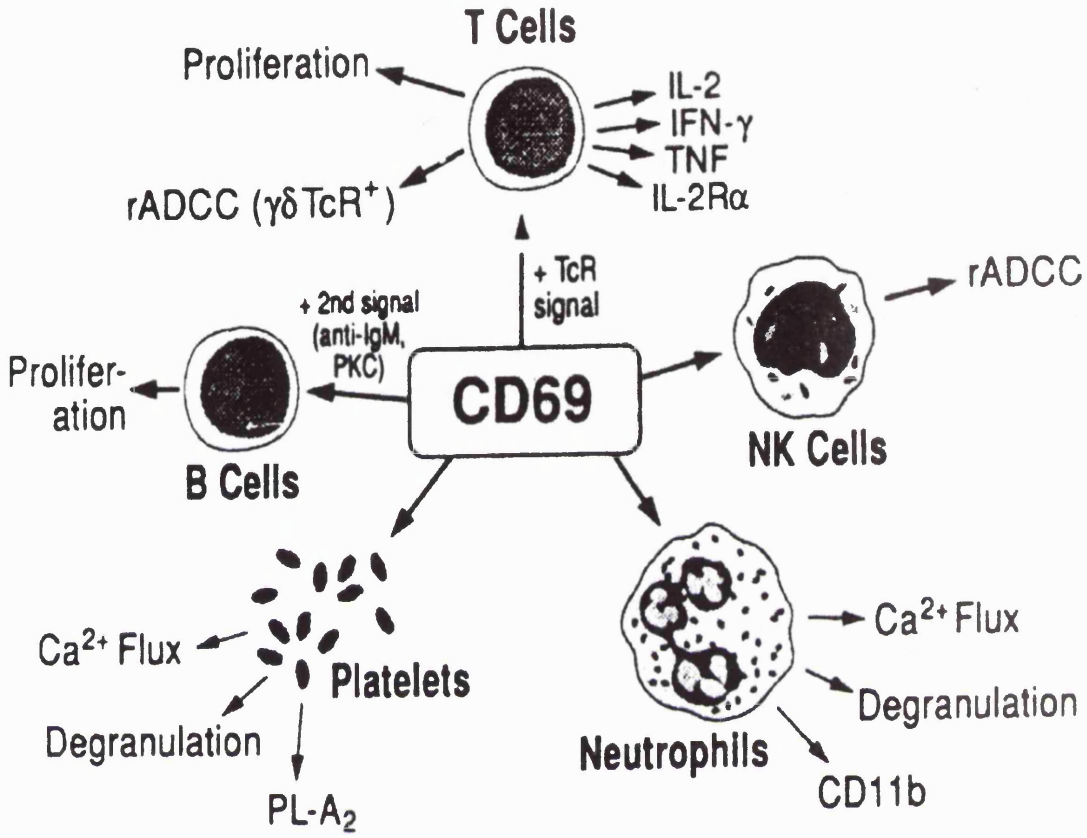
protein contains 3 putative N-linked glycosylation sites in contrast to one in the human (Ziegler et al. 1994a).

Chromosomal mapping of the mouse and human CD69 has suggested a closer relationship with the NK lectin families. The murine CD69 gene maps to the distal end of chromosome 6, linked to the NKR-P1 and Ly-49 gene families in what has been termed the "NK gene complex" (Ziegler et al. 1994b). Similarly, the human gene maps to chromosome 12p13.2-13.3 which is the equivalent "NK gene complex" with CD94 and the NKG2 family. Though all members are capable of signal generation, the difference is that Ly-49, NKR-P1 and NKG2 are all exclusively expressed on NK cells in a constitutive fashion and display allelic polymorphism which provides a role in target recognition by NK cells (Yokoyama 1995). In contrast, CD69 is the only product of a single nonpolymorphic gene inducibly expressed in a wide variety of haematopoietic cells (Ziegler et al. 1994b).

1.5.6 Function of CD69:

CD69 is inducible in a variety of cell types and its functions differ accordingly as illustrated by Fig. 1.4 (Ziegler et al. 1994a). Many of these functions were demonstrated by cross-linking of CD69 with monoclonal antibodies. With regards to T cells, CD69 enhances T cell activation in conjunction with either the PMA, or with anti-CD3 antibody resulting in Ca^{2+} mobilisation, increased expression of CD25, increased proliferation and production of IL-2, TNF-α and IFN-γ (Ziegler et al. 1994a), (Nakamura et al. 1989). It also results in enhanced binding activity of the transcription-activating protein complex AP-1 which has a crucial role in IL2 production (Tugores et al. 1992).

Figure 1.4: CD69 mediated interactions in a variety of haematopoietic cells (Zeigler et al. 1994a)



rADCC: redirected antibody dependent cell cytotoxicity
PL-A2: phospholipase A2

Anti-CD69 monoclonal antibodies have also been demonstrated to redirect killing of γδ T cells and NK cells to target cells bearing F_c receptors (Moretta et al. 1991). The triggering of cytolytic activity mediated by anti-CD69 mAbs can only be detected in experimental conditions that allow redirected killing.

Its possible role in thymocyte positive selection and maturation should also be mentioned although its expression here is constitutive rather than activation-induced. In

circulating monocytes, CD69 cross-linking induces cytotoxic activity mediated by nitric oxide generation (De Maria et al. 1993).

1.5.7 Activation marker OX40 (CD134)

Ox40 belongs to the tumour necrosis factor receptor (TNFR) superfamily which also includes CD95, CD40 and CD30. These are structurally related type I transmembrane proteins with homology restricted to the cysteine-rich extracellular domain (Mallett et al. 1990). Its ligand is a type II transmembrane protein OX40L. OX40 was first identified on activated CD4+ T cells in the rat and has since been detected on human and murine CD4+ T cells and to a much lesser extent CD8+ T cells (Baum et al. 1994). Its ligand has a broader cellular distribution on activated T and B cells, endothelial cell lines and dendritic cells (Murata et al. 2000). Signalling through OX40 generates costimulatory signals, resulting in enhanced Concavalin A induced T cell proliferation and enhanced cytokine production after TCR engagement (Godfrey et al. 1994). It also results in activation of NFκB. Signalling also occurs through cross-linked OX40L which is thought to be important in T cell dependent differentiation of activated B cells into highly immunoglobulin-producing cells (Stuber et al. 1996). OX40 and OX40L interaction has been found to play a critical role in antigen specific T cell responses and cytokine secretion in vivo though APC:T cell interaction (Chen et al. 1999), (Springer 1990).

Activated CD4+ cells is thought to play a central role in the pathogenesis of experimental allergic encephalomyelitis (EAE), a model of multiple sclerosis. OX40 has been found to be selectively upregulated on encephalitogenic myelin basic protein

(MBP)-specific T cells at the site of inflammation during the onset of EAE (Weinberg et al. 1996). Use of a ricin-A anti-OX40 immunotoxin selectively depleted the myelin reactive T cells and ameliorated the disease (Weinberg et al. 1996). An OX40-IgG fusion protein demonstrated efficacy in a murine model of inflammatory bowel disease (Higgins et al. 1999). Expression of the OX40 antigen was found both in a rat GvHD model (Tittle et al. 1997) and in the peripheral blood of patients with acute GvHD (Lamb, Jr. et al. 1999), (Tittle et al. 1985). It was also expressed in target tissues including the liver, spleen and lymph nodes (Higginns et al. 1999). These findings however were not confirmed by other investigators (Gadisseur et al. 1999). The relationship between OX40 and GvHD has been examined in a rat model of GvHD. At baseline, less than 1% of rat CD4+ T cells express OX40 but rises to 18-36% 10-12 days post transplant with early signs of GvHD. In vitro studies confirmed that the OX40+ cells represented the alloreactive population. However, only a small population of CD8+ cells was OX40+.

1.5.8 CD25:

The high-affinity IL-2 receptor (IL-2R) is a heteromultimer composed of the α (p55), β (p75) and γ (p64) chains (Waldmann 1986). CD25 is the 55KD α subunit of this complex. The β (CD122) and common γ chains (CD132) are expressed on the surface of most lymphocytes as an intermediate-affinity receptor. It is only when these T, B and NK cells become activated that they form the high affinity trimolecular IL2 receptor complex (Uchiyama et al. 1981). Interaction of IL2-R with its ligand IL2 triggers initiates signalling pathways that cause the T cell to transit from the G_1 to the S phase of the cell cycle and progress to cell division. Among the IL2 regulated

molecules are c-jun, c-fos, c-myc and the anti-apoptotic protein bcl-2 (Waldmann 1986).

1.5.9 HLA-DR:

This forms part of the MHC class II molecule and as such is expressed on dendritic cells, B cells, monocytes, macrophages and some epithelial cells. It is not expressed on resting T cells but upregulated as an activation antigen (Korman et al 1985).

1.6 Aims of the thesis:

The understanding that the initial process in the generation of the GvH reaction involves T cell allorecognition and activation frames the entire work presented in this thesis. The hypothesis was that if these activated, alloreactive cells could be selectively removed ex-vivo, the entire cascade leading to a GvH reaction would not occur, thereby preventing GvHD following allotransplantation. This engineered allodepleted graft would differ from conventionally pan TCD grafts in the numbers of T and NK cells infused. The population of non-alloreactive lymphocytes remaining in the graft and infused into the patient would hopefully provide adequate anti-viral activity especially against CMV and EBV as well as hasten immunological recovery. In patients with leukaemia, this may allow the preservation of anti-leukaemia activity, in other words: the separation of GvHD from the GvL effect.

To achieve this overall aim, the following questions were addressed:

i) Can we identify the alloreactive, activated lymphocytes ex-vivo by virtue of cell surface activation markers?
ii) Is there an optimal activation marker to use and an effective method of removing these lymphocytes?
iii) Is there preservation of anti-viral and anti-leukaemia activity in the engineered graft?
iv) Will the in-vitro graft engineering strategy translate effectively into an animal model of GvHD for issues of safety and feasibility?

Ultimately, the results of this thesis would provide the scientific basis for a clinical trial in patients undergoing allogeneic transplantation with the hope of improving long term disease free survival.

Chapter 2. Materials and Methods

2.1 General cell culture work

2.1.1 Isolation of human peripheral blood mononuclear cells (PBMCs) and bone marrow cells:

Whole blood or bone marrow specimens were collected in tubes containing preservative free heparin. Peripheral blood mononuclear cells (PBMCs) were isolated by discontinuous density gradient centrifugation. The samples were diluted 1:1 in Roswell Park Memorial Institute hydrogen-carbonate-buffered medium (RPMI1640, Gibco/Life Technologies Ltd, Paisley, UK). The diluted sample was layered over an equal volume of Lymphoprep (Nycomed Pharma AS, Oslo, Norway). and spun down without the brake at 400 g (1500 rpm) for thirty minutes. The mononuclear fraction was recovered from the Lymphoprep interface, diluted in RPMI1640 and centrifuged at 400g for 10 minutes to remove any excess Lymphoprep and to pellet the PBMCs. A further wash was then done at 200g (1000 rpm) for 10 minutes with brake and the cells recovered resuspended in an appropriate volume of complete medium (see section 2.1.3). The cells were then counted using a Neubauer haemocytometer and phase contrast microscopy after mixing with trypan blue solution (0.4%, Sigma) at a 1:1 ratio to distinguish live from dead cells. The trypan blue was made up in phosphate buffered saline (PBS, 171mM NaCl, 3.4 mM KCL, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH7.2). The cells were either used fresh or cryopreserved for use at a later date.

2.1.2 Cryopreservation of cells and subsequent thawing:

Where appropriate the cells were cryopreserved. The cells were centrifuged at 200g for 10 minutes and the cell pellet resuspended in "complete medium" at a concentration of at least $2x10^7$ cells/ml. An equal volume of freezing solution consisting of 80% FCS and 20% dimethylsulphoxide (DMSO, Sigma) which had been cooled to 4^0C was then added drop by drop to the cell suspension. The entire procedure was carried out in ice and the tube containing the cell culture was manually rotated as the freezing solution was added to ensure an even distribution of heat generated from the process. The final cell suspension in RPMI 1640 / 45% FCS / 10% DMSO was transferred into freezing vials (Nunc, Inc) in 1 ml aliquots. The vials were placed inside small insulated polystyrene boxes and placed initially at -70^0C, followed by transfer to liquid nitrogen at -192^0C the following day.

Prior to use, the cells were thawed rapidly in a 37^0C water bath until only a small particle of ice remained. In order to avoid DMSO toxicity, the cells were immediately diluted with a ten-fold volume of complete medium, centrifuged at 200g for 10 minutes and the cell pellet then resuspended at the desired concentration in "complete medium". The cells were then allowed to "rest" for 30 minutes in the fresh "complete medium" before use in cell culture.

2.1.3 Culture of cells in suspension:

All human cells (PBMC or marrow derived) were cultured in RPMI 1640 containing 2.5mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100*u*g/ml) (Gibco/Life Technologies) and supplemented with 10% (v/v) foetal calf serum (FCS) (Myoclone)

(Boiwhittaker, Walkersville, Maryland) which was heat inactivated at 56^0C for 20 minutes. This was also termed as "complete medium" (CM). Cells were grown in tissue culture flasks (Falcon) in a humidified atmosphere of 5% CO_2 in air at 37^0C.

2.1.4 Generation of PHA blasts:

PBMC stimulators from a matched pair were incubated at $1x10^6$ cells/ml in the presence of 2*u*g/ml of phytohaemaglutinnin (PHA, Sigma, Poole, Dorset) in a 6 well plate. The following day, cells were washed twice in RPMI 1640 to remove the PHA before resuspending in "CM" at $1x10^6$ cells/ml. The PHA treated blasts were then irradiated for 6 1/2 minutes (33Gy) and used as stimulators in a one way mixed lymphocyte culture (MLC).

2.1.5 Gamma-irradiation:

All gamma-irradiation of cells was done in a Gammacel 3000 ELAN irradiator (Nordion International, Inc) which emits 503 rad per minute (5.03 Gy per min).

2.1.6 Proliferative assays:

Both responder and allogeneic stimulator populations were resuspended at a concentration of 10^6/ml. 100*u*l of each population were mixed together in triplicate in round-bottomed 96 well microtiter plates (Nunc) with the stimulators irradiated for 6 1/2 minutes (33Gy) in a mixed lymphocyte culture (MLC). The autologous control consisted of 100 *u*l of responder cells mixed with 100 *u*l of autologous irradiated cells which would measure the baseline proliferation of the responder population. To ensure

that the stimulators were adequately irradiated, the negative control consisted of irradiated stimulators alone. Another control was responder cells alone. All samples were incubated in triplicate at 37^0C / 5% CO_2. At 96 hours, 1*u*Ci of tritiated thymidine (^{3}H-thymidine) (Amersham, Buckinghamshire, UK) diluted in 20*u*l of "CM" was added to each well. 18-24 hours later, the plates were harvested and tritiated thymidine incorporation measured in a Wallac 1205 Betaplate (Wallac, Turku, Finland). Consistency was maintained by ensuring that there was <10% difference in counts per minute (cpm) between triplicate cultures.

The results obtained could be expressed in 2 ways:

1. subtracting the arithmetic mean cpm of the autologous control from the arithmetic mean cpm of the MLC. This was referred to as the "change in cpm".
2. Dividing the arithmetic mean cpm of the MLC by the arithmetic mean cpm of the autologous controls. This was referred to as the "stimulation index "(SI).

2.1.7 PKH26 labelling of stimulator cells:

Cells to be labelled were harvested into a universal container and centrifuged at 200g for 10 minutes. The supernatant was discarded and the cells resuspended in Hanks buffered saline solution (HBSS). To wash off all traces of FCS which may be present in the original cell culture medium and would interfere with the labelling process, the centrifugation step was repeated and cells resuspended in 1 ml of the "diluent" (Sigma) provided as part of the PKH26 fluorescent cell linker kit. This 1 ml of cell suspension was then divided into 2 aliquots of 500*u*l each in an Eppendorf tube. 6*u*l of the PKH26 red fluorescent dye stock (concentration $1x10^{-3}$M) (Sigma, UK) was mixed with 1 ml of "diluent" in an Eppendorf tube (Eppendorf) to obtain the optimal concentration for

labelling. Previous labelling had been done to compare 2*u*l of PKH-26 vs 4*u*l vs 6*u*l and the latter was found to produce optimal labelling.

500*u*l of the PKH-26 labelling reagent was added to each aliquot of cells and incubated for 2 minutes at room temperature. At the end of 2 minutes, 500ul of sterile FCS was added to the cell-dye mixture to stop the process and incubated for an additional 1 minute at room temperature. The tubes were then spun in a microcentrifuge at 5000rpm for 30 seconds and the cells resuspended in 1 ml of "complete medium". The cells were spun and washed again and resuspended in "complete medium" at a density of 10^6 cells/ml. The PKH-26 labelling was checked using the flow cytometer with fluorescence in the high FL2 region. Labelling was found to be stable for up to one week which was within the time frame of the intended experiment.

2.1.8 PKH26 labelled alloantigen stimulation of lymphoid cells in an MLC to examine the temporal dynamics of CD69 expression:

PBMCs from two unrelated normal donors were used for each experiment - one labelled with PKH-26 as described above and termed the stimulator population, the other being the responder population. Stimulator and responder cells were mixed in an equal ratio. Non-stimulated responder cells were used as controls in each experiment. In a single experiment responder cells were incubated with PKH-26-labelled autologous PBMCs to exclude the possibility that the membrane dye could be responsible for the stimulation. Samples were incubated at 37^0 C/5%CO_2 and analysed at 4, 24, 48, 72, 96,120,144, 168 and 192 h. At the completion of each incubation the cells were labelled with monoclonal antibodies in the following three colour combinations - CD69 / CD56 / CD3 and CD4 / CD69 / CD8 where the antibodies were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin

chlorophyll protein (PerCp) respectively. Ten thousand responder cells were acquired as list mode data with 1024 channel resolution. Stimulator cells were excluded from the analysis by electronic gating of high FL2 and FL3 signals as shown in section 3.2.3 and figure 3.2 from chapter 3. This technique permitted analysis of a one-way mixed lymphocyte reaction.

Data were analysed at each time interval by quadrant analysis of 2-dimensional dot plots after electronic gating on PKH-26 negative events. The percentage of CD69+ve cells within both the CD3+ and CD56+ subpopulations were determined both for the non-stimulated control and for the mixed lymphocyte culture. Percent T cell activation in both cell suspensions was defined as the proportion of activated CD3+ cells (CD3+CD69+) divided by the total proportion of CD3+ cells (CD3+ CD69+ and CD3+CD69-ve). NK cell activation was assessed in the same fashion. The difference in activation between the MLC (responder population in the presence of stimulator cells) and the control responder population alone was calculated and the percent difference in activation was equal to % activation in MLR -% activation in control.

2.1.9 Antibody staining protocol for flow cytometric analysis:

Polystyrene tubes (Falcon Tubes) were used. 100µl of the PBMC suspension (conc. 10^6 cells/ml) was incubated with 10µl of the relevant fluorochrome conjugated monoclonal antibody for 15 minutes at 21°C in the dark. The cells were then washed once in 2 mls of Phosphate Buffered Saline (PBS) (Microgen Bioproducts Ltd., Surrey) and resuspended in 0.5ml PBS. All samples were analysed by flow cytometry (FACScan - Becton Dickinson Immunocytometry Systems) within 4 hours of preparation. Lymphocytes were isolated by electronic gating on the basis of forward and side angle light scatter signals and at least 10000 events collected as list mode data

(Lysis II or CellQuest - Becton Dickinson Immunocytometry Systems). The instrument photomultiplier gain and compensation for three colour work was determined using PBMCs labelled with one or two fluorochromes. If whole blood samples were used, 2ml of a proprietary red cell lysis buffer (FACSLyse - Becton Dickinson) was added to the tubes after the appropriate incubation time with the relevant antibodies and left for 10 minutes at room temperature. The cells were then washed twice in PBS, resuspended in 0.5mls PBS and ready for analysis in the same way.

2.1.10 Comparison of PKH26 labelled stimulators versus irradiated stimulators in an MLC:

To ensure that the alloantigen stimulatory capacity of cells was not altered by PKH26 labelling, an MLC was set up with dye labelled stimulators. The upregulation of activation antigens was compared with another MLC set up in parallel with the same responder-stimulator pair but with the latter irradiated for 33 Gy as in a traditional one-way MLC. The responder cells were then analysed over a time course and using flow cytometric analysis as described above. For analysis of the 24 and 48 hour time points, stimulator cells that had been irradiated 48 hours prior to setting up the MLC were used to compensate for the time taken for irradiated cells to move out of the lymphoid gate (discussed in section 3.2.2 and figure 3.1).

2.1.11 Comparison of the temporal dynamics of activation markers CD69, CD25,OX40 and HLA-DR in a one way MLC:

A one-way MLC was set up using equal numbers of responder cells and HLA-mismatched irradiated (33Gy) stimulator cells. The baseline autologous control consisted of responder cells and equal numbers of irradiated autologous cells. A negative control consisted of irradiated stimulator cells alone. All cells were resuspended at 10^6 cells/ml in "complete medium" and cultured in 24 well plates (Nunc, UK). Cells were analysed at the following time points: 0, 24, 48, 72, 96, 120, 144 and 192 hours.

The following panel of antibodies was used:

TUBE	**FITC**	**PE**	**PerCp**
1	CD25	CD69	CD3
2	CD4	OX40	CD8
3	HLA-DR	CD4	CD8
4	CD4	CD69	CD8
5	CD25	CD4	CD8

2.2 HLA-mismatched & matched in vitro study on the selective depletion of alloreactive lymphocytes:

2.2.1 Patients and subjects:

In the initial feasibility study involving mismatched pairs, blood was obtained from HLA mismatched normal volunteer donors. For the matched study, the 1st party stimulators were patients (recipients) who were undergoing subsequent HLA-matched allogeneic bone marrow transplants. The responders (donors) were either their HLA-identical siblings or matched unrelated donors from a donor registry (Anthony Nolan Research Centre or Bristol Bone Marrow Registry). Peripheral blood was collected from the donors and remission peripheral blood or bone marrow from the patients after informed consent. For the 3rd party stimulators, blood was collected from HLA mismatched normal volunteer donors.

2.2.2 HLA typing:

In each case, donor and recipient pairs for the matched study were serologically typed for HLA-A and HLA-B locus antigens and high resolution sequence specific oligonucleotide typing for HLA-DRB1, DRB3, DRB4, DRB5 and DQB1 alleles. (performed at the Anthony Nolan Research Centre, London, UK)

2.2.3 HLA-mismatched study:

Section 4.2.1 and Figure 4.1 describes in detail the protocol developed for the HLA-mismatched study and the rationale for devising this approach. This was based on the

one way MLC where responder PBMCs were mixed in an equal ratio with irradiated (33Gy) stimulator cells. All cells were resuspended to a density of 10^6 cells/ml in "CM". To assess the efficacy and specificity of the procedure, the following calculation was made. The CD69 expression of the unsorted cells against either 1st party stimulator or 3rd party was normalised to 100%, and the degree of depletion achieved was determined by:

$$\frac{\text{(CD69 expression in secondary MLC) - (CD69 expression in depleted fraction)}}{\text{(CD69 expression in primary MLC) - (CD69 expression in responder alone)}} \times 100\%$$

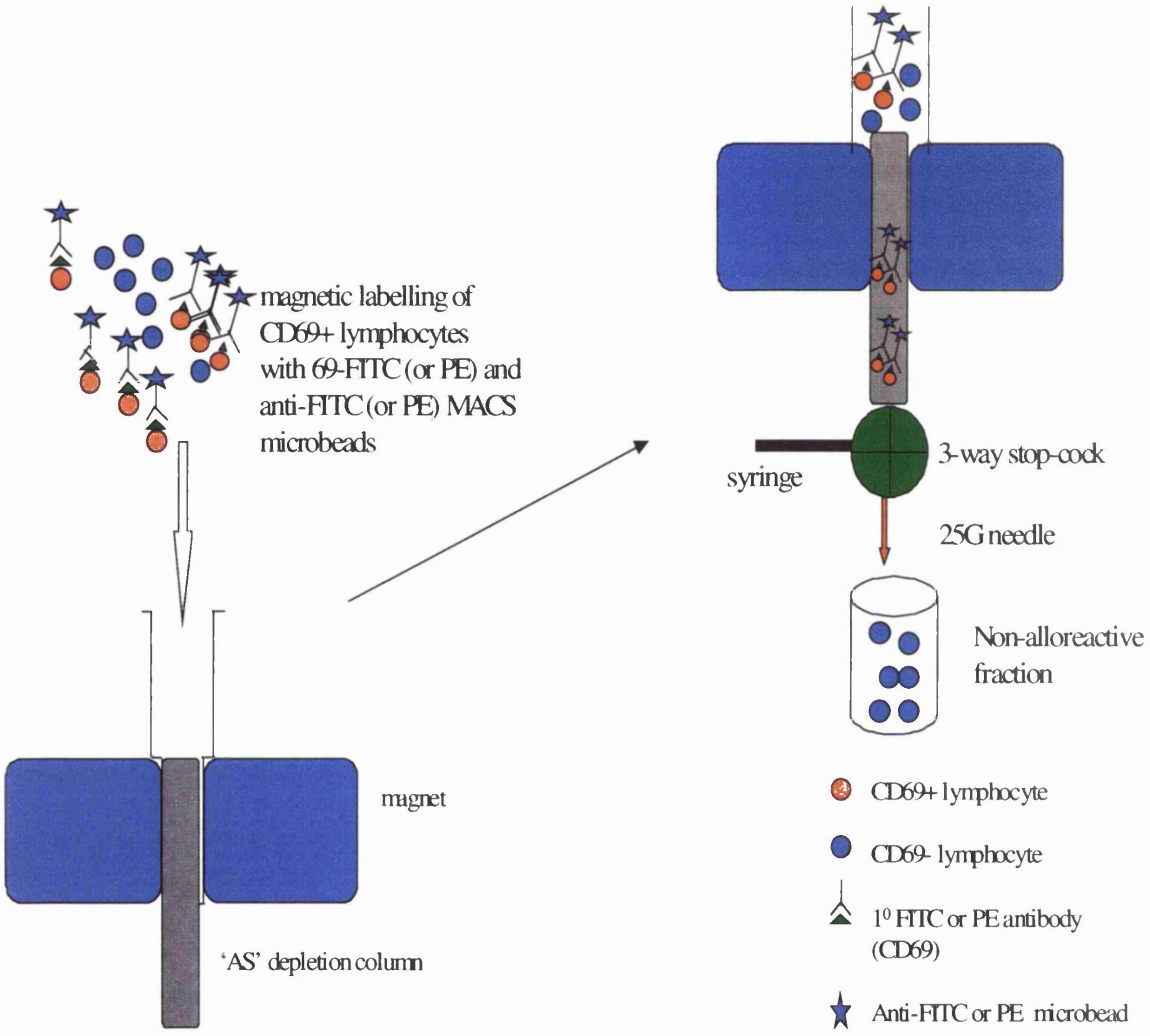
The degree of depletion to both the 1st party stimulator and the 3rd party was compared as a percentage of the unsorted response.

2.2.4 Magnetic bead sorting:

This is a sterile technique of cell separation involving the passage of cells labelled with iron dextran microparticles (50nm diameter) through a ferrous matrix column within an intense magnetic field. The magnetic cell sorting (MACS) 'AS' depletion column' (Miltenyi Biotec GmbH. Cologne) was used for this purpose. Figure 2.1 illustrates diagrammatically the separation and removal of CD69+ alloreactive cells using this technique. The 'AS' columns have a maximum capacity of $3x10^7$ positive cells. Cells were labelled with saturating amounts of anti-CD69 FITC or anti-CD69 PE in later experiments which was found to be 10 *u*l of antibody for every 10^6 responder cells. This was incubated for 10 minutes at room temperature in the dark; excess antibody was washed off by centrifuging at 200g for 10 minutes and the cells resuspended in 90*u*l of Dulbecco's PBS supplemented with 2mM EDTA and 0.5% bovine serum albumin (BSA) (MACS buffer) per 10^7 cells. The cells were then incubated with the

appropriate anti-FITC or anti-PE MACS colloidal super-paramagnetic micro-beads for 30 minutes at 4^0-8^0 C. 10*u*l of anti-FITC or 20*u*l of anti-PE beads were added for every 10^7 total cells. The cell suspension was then diluted to 1 ml after which the labelled cells were passed down an AS column with a 25G capillary that had been primed with buffer. With this, a flow rate of 0.3 ml/min was achieved. After the cell suspension had passed through, the column was washed with 3 column volumes of cold buffer. The effluent or non-bound cells collected was the negative fraction depleted of magnetically labelled cells, termed the "depleted fraction". The column was now removed from the magnetic separator and the retained cells back flushed to the top. With a 23G capillary (1ml/min), the cell suspension was allowed to run through the column and further rinsed with 3 column volumes of cold buffer. The effluent collected was termed the "wash fraction". The flow resistor was discarded, the column removed from the separator and the bound cells back flushed to the top of the column. This was allowed to run through the column and rinsed with 3 column volumes of cold buffer. The eluted cells collected was termed the "enriched fraction". The FITC labelling system was used initially but when the PE system was introduced by Miltenyi and proved to be more effective and consistent in depletion, all further experiments were done using the PE system.

Figure 2.1 Separation and selective removal of CD69+ alloreactive cells using the MACS "AS" depletion columns



2.2.5 HLA-matched pair study:

Section 4.3.1 and Figure 4.5 describes in detail the protocol developed for the HLA-matched study which is similar to that of the mismatched pairs but with the following modifications made as detailed in section 2.2.5.1 and 2.2.5.2. This was to circumvent the problem that no appreciable CD69 expression or ^{3}H thymidine uptake was detected above baseline in HLA matched pairs.

2.2.5.1 Modified MLC:

All stimulator cells (PBMCs from patient, 3rd party donor and the autologous control) were pre-treated with 1000U/ml of γ-IFN (Genzyme) and 1000U/ml of TNF-α (Genzyme) for 24 hours. The cytokines were washed off twice with excess PBS and re-suspended in CM at 10^6 cells/ml. The stimulator cells were then irradiated for a total of 33Gy before being mixed with the donor (responder) cells. To exclude any non-specific effect by the cytokines, an autologous control was set up in parallel consisting of responder cells mixed with cytokine pre-treated autologous cells. 18U/ml of IL-2 or 1000U/ml of IL-4 (Genzyme) was added to this modified MLC

2.2.5.2 Modified proliferation assays:

The ^{3}H-thymidine incorporation was used as an in vitro marker of alloreactivity to compare the results of depletion in matched pairs. In addition to assessing the CD69 expression at 72-96 hours, proliferation assays were set up in triplicate in 96 well round bottom plates using 10^6 donor (responder) and 10^6 cytokine pre-treated irradiated patient (1st party stimulator) or 3rd party cells per well in a total volume of 200μl/well.

18U/ml of IL-2 or 1000U/ml of IL-4 (Genzyme) were added to this modified MLC. The baseline autologous controls were donor cells plus cytokine pre-treated autologous stimulators. The other controls present were irradiated stimulators alone. Cultures were incubated at 37^0C / 5% CO_2 for 5 days, and pulsed for the last 18 hours with 1 μCi/well of ^{3}H-thymidine. Cells were harvested and tritiated thymidine incorporation measured in a Wallac 1205 Betaplate. The specificity of depletion was assessed by comparing the proliferation assays of the depleted fraction against the pre-sorted cells. The ^{3}H-thymidine incorporation of the unsorted cells against either patient or 3rd party was normalised to 100%, and the degree of depletion achieved established from the following formula:

$$\frac{(^3\text{H-thy incorporation in } 2^0 \text{ MLC}) - (^3\text{H-thy incorporation in post sort auto control})}{(^3\text{H-thy incorporation in } 1^0 \text{ MLC}) - (^3\text{H-thy incorporation in auto control})} \times 100\%$$

From Figure 4.5, the relevant calculation was (C) / (A) and (D) / (B).

The ^{3}H-thymidine incorporation in each instance was worked out as the mean of the triplicates.

2.2.6 Cell recovery, purity and depletion efficiency:

The responder cell population was counted before and after sorting via the MACS columns. The responder cells could be easily distinguished from irradiated stimulator cells by cell morphology. Responder cells retained their cell shape and nuclear configuration whereas irradiated stimulator cells had lost their membrane and nuclear integrity. This was additionally confirmed by trypan blue staining.

Cell recovery (yield) was calculated as follows:

$$\frac{\text{Number of CD69 negative responder cells post sorting}}{\text{Number of CD69 negative cells before sorting}} \times 100$$

Cell purity of the engineered graft was defined as (100% - %CD69 positive cells in the depleted fraction).

Depletion efficiency was calculated by comparing the number of CD69 positive cells in the depleted fraction after sorting with the number of CD69 positive cells prior to sorting. This was expressed as follows:

$$\frac{(\%\text{ of CD69 + cells before sorting}) - (\%\text{ of CD69 + cells after sorting})}{(\%\text{ of CD69 + cells before sorting})} \times 100$$

2.2.7 Immunophenotyping for the in-vitro selective depletion study:

The following 3-colour combination was used: CD69-FITC, CD56-PE and CD3-PerCp to differentiate between activated T cells and NK cells. PBMCs were analysed using Lysis II software.

2.2.8 Depletion of alloreactive cells using Dynabeads:

Depletion of CD69 positive alloreactive cells was performed with Dynabeads M-450 (Dynal, UK Ltd) to compare its efficiency of depletion with the MACS method. These are superparamagnetic, polystyrene beads with goat anti-mouse IgG covalently bound to the surface. Prior to their use, the beads were washed three times in HBSS / 2% FCS in a magnetic particle concentrator (MPC) (Dynal UK Ltd), the supernatant discarded and the beads resuspended at a final concentration of $1x10^8$ beads/ml in sterile PBS and cooled to 4^0C. The indirect technique of cell separation was used, similar to that employed in the MACS system. The cell suspension to be separated was labelled with saturating amounts of anti-CD69PE which was found to be 10*u*l of

antibody for every 10^6 responder cells. This was incubated for 10 minutes at room temperature in the dark; excess antibody was washed off by centrifuging at 200g for 10 minutes and the cells resuspended in HBSS at a concentration of 10^7 cells/ml. 75*u*l of Dynabeads M-450 was added to every $3X10^6$ cells to ensure a coating of 3-4 beads per CD69+ cell. This mixture was incubated in polystyrene tubes at 4^0C for 30 minutes with slow tilting and rotation. Following the addition of 500*u*l HBSS/2% FCS, the tubes were placed on the MPC for 2 minutes. The CD69+ cell population remained attached to the magnet while the remaining non-alloreactive CD69 negative cell suspension was recovered from the supernatant and transferred to a fresh tube. This is based on the principle that the alloreactive CD69+ cells would be rosetted with Dynabeads and therefore be attracted to the wall of the test tube by the magnetic field. Enriched and depleted fractions were assessed for purity and viability by flow cytometry.

2.3 A study on the effect of inflammatory cytokines of the "cytokine storm" on expression of molecules involved in allorecognition

2.3.1 Samples and subjects:

Peripheral blood was collected from 12 healthy adult volunteers. PBMCs were isolated from heparinised blood by density gradient centrifugation and resuspended in "CM". All cell densities were adjusted to 10^6 cells/ml and used fresh.

2.3.2 Cytokine combinations used:

Cells were cultured in 24 well tissue culture plates (Nunclon, Nunc, Denmark) in a final volume of 2 ml "CM" ($2x10^6$ cells). Cytokines were added to the cultures at the start of the experiment as detailed in Table 2.1. All cytokines were obtained from Genzyme, UK and concentrations indicated above were worked out from the product sheets provided (TNF-α $5.9x10^7$ U/mg), (IFN-γ $4.57x10^7$ U/mg), (IL4 $5x10^7$ U/mg). Each cytokine combination was tested in duplicate or triplicate and incubated at 37^0C / 5% CO_2 for the proscribed period before analysis.

Table 2.1: Cytokine combinations and concentrations used for the experiment:

TUBE	CYTOKINE COMBINATION
A	none (controls)
B	1000 U/ml IL4 alone
C	500 U/ml TNF-α alone
D	1000 U/ml TNF-α alone
E	2000 U/ml TNF-α alone
F	1000 U/ml γ-IFN alone
G	2000 U/ml γ-IFN alone
H	4000 U/ml γ-IFN alone
I	500 U/ml γ-IFN and 500 U/ml TNF-α
J	1000 U/ml γ-IFN and 1000 U/ml TNF-α
K	2000 U/ml γ-IFN and 1000 U/ml TNF-α
L	1000 U/ml γ-IFN and 1000 U/ml IL4

2.3.3 Antibody combinations used and immunophenotyping protocol:

To assess the changes induced by the various cytokine combinations, PBMCs were incubated with the antibody combinations described in Table 2.2. 4 sets of antibody combinations were used.

Table 2.2: Antibody combinations used to study the change in expression of MHC, costimulatory and adhesion molecules with cytokines:

Tube	FITC conjugated antibody	PE conjugated antibody	PerCp conjugated antibody
1	W632	HLA-DR	CD3
2	CD11a	CD54	CD3
3	CD80	CD86	CD3
4		CD49d	CD3

All antibodies were obtained from Becton Dickinson, Oxford, UK except for W632-FITC (Harlan Sera-Lab), CD80-FITC (Pharmigen, UK) and CD86-PE (Pharmigen, UK). All 4 sets of the 3-flurochrome colour combinations were tested on each PBMC sample incubated with the different cytokine combinations. All samples were tested at the initiation of the experiments and subsequently at t=24 hours and t=48 hours and analysed using Lysis II software.

2.3.4 Flow cytometric analysis using Lysis II software for cytokine study:

At the analysis stage, lymphocytes were differentiated from monocytes using the forward angle light scatter (FSC) vs side angle light scatter (SSC) signals. In a separate experiment, the identification of monocytes based on FSC vs SSC gating was

confirmed by staining positive with CD14 (data not shown). T cells were identified by positive staining for CD3 within the lymphocyte gate, B and NK cells by being CD3 negative and monocytes with a combination of FSC vs SSC gating and CD14 staining. 10,000-15000 events were collected as list mode data in Lysis II after live gating of lymphocytes and lymphoblasts by FSC and SSC signals.

For each cell population (T cells, B cells and monocytes) and each antigen, two parameters were measured:

a) median logarithmic fluorescent intensity (MFI) of the antigen expressing cells which is a measure of the intensity of expression of the antigen on the cell surface and

b) the percentage of cells that express the antigen (% positive).

The mean FSC value was also analysed for each sample as an indication of cell size and the calculated MFI / FSC ratio was used as the best estimate of relative density of expression of the antigen on the mononuclear cell surface; the greater the value, the higher the density of expression. The use of the MFI/FSC ratio allowed comparison of fluorescent intensities between cells of differing size: monocytes, T cells and B cells. As samples were analysed at least in duplicate, the mean value was taken for each parameter, checking also that the individual values differed by no more than 20%.

CaliBrite beads (Becton-Dickinson, Oxford, UK) were used to standardise the instrument settings before each batch of samples to ensure consistency between time points (24 and 48h) and between individual volunteers. This was done by ensuring that the median fluorescent intensities of FL1, FL2 and FL3 were consistent between individual experiments.

2.4 Statistical tests of significance:

The 2-tailed paired t-test was used unless stated otherwise to assess whether the effect of the test sample (allodepletion or cytokine study) compared to the control sample was significant ($p<0.05$). The pairing was also ascertained to be well correlated.

The materials and methods for the work done on the murine model and the retention of anti-viral reactivity are described in their respective chapters for greater clarity.

3. Chapter 3. Temporal dynamics of CD69 and other activation markers with alloantigen stimulation

3.1 Introduction:

The central event in the generation and orchestration of a T cell mediated immune response in GvHD is the activation and clonal expansion of T cells. The aim of the work in this chapter was to determine whether donor anti-host alloreactive lymphocytes could be reliably identified by the cell surface expression of various activation markers. The simple MLC is a functional assay that has been used to evaluate allorecognition and alloreactivity between donor/recipient pairs. This system was therefore used as the basis for investigating how alloantigen stimulation in an MLC would affect the expression of these activation antigens. The proliferative response as measured by ^{3}H-thymidine uptake in an MLC from alloantigen disparity has been well studied and was commonly used as a tool for donor selection and predicting GvHD in BMT patients before the advent of Class II molecular typing (Dupont et al. 1976).

In contrast, the upregulation of different activation antigens in response to alloantigen disparity is not clear. The effects of strong mitogenic stimuli have been well documented and provide some insight into the tempo of response of these activation antigens (Craston et al. 1997). PHA and PMA induce the upregulation of CD69 on T cells as early as 1 hour after exposure while CD25 and HLA-DR are expressed at a later time point (Biselli et al. 1992). Both PHA and PMA however are potent mitogens and

induce a non-specific response in the majority of T cells. The situation with alloantigens is markedly different in that the stimulus is less potent and more specific; only a proportion of alloreactive lymphocytes are likely to respond and this may involve different kinetics of activation. Therefore, it was necessary to study and compare the different activation markers and their temporal dynamics of expression.

Using monoclonal antibodies to label these lymphocytes in a one-way MLC and the subsequent visualisation by flow cytometric analysis offered the advantage over proliferative assays in allowing identification of the alloreactive lymphocyte population from the rest of the non-alloreactive cells. The crucial question remained as to which activation antigen would be the optimal marker to use in developing an allodepletion strategy.

The list of cell surface activation antigens are numerous and include CD69, CD25, OX-40, HLA-DR, CD71, CD54, CD26 and CD97. Of these, CD26, CD54 and CD97 are already present on a significant proportion of resting lymphocytes and further increased upon activation (Gorrell et al. 1991), (Springer 1990). It would be difficult in these instances to differentiate between an 'activated' T cell and a resting T cell that also expressed these antigens, albeit at lower density, a key component of the selective allodepletion strategy. "True" activation antigens can therefore be defined as those that are absent (<5%) on resting lymphocytes but demonstrate specific upregulation of expression only with activation. These "true" activation antigens would be of particular interest to this project and include CD25, CD69, OX40 and HLA-DR.

3.2 RESULTS:

3.2.1 Baseline Expression of CD69, CD25, OX40 AND HLA-DR on resting PBMCs

All healthy volunteers except one used for this study had a baseline (time=0 hours) CD69 expression of <6%. As shown in Tables 3.1 a-b, the mean proportion of CD69+ cells from a series of 31 donors was 2.18% and this was expressed in both CD3+ (0.87%) and CD3- (1.38%) subsets. The corresponding baseline level of CD25 activation was 1% but in contrast, this was expressed exclusively in the CD3+ subset. OX40 was not found on any of the samples tested at baseline while HLA-DR was expressed on 4.21% of CD3+ cells and 10.27% of CD3- cells. The high value in CD3- cells was most certainly due to B cells which constitutively express Class II antigens like HLA-DR on their cell surface. These values were in agreement with previous studies and confirmed that CD69, CD25 and OX40 were "true" activation antigens in that they were expressed on less than 5% of resting cells. The situation with HLA-DR was less clear due to constitutive expression on B cells but it could be regarded as a "true" activation antigen if only T and NK cells were considered.

Tables 3.1a-b also show that despite 6 (144 hours) days of culture in CM containing heat inactivated 10% FCS, the mean level of expression remained at less than 5% in all control autologous cultures. CD69 expression in either the CD3+ (mean 0.79-2.05%) or CD3- (0.41-2.33%) subsets remained constant although the level of expression in both CD3+25+ and CD3+OX40+ subsets exhibited a tendency for a small increase with 6 days in culture. The marked decrease in the percentage of HLA-DR expressing cells was due to the progressive loss of B cells in culture. These results demonstrated that the heat inactivated FCS used in the culture medium did not cause any marked non

Table 3.1 Expression of CD69, CD25, HLA-DR and OX-40 in control autologous cultures

a)

Time(h)	%CD69+ total	%CD3+69+	%CD3-69+	%CD3+25+	%CD3-25+
0	2.18±2.64	0.87±1.14	1.38±1.83	0.9±0.55	0.09±0.07
24	3.31±2.44	1.03±1.34	2.33±1.60	1.92±0.63	0.39±0.23
48	2.91±2.48	1.30±1.76	1.96±1.98	2.84±0.94	0.40±0.28
72	1.73±0.84	0.79±0.60	1.07±0.80	2.48±1.33	0.25±0.22
96	1.76±0.81	1.16±1.05	0.94±0.69	2.54±1.43	0.31±0.20
120	1.85±0.81	1.28±0.73	0.41±0.16	3.49±1.56	0.26±0.12
144	3.78±1.60	2.05±1.82	1.18±1.04	4.96±2.48	0.26±0.19

b)

Time(h)	%CD3+OX40+	%CD8+OX40+	%CD3+DR+	%CD3-DR+
0	0.00±0.00	0.00±0.00	4.21±4.53	10.27±2.92
24	0.02±0.03	0.01±0.01	2.06±1.47	10.22±4.59
48	0.08±0.08	0.01±0.01	1.66±0.87	9.02±6.07
72	0.14±0.11	0.03±0.03	0.93±0.15	4.93±0.84
96	0.38±0.15	0.02±0.01	1.29±0.44	4.59±0.59
120	0.69±0.34	0.21±0.33	1.19±0.12	4.19±0.74
144	2.49±2.58	0.14±0.09	2.86±1.85	4.90±1.36

Autologous cultures: responder cells alone or with autologous stimulator cells.

All values expressed as mean ± s.d.

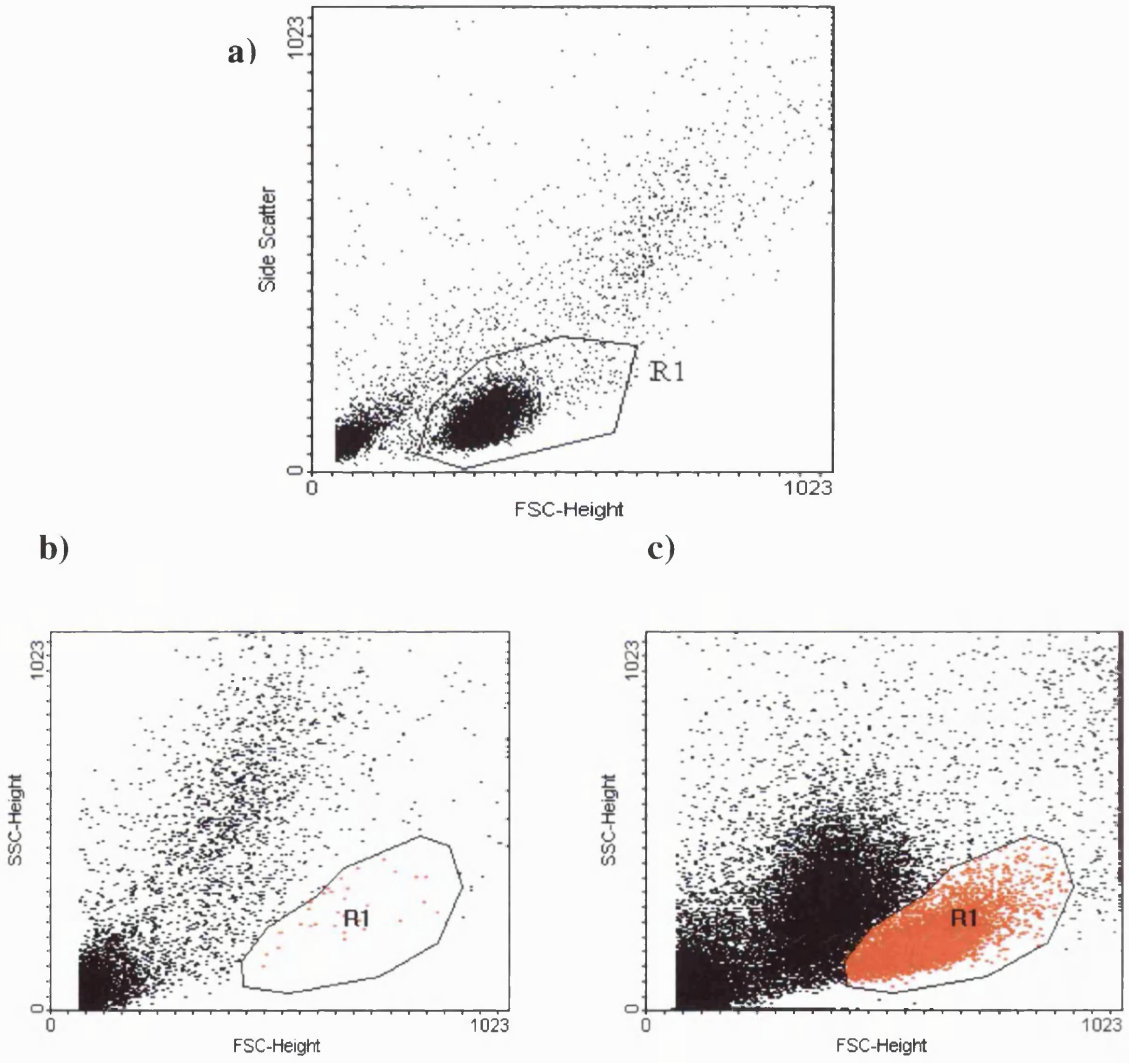
CD69 expression was calculated by determining the percentage of total responder cells in the lymphoid gate that are CD69 positive.

specific increase in activation above background, particularly with CD69. This was however seen only with certain batches of FCS and it was therefore important to ensure that the FCS was batch tested before use in the experiments. Any increase in expression observed in an MLC would therefore imply a specific alloreactive response due to the antigenic disparity between responder and stimulator.

3.2.2 Rationale for using PKH26 labelled stimulators:

Proliferative assays in a one way MLC does not provide any information as to the subset of T cells responding to the alloantigen stimulus or their phenotypic characteristics. Flow cytometric analysis would bypass this problem because by staining the lymphocyte population, alloreactive cells responding to the alloantigen stimulus could be identified by virtue of the fact that they would express activation antigens on their surface. One could therefore distinguish between a proliferating cell from a quiescent one or an activated cell from a non-activated one. The problem however with using flow cytometric analysis of an MLC was that it would not be possible to differentiate responder from stimulator PBMCs as both cell populations were similar with regards to their FSC and SSC characteristics up to 48 hours of culture, falling within the same "lymphocyte gate" as shown in Figure 3.1a. Initial and subsequent experiments showed that it was only at 72h that radiation induced apoptosis of the stimulator cells became evident for the majority of stimulator lymphocytes and it was possible to resolve the responder from the stimulator population by virtue of FSC/SSC signals. This change to an apoptotic morphology led to alterations of cell size and granularity and was reflected as a shift to the left on a FSC vs SSC plot as shown in Figure 3.1b and 3.1c. However, as the responder cells may upregulate various activation antigens before 48 hours, another method had to be used to

Figure 3.1 Flow cytometric dot plots (FSC vs SSC) of responder PBMCs plus irradiated stimulator PBMCs in a one way MLC at time 0 and 72 hours.



a) Flow cytometric dot plot (FSC vs SSC) of responder PBMCs plus irradiated stimulator PBMCs in a one way MLC at time 0h. It can be seen that both populations fall into region 1 (R1) and cannot be differentiated on flow cytometric analysis.

c) Flow cytometric dot plot (FSC vs SSC) of an MLC 72 hours after irradiation of the stimulators. Region 1 delineates the area in which live, non-apoptotic responder PBMCs fall (red) and show the resolution of responders from stimulators.

b) Flow cytometric dot plot (FSC vs SSC) of irradiated stimulators alone at 72 hours showing that the apoptotic stimulator cells have moved out of region 1, allowing electronic gating of the live responder cells in R1 for analysis.

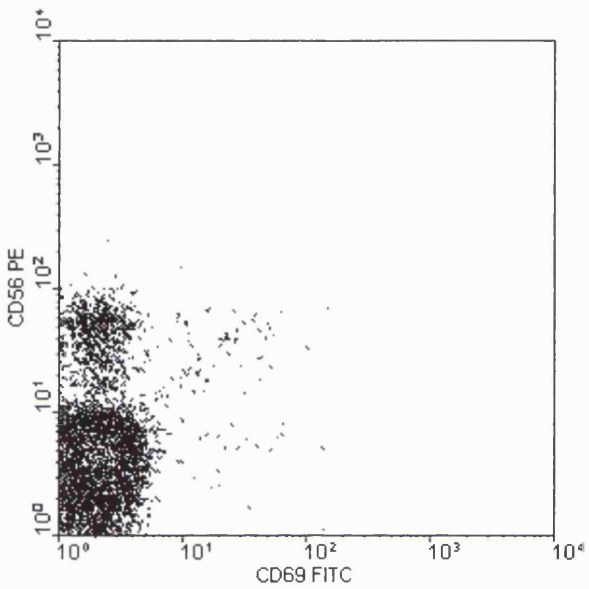
discriminate between responder and stimulator lymphocytes. This was done by staining the stimulator cells with a membrane dye PKH26 (Craston et al 1997). As demonstrated in Figure 3.2a and 3.2b, the responder cells could now be easily discriminated from PKH26 labelled stimulator cells even at the start of MLC. This would enable analysis of the responder population alone reacting to any alloantigen disparity present in the stimulator cells. It was also found that the dye remained without loss of intensity for the whole duration of the MLC. PKH26 fluoresces in the FL2 region similar to PE but while the PE-conjugated antibodies always fell within the first 500 channels of a 1024 channel distribution, the PKH-26 signals consistently appeared above channel 800. Similar differences in fluorescence signals were observed in FL3 between CD3PerCp or CD8PerCp and PKH-26.

3.2.3 Results of temporal dynamics of CD69 expression in a one way MLC using PKH26 labelled stimulators:

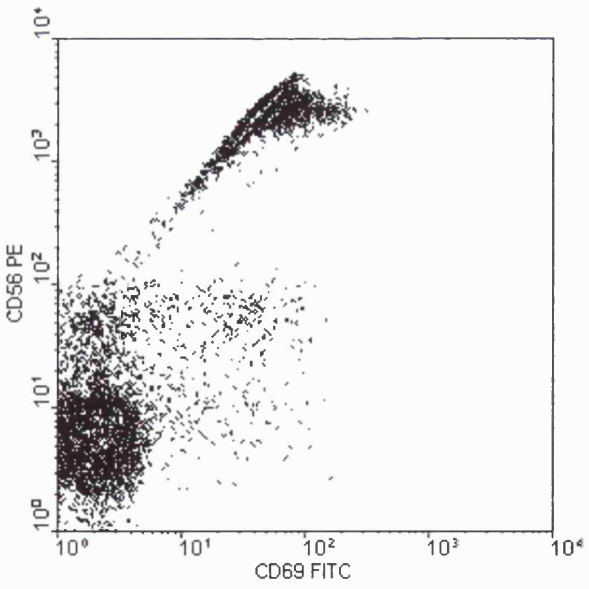
A one way MLC was set up using MHC mismatched responder and PKH26 labelled stimulator pairs. Antibody staining followed by flow cytometric analysis (gating out the stimulator cells) was performed to examine the expression of CD69 on the responder population. Figure 3.3 shows the effect of MHC mismatched alloantigen stimulation on the temporal expression of CD69 in lymphocytes while figure 3.4 examines the effect on the CD3+ T cells in the MLC. Both graphs display broadly similar temporal dynamics of CD69 expression, indicating that the upregulation of CD69 expression in response to alloantigen stimulation was similar in both the responder CD3+ T cell subset and the CD3- subset (which includes NK cells). The proportion of CD3+ and CD3- cells in the control cell cultures expressing CD69 was low and remained relatively constant over the time course. There was a detectable

Figure 3.2 Resolution of responders from stimulators in a MLC with PKH labelling

a)



b)



Resolution of PKH26 labelled stimulator cells from responder cells at time=0h. Both populations fell into the same lymphocyte gate. A) responder cells alone labelled with CD69 FITC and CD56 PE. B) responder and stimulator cells mixed at a 1:1 ratio showing clear resolution between the two due to PKH fluorescence in the high FL2.

increase in CD69 expression first seen from 24 hours post stimulation (1.2% total CD69 and 0.7% in the CD3+ subset), steadily rising to a peak at 120 hours (10.1% total CD69 and 5.5% in the CD3+ subset) and maintaining a plateau thereafter until 192 hours. This progressive rise in CD69 expression was associated with an increasing proportion of lymphocytes moving from region 1 (R1: the lymphocyte gate) to region 2 (R2), as shown in Figure 3.5. R1 or the "lymphocyte gate" denoted the area in which live, non-apoptotic responder PBMCs fell while R2 or the "blastoid gate" delineated the area in which live responder cells have increased in cell size and prior to proliferation. This increase in cell size and a change into a "blastoid form" was reflected in alterations to the FSC vs SSC characteristics. These "blastoid cells" were largely CD69+, indicating that they were alloreactive cells which had become activated and were now committed to proliferation. By measuring the proportion of cells in the R1 gate as compared to proliferating cells in the R2 gate, the R1:R2 ratio would identify trends in the MLC over time and highlight any relative skewing of the responder cell population. As shown by the blue dots in figure 3.5, there was a progressive increase in the number of alloreactive proliferating cells in the R2 gate compared to more constant numbers in the R1 gate. This was reflected in the serial decrease in the R1/R2 ratio. For the first 72 hours, there was relatively little proliferation as reflected in the R1/R2 ratio (75:1 at t=24h and 57:1 at t=72h). At 96 hours, some proliferation had occurred with a corresponding drop in the R1/R2 ratio (30:1) although it was effectively still small. However at 120 hours and beyond, there was a sharp and marked increase in proliferation such that the activated proliferating cells accounted for between 17-45% of the total responder lymphocyte population (R1/R2 ratio 5:1 to 1.8:1). These results implied that up to 96 hours, the responder cell characteristics remained relatively unchanged but after that, a marked shift occurred. This was specific, reproducible and not due to the time spent in culture as contrasted

against a dot-plot of a baseline autologous culture (responder and autologous irradiated stimulators) at 144 hours. Without an alloantigen stimulus, the proportion of activated cells that moved on to proliferate and occupy R2 gate remained relatively constant and small despite 6 days in culture.

3.2.4 Analysis of CD69 expression in various lymphocyte subsets

Having established that CD69 was expressed on CD3+ T cells, the spectrum of CD69 expression in the various lymphocyte subsets was analysed in more detail, specifically the CD4+, CD8+ and NK cell subsets which have been implicated in the GvH reaction. Figure 3.6 illustrates the CD69 expression over time in the CD4+ and CD8+ T cell subsets and figure 3.7 the CD56+ NK cell subset. The baseline expression of CD69 was low in the control cultures although it was higher in the CD56+ve cell population (mean 3.96 $\pm$ 2.36%) compared to the T cell population. Following alloantigen stimulation, it can be seen that CD69 upregulation was seen in CD4+, CD8+ and CD56+ lymphocyte subsets. The temporal dynamics of CD69 expression was also similar with the first response seen at 24 hours and rising steadily to near peak levels at 120 hours and sustained thereafter. This was again associated with increasing cell size and a skewing of the MLC to the proliferating cells. The proportions of CD69+ activated CD4+ and CD8+ T cells were roughly similar although at 120 hours and beyond, the proportion of activated CD4+ cells exceeded that of CD8+ cells. The level of activation in the CD56+ NK cell subset was always consistently more marked than in the T cell population, reaching a level of 17.0% at 72 hours, 30.2% at 96 hours and 35.1% at 120 hours.

Figure 3.3 Temporal expression of CD69 in lymphocytes after allogeneic stimulation

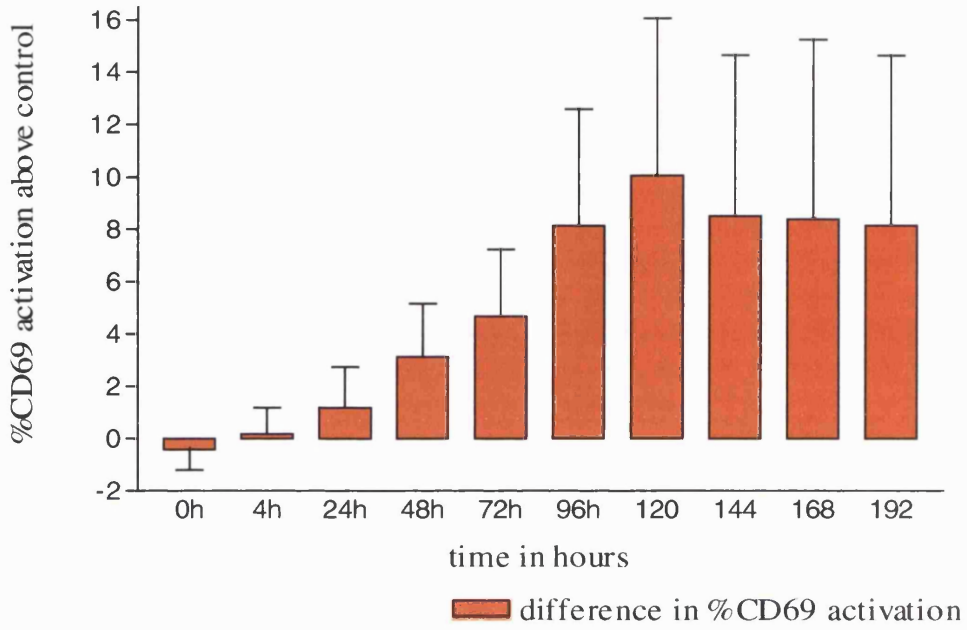
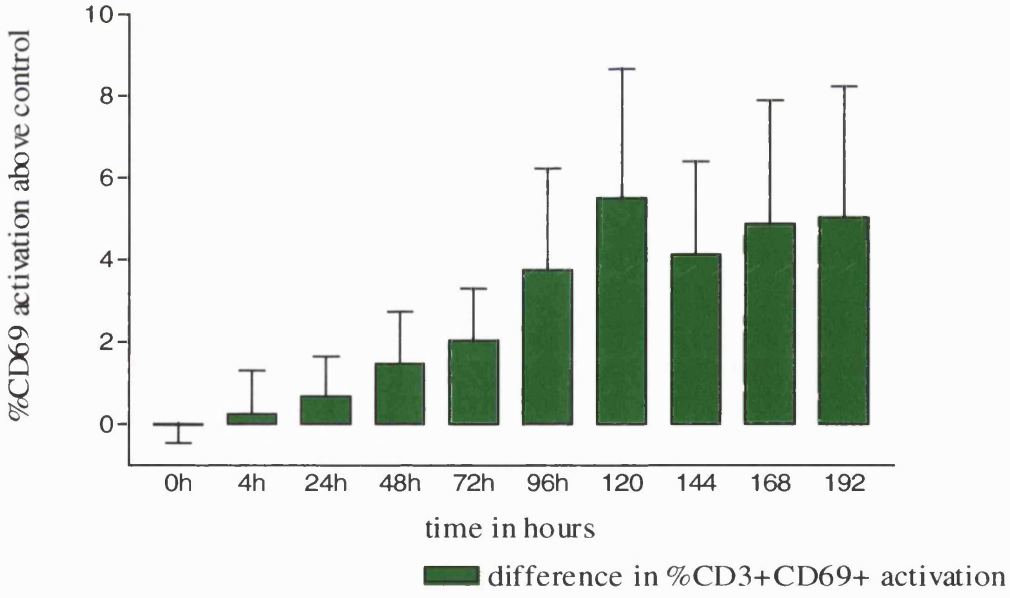
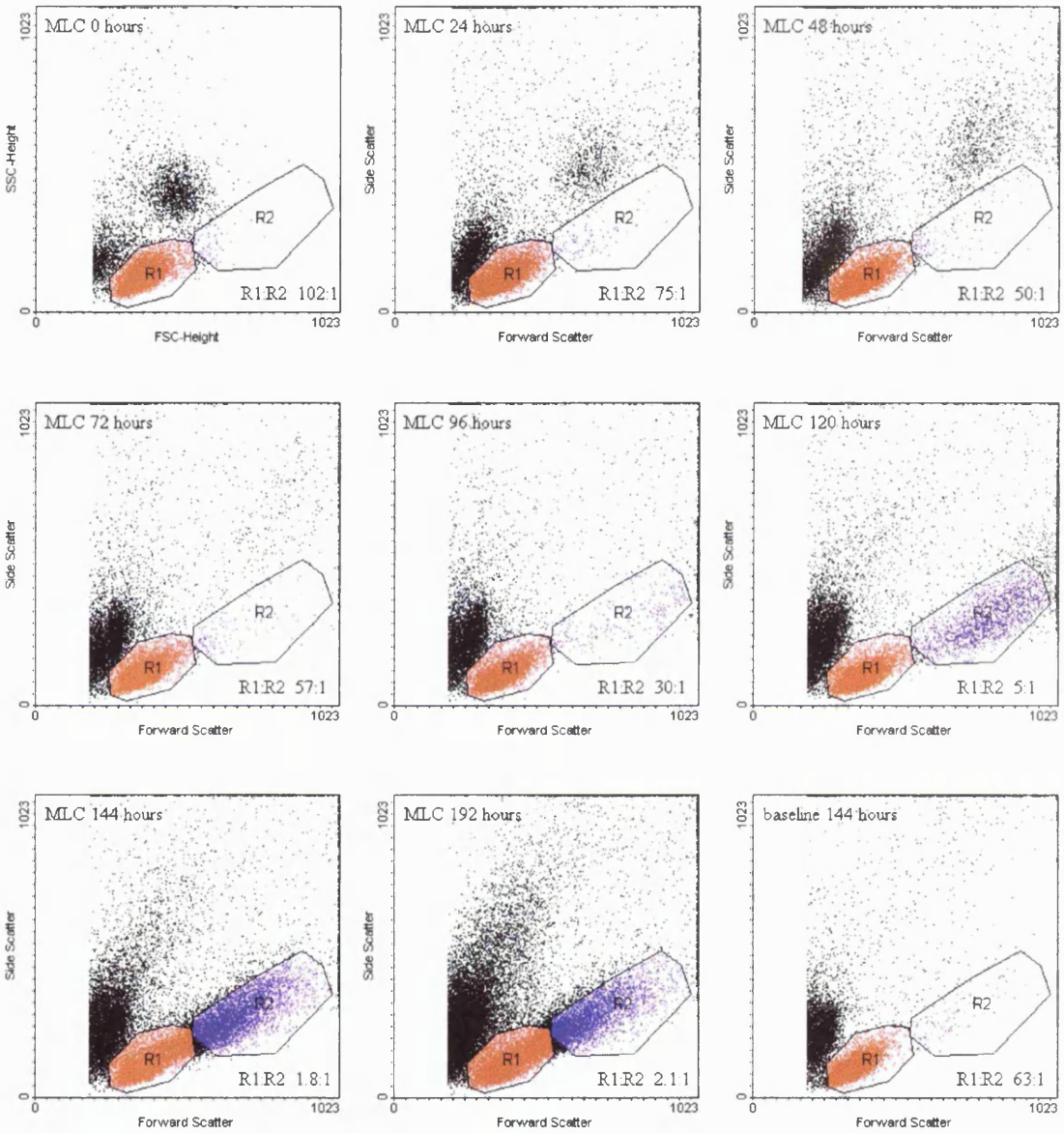


Figure 3.4 Temporal expression of CD69 in CD3+ cells after allogeneic stimulation



control cultures: responder cells alone or with autologous PKH labelled stimulators. %CD69 expression was calculated by determining the percentage of total responder cells in the lymphoid gate that were CD69+. For CD3+ cells, the %CD69 expression was defined as the proportion of CD3+CD69+ cells divided by the total proportion of CD3+ cells (CD3+CD69+ and CD3+CD69-). All data are expressed as mean $\pm$ s.d.

Figure 3.5 Skewing of the responder cell population over time in the MLC:



The flow cytometric dot plots with forward angle light scatter vs 90^0 light scatter shown in figure 3.5 document the changes in responder cell characteristics over time (0-192 hours) in the MLC. R1 or the "lymphocyte gate" denotes the area in which live, non-apoptotic responder PBMCs fall (red) while R2 or the "blastoid gate" denotes the area where live responder cells have increased in cell size and are proliferating (blue). Cells to the left of R1 are irradiated apoptotic stimulators (black). The R1:R2 ratio indicates the relative proportion of cells in the R1 gate compared to proliferating cells in the R2 gate and demonstrates the relative skewing of the responder cell population. This is contrasted against a dot-plot of a baseline autologous culture at 144 hours.

Figure 3.6 Temporal expression of CD69 in CD4/8 cells after allogeneic stimulation

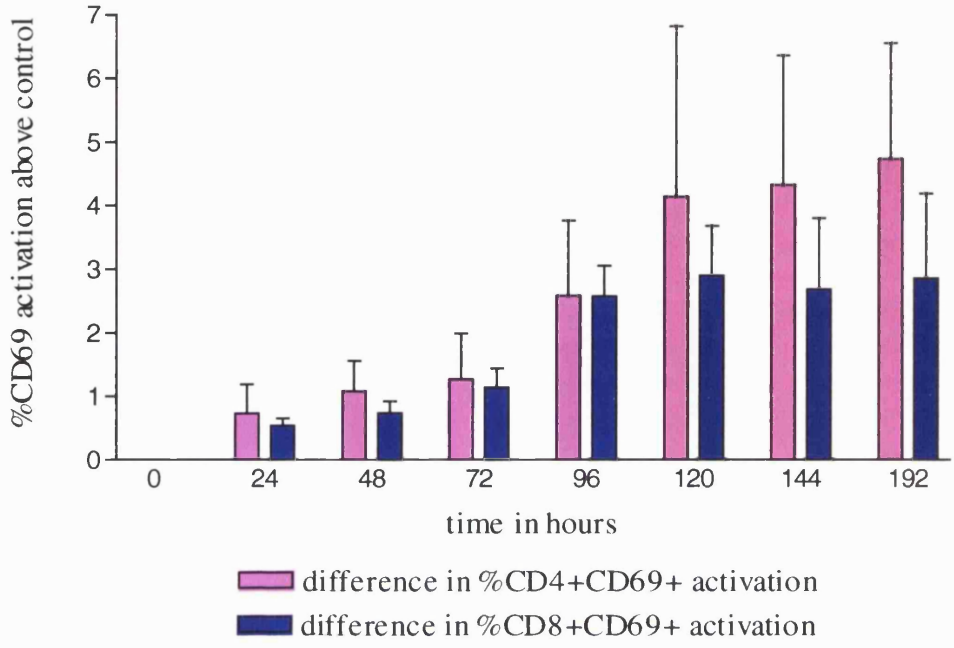
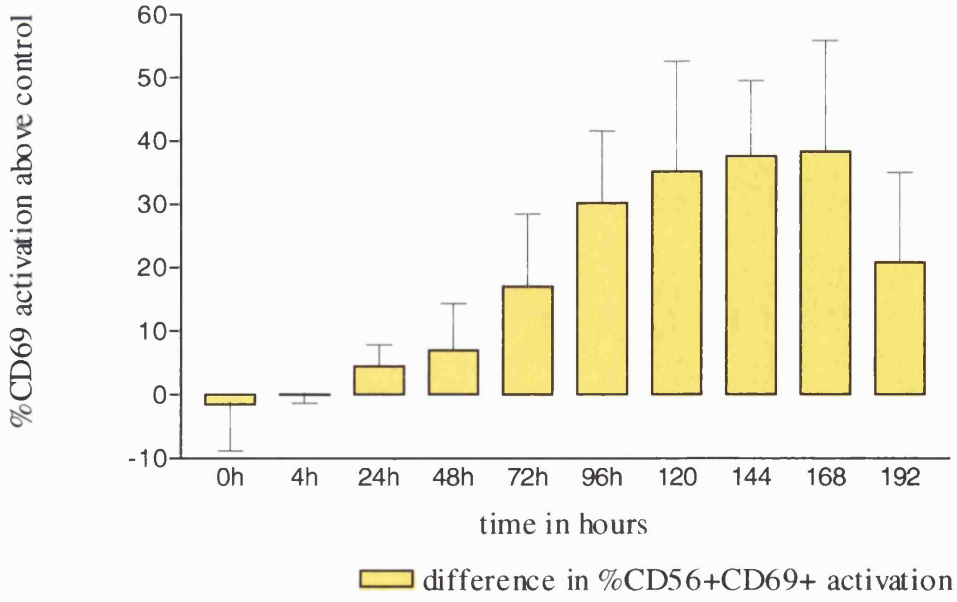


Figure 3.7 Temporal expression of CD69 in CD56+ cells after allogeneic stimulation



control cultures: responder cells alone or with autologous PKH labelled stimulators. For CD56+ cells, the %CD69 expression was defined as the proportion of CD56+CD69+ cells divided by the total proportion of CD56+ cells. The same was done for CD4+ and CD8+ cells. All data are expressed as mean $\pm$ s.d.

3.2.5 PKH26 does not alter the allostimulatory capacity of the MLC

To exclude the possibility that PKH26 labelling might directly affect CD69 expression, a time course experiment was performed directly comparing control responder cells alone or with PKH 26 labelled autologous stimulator cells. This showed no non specific increase in CD69 expression using the PKH26 labelled cells as compared to the control unlabelled responder population (data not shown).

It was also necessary to demonstrate that PKH26 labelling did not reduce the allostimulatory capacity of the allogeneic PBMCs. To do this, CD69 expression in a one way MLC with similar donor-stimulator pairs were compared using irradiated versus PKH26 labelled stimulators. At 72 hours and beyond, both irradiated and PKH labelled stimulators could be easily resolved from the responders and for the earlier time points, stimulator PBMCs were irradiated 48 hours earlier. No significant difference was found in the allostimulatory capacity between the two. In subsequent experiments using irradiated stimulators, the temporal dynamics of CD69 expression was also found to be broadly similar. This showed that PKH26 labelling did not affect CD69 activation in an MLC. It also confirmed that effectiveness of PKH labelling in allowing the accurate resolution of responder and stimulator PBMCs.

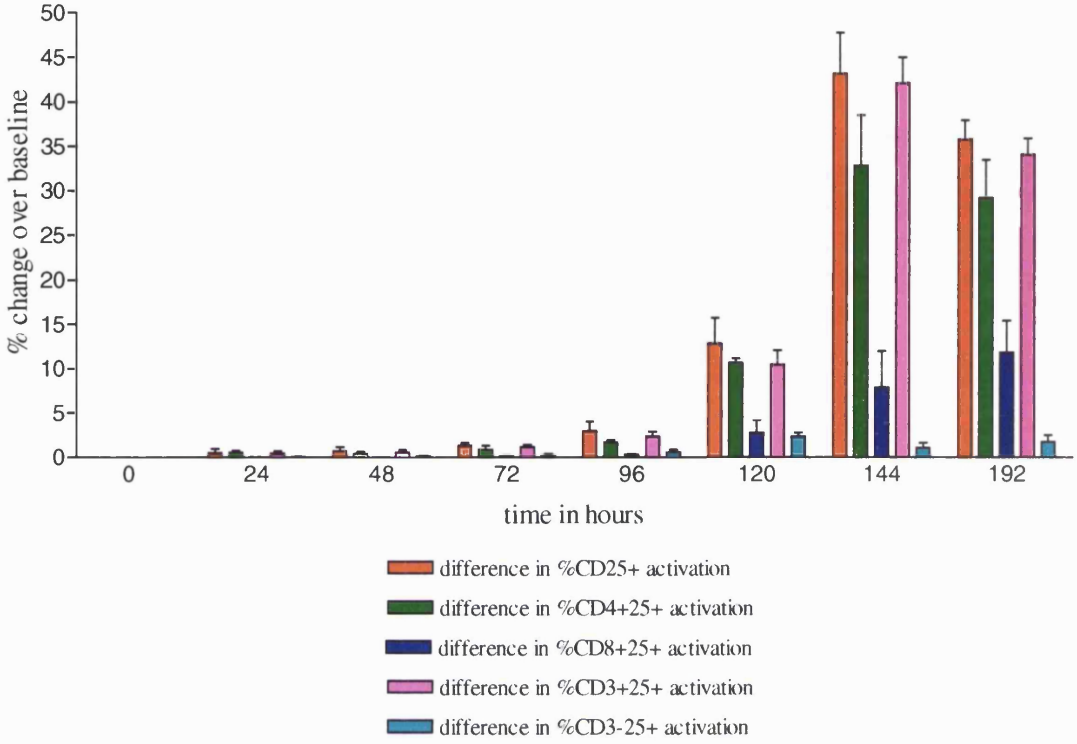
3.2.6 Temporal dynamics of activation marker CD25 in a one way MLC

Initial experiments performed with PKH labelled stimulators showed no CD25 expression above baseline control up to 48 hours. A time course study was therefore done using responders and MHC mismatched irradiated stimulators. The activation profiles of CD25, CD69, OX40 and HLA-DR were compared using the panel of antibodies described in material and methods. Figure 3.8 shows the expression of CD25 in various lymphocyte subsets after alloantigen stimulation. In contrast to CD69, there was a detectable but small upregulation of CD25 (1.3%) above baseline control only at 72 hours. There was a further small rise at 96 hours (mean CD25 expression above baseline of 3.0%). At later time points however (120 hours and beyond), there was a sharp rise in CD25 expression to a peak of 43.2% indicating sustained level of expression. This was associated with increasing cell size and a skewing of the MLC to the proliferating alloreactive cells. CD25 expression was restricted only to the CD3+ T cell subset with no expression on the CD3- lymphocytes including NK cells. Within the CD3+ T cells, this was largely a CD4+ response with only a small proportion of CD8+ cells expressing CD25 and only at very late time points (144 hours and beyond). CD25 was expressed at sufficient density to allow for clear distinction between the positive (CD25 expressing) and negative population on a dot-plot.

When cells were double stained for CD25-FITC and CD69-PE simultaneously, there were no double positive activated cells seen for the first 72 hours of the MLC. Only CD69 was expressed on activated cells. Significant numbers of CD25+CD69+ cells were seen (1.5% above baseline) only at 96 hours which corresponded with the temporal dynamics of CD25 expression. The proportion of double positive activated cells rose thereafter to a peak of 4.6% at 144 hours which was in keeping with maximal

expression of CD25 at this time point. This also indicated the stability of CD69 expression in that it was still expressed past peak levels. If only the "blastoid" or proliferating cells were analysed, at 72 hours, 80% of the cells were CD69+ but only 5% CD25+; at 96 hours, the proportion of CD25+ cells had increased to 54% while the CD69 expression remained stable. At 120 hours onwards, 70% of the cells were CD25+CD69+ and by 168 hours, nearly all proliferating cells expressed CD25 while CD69 expression was beginning to diminish (46%).

Figure 3.8 CD25 expression in lymphocytes with alloantigen stimulation



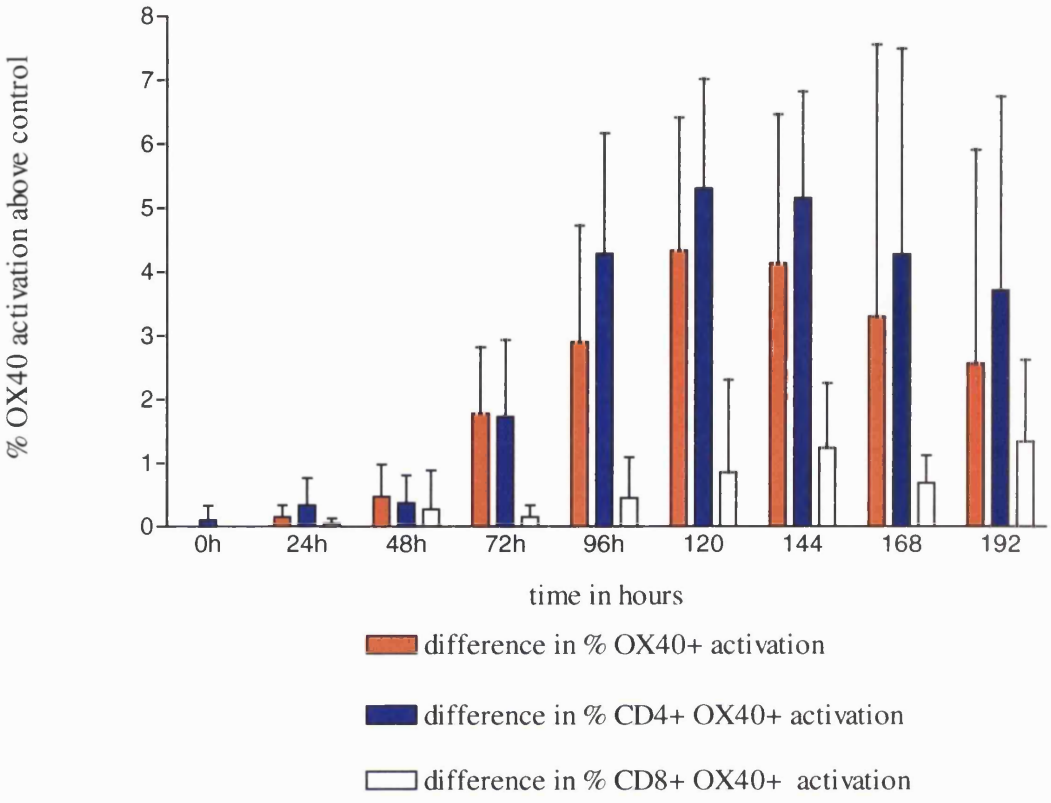
The percentage change of activation antigen CD25 above baseline autologous control (y-axis) was analysed against time in hours from the start of the one way MLC (x-axis). The percentage change was calculated at each time point using the following equation: (% of lymphocytes expressing the relevant activation marker in the MLC - % of lymphocytes expressing the same marker in the autologous control culture). The percentages were calculated at each time point using quadrant analysis of dot-plots. CD25 expression was also analysed for the following subsets: CD3+, CD4+, CD8+ and CD3- lymphocytes. Only lymphocytes which fell inside the lymphocyte gate were considered in the analysis. All bar columns in the figure are expressed as means + s.d.

3.2.7 Temporal dynamics of activation marker OX40 in a one way MLC

As anti-OX40 PE was only available as a PE-conjugate, labelling of stimulator cells with PKH26 in this case was not the appropriate choice. Activation involves the upregulation of only a small proportion of alloreactive cells and as PKH26 fluoresces in the high FL2 region, the possibility of a significant error in results could arise if any of the PKH26 labelled stimulators were erroneously included as an activated OX40PE+ cell. PKH2 labelling of stimulator PBMCs which fluoresces in the FL1 region was attempted but the resolution of FITC-labelled responder PBMCs with PKH2 labelled cells was poor. This was because unlike PKH26, there was a gradient of labelling with PKH2 and this overlapped with FITC positive cells. Increasing the concentration of the dye could not rectify this problem. F18 labelling, which also fluoresces in the FL1 region was attempted with no improvement in resolution. Irradiated 2-day-old stimulator cells were then used to allow for analysis of responder cells at 24 and 48 hour time points and freshly irradiated cells for time points thereafter.

Figure 3.9 shows the temporal dynamics of OX40 expression with allostimulation. The earliest time point when OX40 expression was first detected was at 72 hours (1.8%), similar to CD25 and in contrast to CD69 where expression was seen as early as 24 hours. Thereafter, there was a steady increase, reaching a peak at 120 to 144 hours (mean 4.4% above baseline). The decrease in levels thereafter signified that the expression of OX40 might not be sustained. OX40 was only seen on CD3+ T cells and not at all on CD3- lymphocytes including NK cells. Within the CD3+ T cell subset, nearly all cells expressing OX40 were CD4+ cells, with minimal expression on CD8+ cells (fig 3.9). OX40 was expressed at sufficient density to allow for clear distinction between the positive (OX40 expressing) and negative population on a dot-plot.

Figure 3.9 OX40 expression in T cells with alloantigen stimulation in an MLC

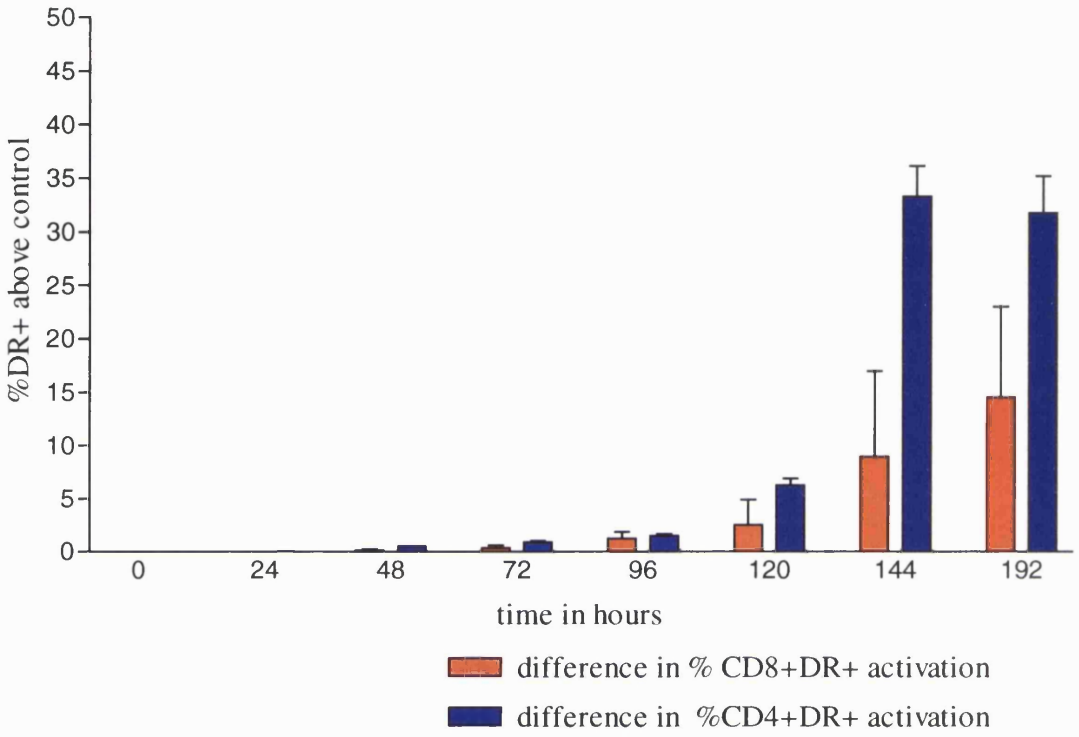


The percentage change of activation antigen OX40 above baseline autologous control (y-axis) was analysed against time in hours from the start of the one way MLC (x-axis). The percentage change was calculated at each time point using the following equation: (% of lymphocytes expressing the relevant activation marker in the MLC - % of lymphocytes expressing the same marker in the autologous control culture). The percentages were calculated at each time point using quadrant analysis of dot-plots. The % activation for each subset (e.g. CD4+) was defined as the proportion of CD4+OX40+ divided by the total proportion of CD4+ cells x100. OX40 expression was analysed for the following subsets: CD3+, CD4+ and CD4- lymphocytes. Only events which fell inside the FSC/SSC lymphocyte gate were considered in the analysis. All bar columns in the figure are expressed as means + standard deviation.

3.2.8 Temporal dynamics of activation marker HLA-DR in a one way MLC

The Class II MHC molecule HLA-DR is regarded as an activation marker on human T lymphocytes. It is constitutively expressed on antigen presenting cells, including B cells in baseline control cultures. The expression of HLA-DR was therefore analysed specifically with regards to CD4+ and CD8+ T cells. Compared to CD69, CD25 and OX40, HLA-DR was the last activation antigen to be expressed on the lymphocyte surface after alloantigen stimulation (Figure 3.10). Both CD4+ and CD8+ T cells exhibited similar tempos of activation with a small increase seen after 96 hours in the MLC (mean expression above baseline 1.5% for CD4+ and 1.3% for CD8+ lymphocytes). Thereafter, there is a progressive sharper rise in HLA-DR expressing CD4+ cells to a peak of 33.3% above baseline and a more gradual increase in the proportion of HLA-DR+CD8+ cells (peak 14.5%). There was no expression of HLA-DR on NK cells (data not shown). HLA-DR was expressed at sufficient density to allow for clear distinction between the positive (DR expressing) and negative population on a dot-plot.

Figure 3.10 HLA-DR expression in T cells with alloantigen stimulation in an MLC



The percentage change of activation antigen HLA-DR above baseline autologous control (y-axis) was analysed against time in hours from the start of the one way MLC (x-axis). The percentage change was calculated at each time point using the following equation: (% of lymphocytes expressing the relevant activation marker in the MLC - % of lymphocytes expressing the same marker in the autologous control culture). The percentages were calculated at each time point using quadrant analysis of dot-plots. DR expression was analysed for CD4+ and CD8+ lymphocytes. Only lymphocytes which fell inside the lymphocyte gate were considered in the analysis. All bar columns in the figure are expressed as means + standard deviation.

3.3 Discussion:

The results presented in this chapter describes the effect of alloantigen stimulation on the expression profile of various activation markers on the responding lymphocyte cell surface, namely CD69, CD25, OX40 and HLA-DR. The parameters analysed which would be of particular relevance to a selective depletion strategy and to the pathophysiology of GvHD were the time course of upregulation, peak levels and density of expression as well as the pattern of distribution among the various lymphocyte subsets.

All the activation antigens studied demonstrated selectivity of response in that expression remained low without an alloantigen stimulus while in the presence of allogeneic PBMCs in an MLC, there was a consistent upregulation of cell surface activation markers. This specificity was further confirmed when it was shown that the allogeneic response was not affected by either the FCS used in the culture or by the process of PKH26 labelling. All this suggested that it was possible to identify alloreactive responder cells by virtue of their expression of activation antigens. Of the four activation antigens studied, CD69 was the first to be expressed following alloantigen stimulation at 24 hours, followed by OX40 (72 hours) and CD25 (72 hours) while HLA-DR was the last activation antigen to be expressed. The time taken to peak levels of expression was also in that order, with CD69 peaking at 96-120 hours and HLA-DR at 192 hours. If alloreactive cells causing GvHD could be identified using these activation markers, the advantage of choosing CD69 over the others would be the rapidity of its expression and the ability to detect and isolate maximum numbers of alloreactive cells (peak level of expression) in the shortest time interval. This was an important factor bearing in mind that the aim of this work was the translation into a

clinical GvHD prevention strategy. Any procedure that involves the minimal time for manipulation or ex-vivo culture would be advantageous in terms of sterility and logistics involved. Moreover, it was noted that beyond 96 hours, there was a progressive skewing of the responder cell population as the alloreactive cells begin to divide and numbers increase disproportionately. In contrast, the non-alloreactive cell population which contains useful mature cells possibly involved in anti-viral and anti-leukaemia activity would most likely start to undergo apoptosis the longer they remain in ex-vivo culture without receiving any productive stimulus. This skewing of the responder cell population was evidenced by the fact that at 144 hours, over 40% of the cells were activated (CD25+ and HLA-DR+) and within the increasing "blastoid" gate, nearly all were activated. Therefore, it would be important if these alloreactive cells could be identified before considerable skewing of the responder population occurs at 120 hours and beyond. Only CD69 and OX40 were expressed at appreciable levels by 96 hours.

All four activation markers were expressed in sufficient density for clear separation and identification using flow cytometry. As such, this was not a factor in deciding the optimal marker to use although it has been reported that CD69 expression exhibits a higher fluorescence intensity compared to CD25 (Cebrian et al. 1988). With regards to the spectrum of cellular distribution, only CD69 was expressed on CD4+, CD8+ T cells and NK cells which have all been implicated in the pathogenesis of GvHD. CD25 was also expressed on both CD4+ and CD8+ T cells but there was no evidence of expression on NK cells in the MLC system that was used. In contrast, OX40 was found to have a restricted cellular distribution, confined mainly to CD4+ T cells alone and minimal expression on CD8+ cells which is consistent with other reports in the literature (Tittle et al. 1997). HLA-DR was found to have a wider range of distribution

but the problem remains that HLA-DR is constitutively expressed on B cells, monocytes and dendritic cells. Any selective depletion strategy based on the removal of HLA-DR expressing cells would also remove those cells in addition to activated T cells from the graft. This might have profoundly detrimental effects on post-transplant immune reconstitution.

For all those reasons mentioned above, CD69 was selected as the most suitable marker for the allodepletion strategy. Although the lymphocyte response in terms of CD69 expression to potent stimuli like PMA and PHA have been well studied (Craston et al. 1997), (Caruso et al. 1997), there have been only a few reports regarding the upregulation of CD69 in response to an alloantigen stimulus. Potent stimuli like PHA induce a non-specific response from the majority (60-90%) of T cells while the data presented in this chapter show that with alloantigens, only a minority of T cells express CD69. This small proportion of cells in the MLC (%) that activated in response to the alloantigen was in keeping with previous estimates of the frequency of alloreactive T cells (1-5%)(Detours and Perelson, 1999), (Sherman and Chattopadhyay 1993). The dynamics of the CD69 alloantigen response was also markedly different from the mitogen responses of both T and NK cells where expression of CD69 was seen as early as 1 hour after stimulation and peaked 4-24 hours later. In all cases CD69 expression in response to alloantigen stimulation was delayed with upregulation first seen only at 24 hours. The results of the early time points (24-72 hours) were corroborated by one group (Fehse et al. 2000) while another study of CD69 expression in response to alloantigen stimulation by irradiated cells detected expression above baseline but the earliest time point studied was 60 hours post initiation of the MLC and the response peaked at 108-156 h with 21% of the cells were CD69+, comparable to the time point of maximal response in this study (Hara et al. 1986). One other group reported

different dynamics of CD69 expression with a peak response observed at 48 hours. However PBMC sonicates were used rather than whole stimulator cells and this could possibly alter the time frame of alloreactive recognition and activation (Leiva et al. 1997). Paglieroni used monocytes as stimulators (1:1 responder:stimulator ratio) but examined only the CD4 response (Paglieroni et al. 1999). A response was first seen at 12 hours, a peak expression at 24h with 2-21% of CD4+CD3+ cells CD69+, and tapering off at 96h. On the other hand, one report has suggested that the use of CD69 as a method of assessing lymphocyte activation may only be restricted to potent stimuli with no increase seen in response to alloantigen (Simms & Ellis 1996). However, the same group failed to detect a CD69 response to tetanus toxoid in contrast to the findings of at least two others groups (Caruso et al. 1997), (Mardiney et al. 1996). It may be that in some culture systems failure to detect a CD69 response to specific stimuli is due to high levels of background cell activation, presumably in response to cytokines within the foetal calf serum. It is possible that such background activation may screen the low level alloantigen-specific activation and explain the negative findings. The experimental systems described in this chapter ensured that the batch of FCS used did not affect CD69 expression non-specifically.

Another advantage in using CD69 was its stability of expression. Although upregulated early after allostimulation, sustained expression was observed at least up to 144 hours. Double staining showing simultaneous expression of CD25 and CD69 at 144 hours confirmed the finding that CD69 continues to be stably expressed on the cell surface to overlap with the later expression of CD25.

The results noted that a higher proportion of NK cells responded to the alloantigen stimulus than T cells. This was interpreted to be a reflection of the high incidence of

mismatches between the responder-stimulator pair at the appropriate HLA-C and HLA-B loci involved in NK cell regulation (Colonna & Samaridis 1995). This observation supports the concept that NK cells are involved in graft rejection and graft-versus-host disease after allogeneic bone marrow transplantation (Murphy et al. 1993).

This chapter has also described a novel method of establishing a one-way MLC to circumvent the issue of responders and stimulators falling into the same lymphoid gate and making the enumeration of alloreactive cells difficult. This method of using PKH26 to label stimulator PBMCs is especially useful when examining early lymphocyte responses. This problem was tackled in a different way by (Leiva et al. 1997) who used PBMC sonicates rather than whole cells. The problem here was that it was found that only freshly used sonicates were able to stimulate an allogeneic reaction and this capacity was lost if cells were cryopreserved. This would therefore not be applicable in a clinical allodepletion strategy as recipient and donor samples may not necessarily be obtained at the same time.

The baseline expression of CD69 in autologous cultures was 2.18% and this was supported by the findings of other groups ($0.8\pm0.4\%$) (Cebrian et al. 1988), (Paglieroni et al. 1999). As CD69 is expressed at low levels on resting lymphocytes, the percentage of cells that were induced to express CD69 after an alloreactive stimulus was therefore used a measure of the strength of the response. The other parameter that may be important is the density of expression as measured by fluorescence intensity. From the responder-stimulator pairs used, there appeared to be no appreciable difference in fluorescence intensity from one pair to another but clear distinction could be seen between CD69 positive and negative cells. In normal donors, the density of expression to pokeweed mitogen (PWM) was also found to be broadly similar (Maino

et al. 1995) and would suggest that the use of percentage positive values was a valid index of activation with samples from normal donors.

Standard in vitro methods for assessing T cell activation have typically measured either the proliferative responses of PBMC cultures to various provocative stimuli employing tritiated thymidine incorporation or the secretion of specific cytokines. However, these bulk assay methods suffer the drawback of being lengthy assays and in addition, they do not provide information about functional responses of individual lymphocyte subsets. The development of intracellular cytokine staining has allowed the visualisation of individual cytokine secreting cells. The detection of cell surface activation antigens is another.

It has been demonstrated that the expression of CD69 on multi-parametric flow cytometry at 4 hours to a potent stimulus like the comitogenic monoclonal antibodies CD2/CD2R mirrored closely the dose-response patterns observed with lymphocyte proliferation as measured by thymidine incorporation (Maino, Suni, & Ruitenberg 1995). Mardiney has also found agreement between percentage of cells expressing CD69 and proliferation (Mardiney et al. 1996), as did Lamb LS (Lamb, Jr. et al. 1997) while others report (Caruso et al. 1997) overall agreement between the two in distinguishing a positive from a negative response but no actual correlation between percentages and the amount of ^{3}H-thymidine incorporation. In addition, it has been (Paglieroni et al. 1999) found that not only did CD69 expression compare favourably with thymidine uptake but also with intracellular cytokine production to an alloantigen stimulus. Multi-parameter flow cytometry employing FITC-labelled antibodies to specific Vβ T cell receptor antigens found that CD69 expression in response to SEB was consistent with the expected specificities of Vβ6+ subsets reported for SEB

(Kappler et al. 1989). This suggested that CD69 expression on activated T cell subsets also demonstrate specificity at the level of the T cell receptor. The induction of CD69 also strictly correlated with the extent of CD3/TCR cross-linking (Testi et al. 1988).

All these findings imply that CD69 expression is antigen-specific and part of the integral set of events beginning with TCR engagement and leading to proliferation and cytokine secretion: for a cell to proliferate, it needs to be activated first (Testi et al. 1989b), (Lanier et al. 1988). The converse however may not hold true in that not all activated cells would finally proceed to proliferation. PMA alone for example induces all cells to express CD69 but does not stimulate significant DNA synthesis in the absence of calcium ionophore. Moreover, if the amount of stimulus was lowered, CD69 upregulation still occurred but in the absence of proliferation (Caruso et al. 1997). Nonetheless, within the first hours of activation, a number of committed differentiation pathways consequent to T cell activation become evident (cytokine expression, apoptosis, anergy) before DNA synthesis and cell division occur (Germain & Stefanova 1999). Factors that influence this T cell activation response as measured by CD69 expression include the type of stimulus, accessory cell function, avidity of the TCR and the amount of costimulatory and adhesion molecules present (Maino et al. 1995).

CD69 has been used as an informative marker in various disease states. Expression of CD69 has been used to assess the anti CD3 induced proliferative response and predict functionality of the lymphocytes in HIV infected patients (Nielsen et al. 1998), (Prince & Lape-Nixon 1997). Eosinophils isolated from the bronchoalveolar lavage of asthma patients showed abnormally high CD69 cell surface expression compared to normal individuals (Hartnell et al. 1993). Endomyocardium infiltrating CD8+ T cells in heart

allograft rejections selectively express CD69 (Santamaria et al. 1992). CD69 is also expressed by CD8+ infiltrating cells in the livers of patients with chronic active hepatitis (Garcia-Monzon et al. 1990). It has been used as a marker to monitor autoimmune disease like systemic lupus erythematosus activity (Su et al. 1997) and rheumatoid arthritis (Isomaki et al. 1997). Other investigators have also used it to assess T cell function post bone marrow transplantation (Lamb, Jr. et al. 1997). Therefore, the identification of cell surface structures like CD69, minimally expressed on resting PBMCs but broadly upregulated on activated lymphocytes following stimulation would enhance the potential for detection of low frequency responses like alloreactive cell populations.

4. Chapter 4. Allodepletion with CD69: in vitro studies

4.1 Introduction:

The results presented in the previous chapter showed that alloantigen-activated lymphocytes could be reliably identified by expression of various activation markers on the cell surface. In a comparative analysis and for reasons already detailed, CD69 was determined to be the most appropriate activation antigen to use. The work presented in this chapter was aimed at engineering an allodepletion strategy that would effectively remove these activated alloreactive cells by virtue of their CD69 expression while retaining a pool of functional non-alloreactive lymphocytes. The mixed lymphocyte culture (MLC) is the in-vitro correlate of the clinical situation where mature donor lymphocytes recognise the recipient cells as foreign and initiate a sequence of events leading to the GvH reaction. This MLC system has already been used in the work presented in the previous chapter and would form the basis for the new strategy. Initial feasibility studies were done with unrelated HLA- mismatched pairs and extended into the matched setting. The other crucial issues that were addressed were the method of depletion employed, how effective this depletion was and ways of assessing the specificity of the depletion as well as the retention of functional responses.

The crucial role of cytokine cascades in the pathogenesis of GvHD is well accepted and another aim was to investigate the importance of these cytokines and possibly to incorporate them into the allodepletion strategy.

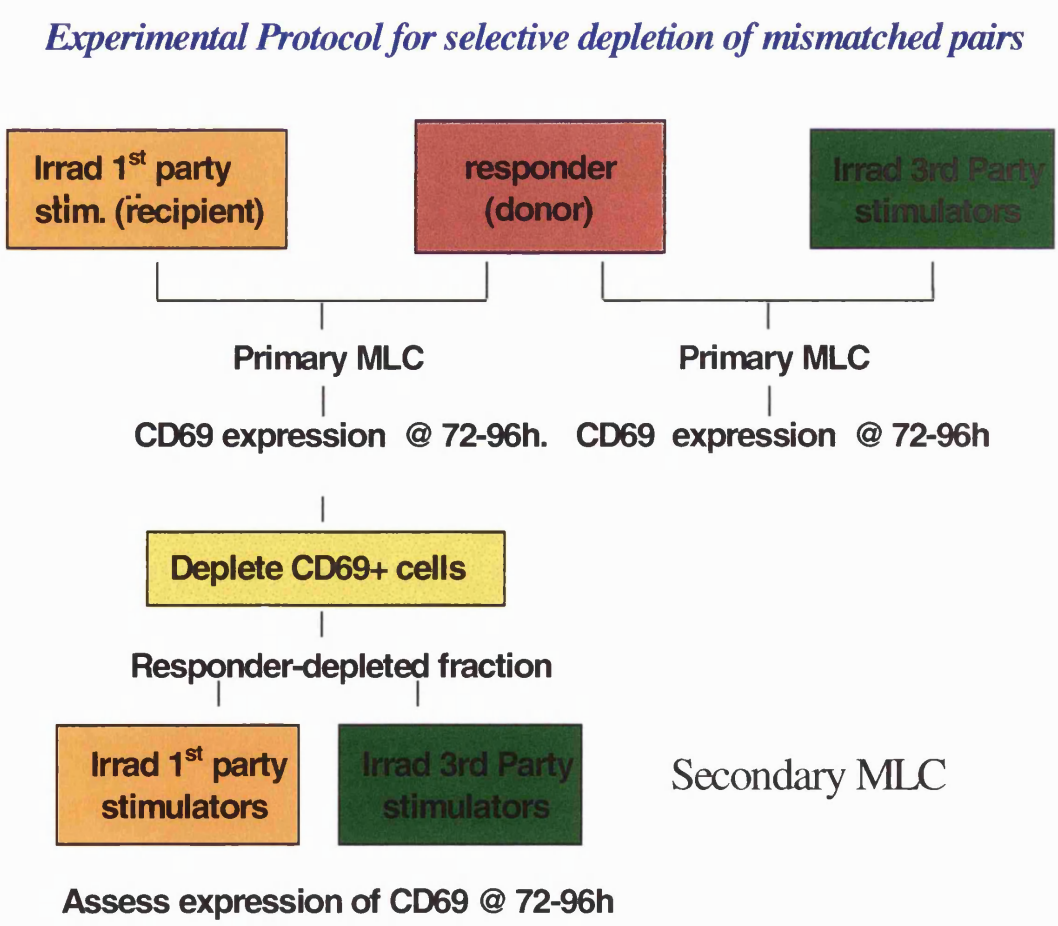
4.2 Results of selective depletion in HLA-mismatched individuals:

4.2.1 Setting up the protocol for the HLA-mismatched study:

Using the MLC as the basis, Figure 4.1 illustrates the protocol that was devised for the selective depletion of alloreactive cells from mismatched pairs. In each case, PBMCs from three unrelated normal donors were used; one termed the responder population, one the 1st party stimulator population and one as the3rd party stimulator to assess specificity of alloreactive cell removal. Responder PBMCs were mixed in an equal ratio with irradiated 1st party stimulator cells in a one way 'primary mixed lymphocyte culture (MLC) '. In a parallel culture, responder cells were also mixed in an equal ratio with an irradiated 3rd party population. All cells were resuspended to a density of 10^6 cells/ml and cultured in tissue grade plastics (Nunclon, Nunc, Denmark). The controls for each experiment were non stimulated responder cells alone. All stimulator cells were irradiated for a total of 33 Gy just prior to mixing. The 'primary MLCs' were incubated at 37^0C / 5% CO_2 and samples analysed at 72-96 hours for CD69 expression in the responder population. Using analysis by flow cytometry (FACScan with Lysis II software-Becton Dickinson, Oxford, UK), responder lymphocytes and lymphoblasts were easily identified by live gating on FSC and SSC signals. By 72 hours, the irradiated stimulator cells had moved out of this lymphoid gate due to apoptosis and could be easily separated from the responder population as already demonstrated in the previous chapter. CD69 expression was assessed for the responder population in both the 1st party primary MLC' and the 3rd party primary MLC'. Alloreactive cells in the 1st party primary MLC' could be distinguished from non alloreactive cells by the expression of CD69. These CD69+ve cells in the 1st party primary MLC were then depleted using a MACS 'AS' depletion column'. The CD69 depleted responder

PBMCs were then re-introduced with equal numbers of 1st party stimulator PBMCs in a 1st party secondary MLC'. The depleted fraction was also mixed in a parallel culture with equal numbers of the same 3rd party PBMCs in a 3rd party secondary MLC'. CD69 expression of the responder population in both secondary MLCs were then assessed at the same time point prior to sorting as in the primary MLC

Figure 4.1: Experimental protocol for selective depletion of mismatched pairs:



A 1:1 ratio of recipient (stimulator):donor (responder) mononuclear cells was used to achieve maximal alloantigen stimulation. This is the normal ratio adopted in an MLC system which has been used as the in-vitro correlate of the GvH reaction. A higher stimulator:responder like 2:1 was not considered as it may not be practically possible to obtain large volumes of blood from patients if the strategy were to be clinically applicable. The optimal time for removal was 72 to 96 hours for three reasons:

i) expression of CD69 at these time points was near peak levels,

ii) it was noticed from the alloantigen time course studies that after 96 hours in culture, the donor lymphocyte profile was beginning to be skewed. This is not surprising as the stimulated alloreactive cells would begin to proliferate while the non-alloreactive cells including NK cells would gradually undergo apoptosis in culture due to lack of stimulation,

iii) if this strategy were to translate into a clinical procedure, an earlier time-point for removal of alloreactive cells, if equally effective would be more attractive than a later time point in terms of logistics and clinical safety.

4.2.2 CD69 expression in HLA-mismatched pairs in 1^0 and 2^0 MLC:

Table 4.1 and 4.2 show the increase in CD69 expression above baseline in both 1^0 and 2^0 MLCs at 72-96 hours. This was derived from analysing FACScan dot-plots the percentage of total responder cells in the lymphoid gate that are CD69 positive. Baseline control cultures were responder cells with irradiated autologous stimulators. There was less than 5% CD69 expression in all control cultures, with a median of 1.4% (range 0.8-4.2%). There was a mean increase of 5% in CD69 expression above baseline in the MLC for both 1^{st} and 3^{rd} party stimulators which is in keeping with the estimate (1-5%) of the frequency of T cells that are alloreactive. Responder cells

alloreactive against the 1st party stimulator were identified by upregulation of CD69. These 1st party alloreactive cells were then selectively removed by MACs sorting and the residual response to both 1st and 3rd party assessed in 2^0 MLCs. Following the depletion strategy, the increase in CD69 expression for the 1st party stimulator MLC fell from 6 ± 2.4% to 1.5 ± 1.2% while that that of the 3rd party MLC was well maintained at 3.9 ± 2.7% in the 1^0 MLC and 4 ± 2.4% in the 2^0 MLC.

4.2.3 Selective depletion in HLA-mismatched pairs:

To calculate the degree of selective depletion, the primary response for both 1st party stimulator and 3rd party was normalised to 100% and the residual response expressed as a percentage. The mean residual response post CD69 depletion was 25.6% ±17.9. The response to 3rd party stimulator cells was retained (mean of 115.7% ± 35.9) (figure 4.2). The median cell recovery was 73% (range 65-75%) with an associated median purity of 97% (range 96-98%). The depletion efficiency was 75% (range 65-84%).

4.2.4 Two different methods of immunomagnetic separation:

The MACs method of separation was directly compared against that of Dynal beads. The same mismatched responder and stimulator pair was used for each procedure. A representative experiment is illustrated by the dot plots in figure 4.3a-d. It can be seen that the pre-depletion CD69 expression in the MLC fell from 11 to 1.2% using the MACs separation device and 11.9 to 2.3% with the corresponding Dynal bead sort. In terms of depletion efficiency, the MACs method was superior (89.1%) compared to the Dynal device (80.5%). Cell purity was also superior with MACs sorting although cell recovery was comparable. The MACs method was therefore chosen over Dynal beads.

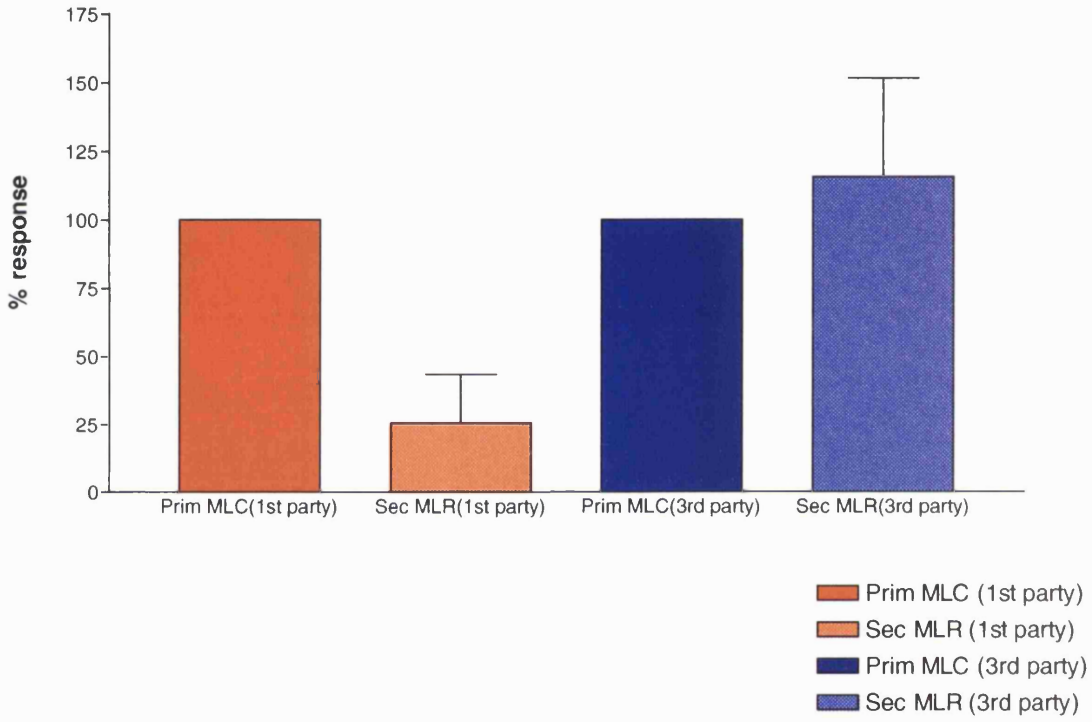
Table 4.1: CD69 expression (% of total responder MNC fraction) in primary MLC

Donor-Recipient Pair	CD69 expression above baseline for 1st party stimulator primary MLC	CD69 expression above baseline for 3rd party stimulator primary MLC
1	6.1	7.2
2	9	1.5
3	8.2	1.4
4	4.9	3
5	2.9	6.1
Mean ± SD	**6.2 ± 2.4**	**3.9± 2.6**

Table 4.2:Residual CD69 expression (% of total responder MNC fraction) in 2^0 MLC

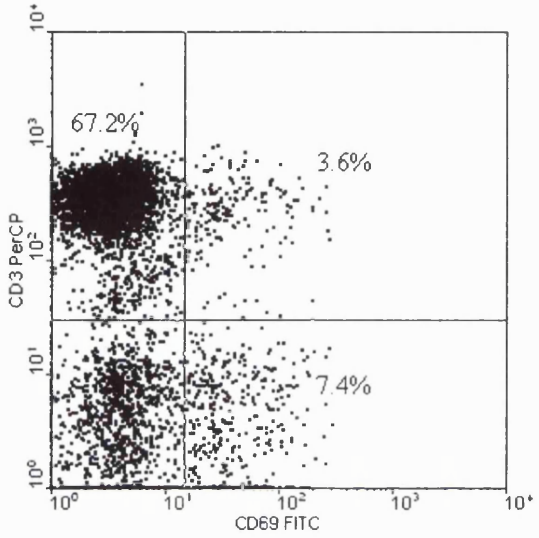
Donor-Recipient Pair	Residual CD69 expression above baseline post depletion for 1st party stimulator secondary MLC	Residual CD69 expression above baseline post depletion for 3rd party stimulator secondary MLC
1	2.5	7.7
2	0	1.5
3	2.7	2.3
4	1.9	4.1
5	0.4	4.3
Mean ± SD	**1.5 ± 1.2**	**4.0 ± 2.4**

Figure 4.2: Residual alloantigen response post depletion in HLA-mismatched pairs

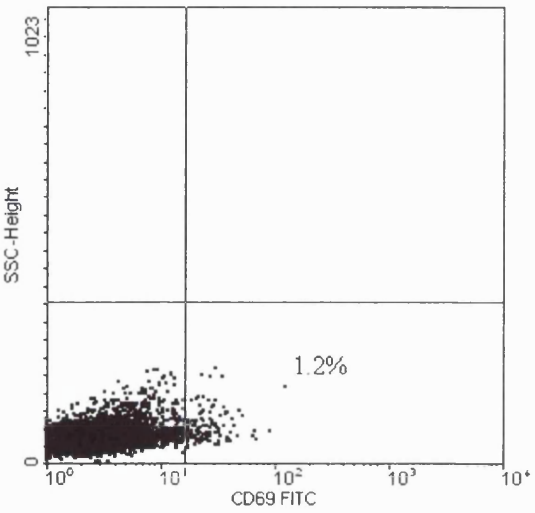


Results are expressed as mean ($\pm$ s.d.) percentage of the original response prior to selective depletion of CD69+ responder cells.

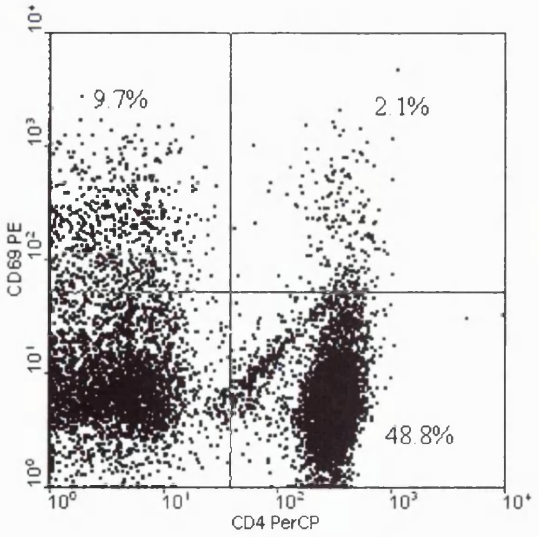
Figure 4.3 Comparison of MACs versus Dynal bead sorting for CD69+ cells



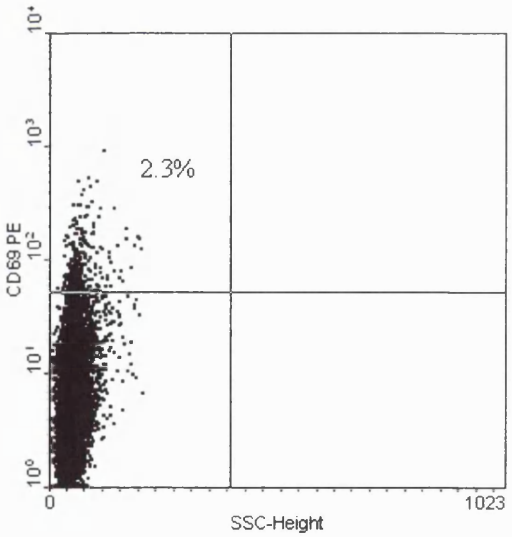
a) Pre MACs sorting



b) Post MACs sorting



c) Pre Dynal bead sorting

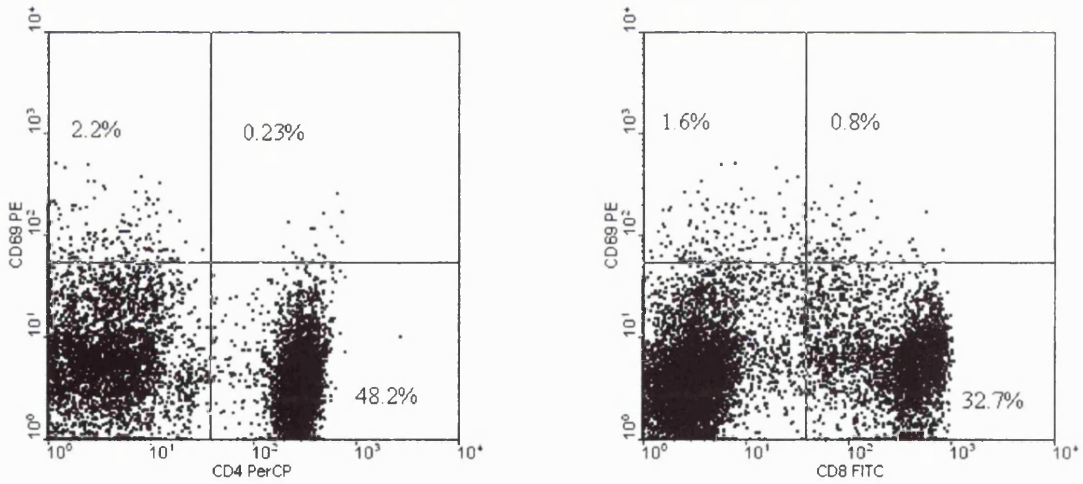


d) Post Dynal bead sorting

4.2.5 Depletion of CD69+ alloreactive cells preserves both CD4 and CD8 subsets

Figure 4.4a and 4.4b illustrates that post allodepletion of CD69+ cells, the depleted fraction retains substantial numbers of both CD4+ and CD8+ T cell subsets. This demonstrated that the strategy did not only target one subset and is in keeping with the fact that both CD4 and CD8 positive T cells are likely to be involved in the induction of GvHD. Immunophenotyping has also demonstrated that the alloreactive CD69+ fraction contained both CD4+CD69+ and CD8+CD69+ lymphocytes.

Figure 4.4 The allodepleted graft contains both CD4+ and CD8+ T cells



a) *b)*

Figure 4.4a shows cells stained with CD69PE and CD4PerCP demonstrating that 48.2% of the allodepleted cells are CD4+CD69-. Figure 4.4b shows cells stained with CD69PE and CD8 FITC demonstrating that 32.7% of the allodepleted cells are CD8+CD69-.

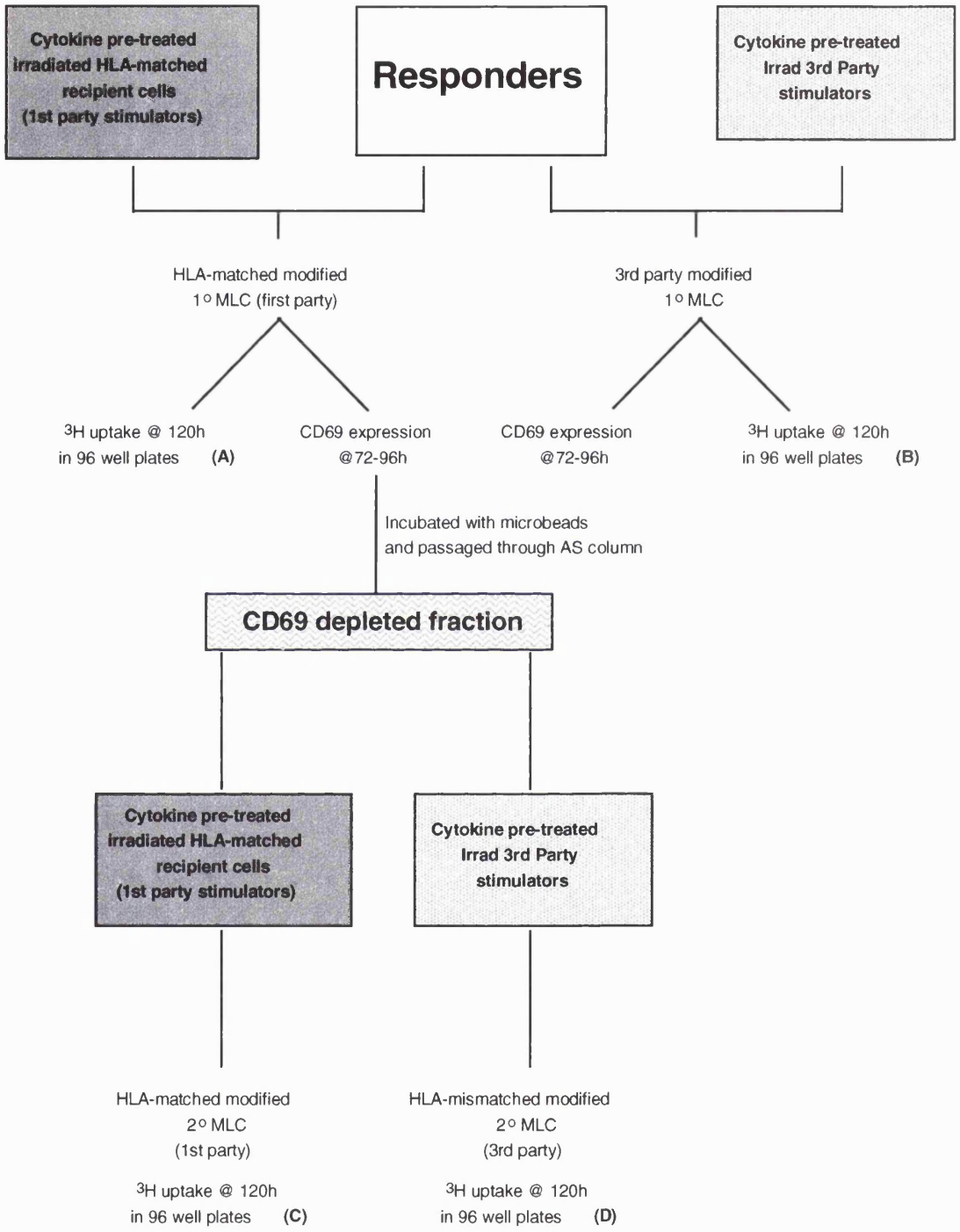
4.3 Results of selective depletion in HLA-matched individuals:

4.3.1 Setting up the protocol for the HLA-matched study:

When conventional MLCs were initially set up with HLA matched responder and stimulator pairs, no increase in CD69 expression or proliferation above baseline was seen. This is due, at least in part, to the failure of the conventional MLC to detect mHag and some MHC Class II mismatches. Although widely used in the past in HLA typing, it is too insensitive to be an accurate indicator of alloreactivity and GvHD in HLA matched pairs (Mickelson et al. 1993). Preliminary work demonstrated no significant upregulation of CD69 above baseline in an MLC using matched pairs. As most clinical allogeneic transplants are still done in the matched sibling or unrelated setting, the experimental protocol developed so far would not be feasible as it crucially relied on an increase in CD69 above autologous control in the MLC. Figure 4.5 illustrates the modified protocol that has been devised for the selective depletion of alloreactive cells from HLA-matched related and unrelated pairs. The major revision was the introduction of cytokines into the depletion strategy as detailed in section 2.2.5.1. Otherwise, the structure and rationale of the procedure remained similar to that described for mismatched study in section 4.2.1 with the patient being the 1st party stimulator and the matched donor (either sibling or unrelated) being the responder.

The importance of the cytokine cascade in the pathogenesis of GvHD has been highlighted. This cytokine based modified MLC was first described by Bishara et al (Bishara et al. 1999) in being able to detect fine antigenic disparities between HLA-identical siblings. It was found to be an accurate predictive tool in correlating reactivity as detected in the modified MLC (mMLC) with the incidence of GvHD.

Figure 4.5 Flow diagram for the experimental protocol in HLA-matched pairs



How the cytokine pre-treatment of stimulator cells allowed the detection of fine antigenic disparities including mHags is further addressed in the next chapter. The original modified MLC assay used as its readout the ^{3}H-thymidine proliferation assay in distinguishing a positive response from a negative one. That has also been incorporated into the protocol by comparing the ^{3}H-thymidine proliferation in the unmanipulated donor cells and the depleted fraction. Proliferation was therefore measured for both the primary matched and 3rd party MLCs. After removal of alloreactive cells via the "AS" column, proliferation assays were again set up for the depleted fraction in the secondary HLA-matched and 3rd party MLC and compared as described in section 2.2.5.2.

4.3.2 Modified MLC:

Following cytokine pre-treatment of stimulator PBMCs, proliferative responses were detected in 5 out of 9 HLA matched pairs tested. This was evidenced in both matched sibling and unrelated pairs. Table 4.3 shows the ^{3}H-thymidine incorporation by each of the 9 donor-recipient pairs in the primary MLC. All stimulator cells were cytokine pre treated in the same way i.e. cytokine pre treated, washed to remove cytokines after 24h, irradiated and added to donor (responder) PBMCs in similar ratios. To control for any possible non-specific effect from the cytokines and to ensure that the washing step (to remove γ-IFN / TNF-α) was thorough, the background ^{3}H-thymidine incorporation in the autologous control setting i.e. donor PBMCs with cytokine pre-treated autologous PBMCs was assessed. As detailed in table 4.3, the addition of cytokines to the modified MLC did not cause a significant increase in the autologous MLC compared to the responder cells incubated alone ($p>0.1$). In contrast, the majority of MLC of the matched donor-recipient pairs did generate a proliferative response above

that of the autologous control. The trend in all 9 experiments for the MLC response was: 3rd party MLC> matched donor-recipient MLC> autologous MLC> responder alone. This is in keeping with the greater degree of HLA disparity between donor and 3rd party as compared to the matched recipient. When the modified MLC was developed as a predictive tool for clinical GvHD, the relative response index (RRI) was used to calculate if the modified MLC response in the matched pair was positive compared to the autologous control (Bishara et al. 1999). Normalising the response against 3rd party as 100%, the RRI was calculated as follows:

$$\text{RRI (\%)}: \quad \frac{\text{(matched MLC cpm)-(autologous MLC cpm)}}{\text{(3rd party MLC cpm)-(autologous MLC cpm)}} \times 100$$

A relative response index >5% was considered positive. Table 4.3 shows the RRI calculated for each experiment.

4.3.3 Effect of cytokines on CD69 expression after HLA-matched MLC:

We have previously established the expression of CD69 in an allogeneic setting and confirmed this in our mismatched study. Initial work done with matched pairs without the use of cytokines showed no upregulation of CD69 expression above autologous control. Like the proliferation seen in the modified MLC, adding cytokines to the system did cause upregulation of CD69 expression in the matched pairs. The difference was that there was an upregulation of CD69 in all pairs tested compared to 5 out of 9 for the proliferative assay as shown in table 4.4.

Table 4.3: ^{3}H-thymidine incorporation in cytokine based primary modified MLCs

(mean counts per minute; n=3)

Donor - recipient pair	Donor alone	Donor cells + autologous stimulators	Donor cells + HLA-matched recipient 1st party cells	Donor cells + HLA-mismatched 3rd party cells	Relative response index (RRI) +ve if >5
1	37199	28744	60444	74490	71-positive
2	8019	13027	25823	60175	26-positive
3	8943	12590	19695	76925	11-positive
4	16370	17152	18152	63594	2.1-negative
5	6356	4690	3159	30139	0-negative
6	891	N.A.	857	N.A.	0-negative
7	25189	29626	36171	71324	12-positive
8	N.A.	1980	17071	31410	51-positive
9	9849	6375	7084	46403	2.6-negative
t-test		**p=0.98**	**p=0.04**	**p=<0.0001**	

N.A.: Not assessed

The upregulation above autologous control was significant for matched pairs and highly significant for the 3rd party MLC ($p<0.0001$) while there was no non-specific proliferation seen from the cytokines ($p=0.98$)

The mean baseline CD69 activation of the autologous control at 72 to 96 hours was 5.0 ±2.5%, that of matched pairs 8.9±3% and 3rd party was 10.1 ± 4.1%. The mean increase in CD69 expression above autologous control for matched pairs was 3.8% and that of 3rd party controls was 5.1% above baseline. This increase in CD69 expression for mismatched 3rd party using cytokines compared well with the data for the mismatched study. Pre-treatment of stimulator cells with γ-IFN and TNF-α and addition of low dose IL-2 did not non-specifically increase the baseline CD69 activation of the autologous controls. In contrast, an appreciable CD69 response with matched siblings was generated above the baseline controls. Similar to the ^{3}H-thymidine incorporation, the trend with CD69 expression was as follows:

3rd party>matched donor-recipient pairs> autologous controls> donor alone.

The main difference however was that unlike the ^{3}H-thymidine uptake where the 3rd party MLCs were much higher than the matched pairs, the CD69 expression in matched pairs approached or equalled that of 3rd party and in 3 pairs, was higher. This could be explained by the data in the next chapter which shows that in some cases, the pre-treatment of cytokines allowed increased expression of MHC, costimulatory and adhesion molecules important in allorecognition. This would then allow for the unmasking of previously undetectable fine antigenic disparities including mHag disparities resulting in an upregulation of CD69 that was comparable to mismatched pairs.

Table 4.4: CD69 expression in primary MLCs expressed as (% total of responder MNC fraction)

Donor-recipient pairs	Donor cells alone	Donor cells + autologous stimulator cells	Donor cells + HLA-matched recipient cells	Donor cells + HLA-mismatched 3rd party cells
1	5.4	5.9	11	10
2	4.6	2.1	7.2	10.3
3	3.6	2.8	6.9	8.9
4	2.2	4.4	10	8.1
5	3.4	4.8	6.2	6.2
6	2.5	2.5	6.1	6.2
7	N.A.	10	11	19.4
8	6.7	6.7	15	13
9	N.A.	6.1	6.7	8.8
mean ± s.d.	**4.1 ± 1.6**	**5.0 ± 2.5**	**8.9 ± 3**	**10.1 ± 4.1**
t-test		**p=0.85**	**p=0.0017**	**p=0.0004**

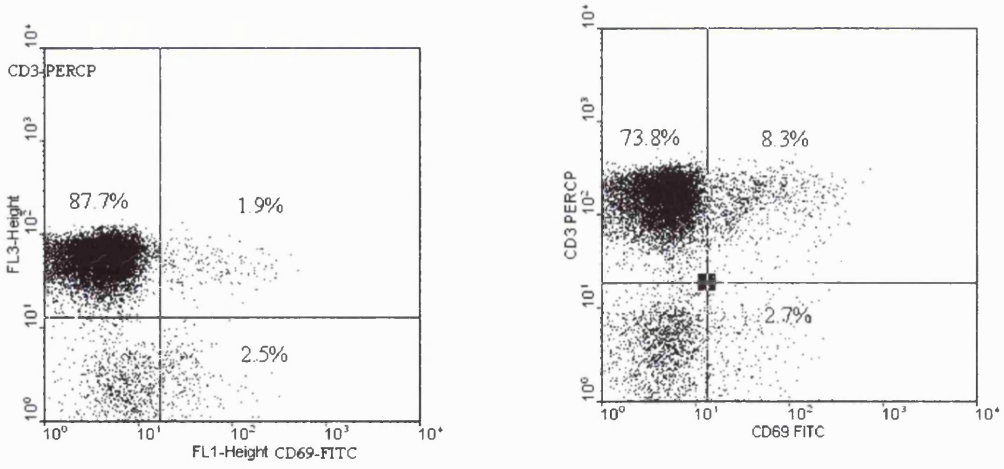
N.A.: not assessed

As in the mismatched study, CD69 expression was calculated by determining the percentage of total responder cells in the lymphoid gate that are CD69 positive. The upregulation above autologous control for both matched and 3rd party MLCs were highly significant as evidenced by the p values while there was no non-specific upregulation from the cytokines (p=0.85)

4.3.4 PHA blasts as stimulators:

The possibility of using irradiated recipient PHA blasts as stimulators in an MLC was initially attempted to maximise the alloantigenic capacity. Moreover, PHA blasts are used in cytotoxicity assays including the CTLp assay. However, it was evident (Fig 4.6) that despite repeated washings, sufficient PHA (a potent mitogen) remained bound on the stimulator cell surface to induce marked non-specific CD69 expression even in the autologous control cultures (donor PBMCs plus irradiated autologous PHA blasts). It was thus not feasible to use PHA blasts as stimulators in the depletion strategy.

Figure 4.6 Non specific upregulation of CD69 by PHA in autologous controls



a) **b)**

Figure 4.6a shows the result of incubating PBMCs with autologous irradiated blasts and 4.6b PBMCs with autologous irradiated PHA blasts. It can be seen that using PHA. results in a non-specific upregulation of CD69 from 4.4 to 11%. Both dot-plots show CD69 FITC on the *x-axis* and CD3-PerCp on the *y-axis.*

4.3.5 Depletion efficiency, cellular recovery and cell purity:

Table 4.5 shows the results of magnetic cell sorting using the 'AS' depletion columns. This gave a median depletion efficiency of 80.3% (range 62-98.6%). The cell purity was consistently more than 94% (range 94.3-99.8%). As the columns used were designed for depletion strategies, not surprisingly, the enrichment process was less stringent ranging from 30-68% CD69 positive cells in the alloreactive enriched fraction. The median cell recovery was calculated as described in materials and methods and was 75% with a range of 46-85%.

4.3.6 Selective depletion of alloreactive cells in matched pairs:

In 5 out of 9 experimental pairs (1, 2, 3, 7 and 8), a positive proliferative result was obtained in the modified MLC. It was therefore possible to compare the ^{3}H-thymidine incorporation of the PBMCs before and after depletion. In the other 4 experiments however, the degree of selective depletion of alloreactive cells could not be assessed since no proliferative response was generated in the primary MLC. Nevertheless, in all cases, we were able to determine whether the 3rd party response had been preserved despite the removal of CD69 positive cells after allostimulation. Table 4.6 shows the ^{3}H-thymidine incorporation of the 5 evaluable pairs post depletion. The residual response was calculated as described in Materials and Methods. A mean depletion of proliferative capacity to 11.5 $\pm$ 9.9% of the original 1st party response was achieved. (figure 4.7). The RRI (Table 4.6) of the depleted fraction was <5% in each instance which is below the threshold reported to be predictive of clinical GvHD.

Table 4.5: CD69 expression in fractions pre- and post-sorting

(% of total responder MNC- depletion efficiency, cell recovery and purity

Donor-recipient pairs	Pre-sort %CD69	Depleted fraction post-sort: %CD69	Enriched fraction post-sort: %CD69	Depletion efficiency (%)
1	11	0.2	68	98
2	7.2	1.2	41	83
3	6.9	1.8	45	74
4	10	2.2	50	78
5	6.2	1.1	63	82.3
6	6.1	1.2	N.A.	80.3
7	11	1.5	79	86.4
8	15	5.7	N.A.	62
9	6.7	1.5	30	77.6

N.A.: Not assessed

Table 4.6: ^{3}H-thymidine incorporation in secondary MLCs after removal of CD69+ cells

(mean counts per minute; n=3)

Donor - recipient pair	Donor CD69 depleted cells alone	Donor CD69 depl. cells + autologous stimulators	Donor CD69 depl. cells + HLA-matched recipient stimulators	Donor CD69 depl. cells + HLA-mismatched 3rd party stimulators	Residual response (%)	RRI
1	49524	37726	39774	89544	6.5	3.9
2	1827	3464	5825	55921	18.5	4.5
3	16194	21058	21170	82987	1.6	0.2
7	3300	8806	10455	42304	25.2	4.9
8	N.A.	707	1569	22485	5.7	4.0

N.A.: Not assessed

depl: depleted

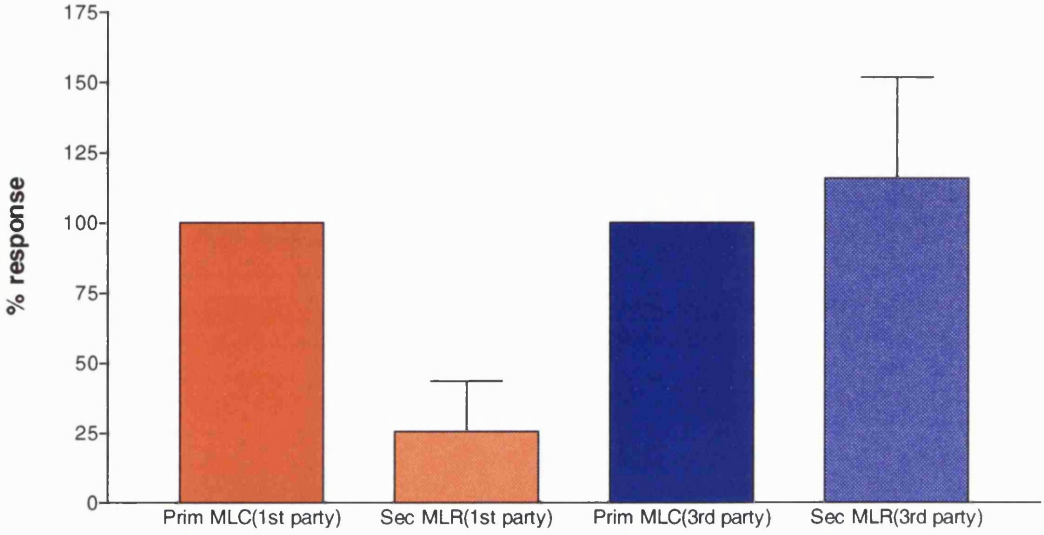
4.3.7 Preservation of 3rd party response:

The residual 3rd party response was assessed to determine if the depletion of alloreactive CD69 positive cells was alloantigen specific. This showed a mean preservation of proliferative response of 77.8% ± 20.9% as illustrated in Figure 4.7.

4.3.8 Association with clinical GvHD:

Table 4.7 shows the relationship between the results of the modified MLC and the incidence of clinical GvHD in the recipients who have undergone an allogeneic transplant from their HLA matched donor. All sibling transplants (6 patients) were non T-cell depleted (TCD) and recipients were given either cyclosporin or cyclosporin and methotrexate as GvHD prophylaxis. 2 patients who had matched unrelated transplants received Campath 1-M ex-vivo TCD marrows for GvHD prophylaxis with no additional immunosuppressive therapy. Both also received Campath 1-G prior to transplant as part of the conditioning treatment. The 9th donor-recipient pair did not proceed on to a transplant. A RRI >5%, indicating a positive modified MLC was associated with subsequent clinical GvHD in 3 of 4 evaluable patients. Only 1 of the 4 patients receiving a bone marrow from a donor showing a RRI <5% (i.e. "negative") showed evidence of clinical GvHD post transplant that was responsive to steroids. The median follow-up period of these patients was 12 months (range 8-13 months).

Figure 4.7 Residual alloantigen proliferative response to HLA identical stimulator cells in the modified MLC after removal of CD69+ responder cells .



Results are expressed as mean + (S.D.) percentage of the original response prior to depletion.

Table 4.7 Use of the cytokine based modified MLC to predict clinical GvHD

Positive if RRI >5%	Clinical GvHD
positive	grade I
positive	grade III
positive	grade II
negative	No GvHD
negative	No GvHD
negative	No GvHD
positive	No GvHD
positive	Not evaluable
negative	grade II

4.4 Discussion:

The work in this chapter describes a selective T cell depletion strategy that preferentially removes cells recognising alloantigens by in vitro stimulation of donor lymphocytes with irradiated recipient PBMCs and the subsequent selection of activated cells on the basis of CD69 expression. This was initially performed using completely mismatched pairs and the procedure successfully transposed to HLA-matched individuals with the addition of a cytokine based modified MLC. By removing the CD69+ cells using a MACS column, it was possible to deplete approximately 90% of the alloreactive component while maintaining >70% of the residual immunity as measured by a 3rd party alloantigen response. Significantly, as the alloreactive component consisted of less than 10% of the total lymphocyte pool, this meant that about 90% of the original lymphocytes were retained and infused with the graft. Although graft rejection is now less of a major problem in view of improved conditioning regimens and infused higher stem cell doses, the increased lymphocyte content of the graft could further improve haematopoietic engraftment rates post transplant. Immune reconstitution might be enhanced since this has been shown to be directly proportional to the number of T cells in the infused graft (Lowdell et al. 1998). The diversity of the regenerating T cell repertoire would also improved (Roux et al. 1996). It has also been shown that T cells of donor origin, devoid of anti-recipient activity, may contribute to engraftment either by the production of cytokines that promote haematopoiesis or by the exertion of a veto effect on host CTLs (Lapidot et al. 1990), (Rammensee 1988). This method may exploit antigen differences on normal tissues and leukaemia cells in that donor responses to recipient MHC and ubiquitous mHag are eliminated while conserving responses to lineage restricted or even

leukaemia specific antigens. This would translate into a graft mediated anti-leukaemia activity.

This selective depletion strategy contrasts with a non-selective pan T cell depletion method which does not discriminate between useful and GvHD inducing lymphocytes. It is also a step ahead of depletion techniques which removes T cell subsets such as CD8 depletion as it has convincingly been demonstrated that both CD4+ and CD8+ cells can induce GvHD; its relative importance probably dependent on disease and type of transplant as discussed in section 1.2.3. The CD69 depletion strategy removes both activated CD4+ and CD8+ T cells as well as activated NK cells.

It is well established that in an HLA- matched setting, a conventional MLC is non reactive and unable to detect minor antigenic differences that exist. mHags are the main source of disparity between HLA matched siblings and contribute a significant part to the antigenic disparity between HLA-matched but unrelated pairs. Bishara et al (Bishara et al. 1999) have demonstrated that pre-treatment of stimulator cells with cytokines and the subsequent addition of cytokines to the MLC can detect fine antigenic disparities that exist between HLA-identical siblings. The data presented here supported this. In 5 out of 9 pairs (3 sibling, 2 unrelated), a positive MLC was generated while 4 (3 sibling, 1 unrelated) remained negative despite the addition of cytokines. Furthermore, the results of the modified MLC were, in general, predictive of the incidence of clinical GvHD following transplantation. Although numbers are small, this does imply some clinical association and a negative modified MLC may predict absence of GvHD. Another valid issue is the level of depletion necessary to achieve a threshold below that for clinical GvHD which has been estimated at $<1x10^5$ T cells/kg for pan TCD grafts (Lowenberg et al. 1986). This figure would probably not

be applicable for a selective TCD graft but it is encouraging that this method of alloreactive cell removal has depleted the response to below the predictive threshold for clinical GvHD (RRI <5%).

In terms of CD69 expression, the addition of cytokines did not induce any non-specific increase above the baseline controls. The matched pairs and 3rd party MLCs showed a significant increase in CD69 expression above background. Interestingly, the CD69 expression in the modified (with cytokines) 3rd party MLCs and autologous controls were no higher than in the simple 3rd party and control MLCs (without cytokines) in the mismatched pair study. What the cytokines did was to enhance the response mainly in the matched pairs, some almost to the same degree as in the 3rd party pairs. These data did contrast with the ^{3}H-thymidine incorporation data in the MLCs where the 3rd party MLCs was consistently and considerably higher than the matched pairs. One must however remember that CD69 is a marker for activation and is not equivalent to the in vitro assessment of proliferation. The addition of cytokines, by amplifying minor antigenic differences might be sufficient to trigger a CD69 response, almost equivalent to a 3rd party response in some instances.

4.4.1 Other all-depletion strategies:

Variations of this allodepletion strategy have been attempted by other groups, with differences in the activation antigen used, type of stimulator cell employed, method of depletion, the in vitro or in vivo readout for depletion and the time frame involved (Fehse et al. 2000), (Mavroudis et al. 1996), (Harris et al. 1999), (Garderet et al. 1999), (Mavroudis et al. 1998), (van Dijk et al. 1999), (Datta et al. 1994), (Rencher et al. 1996), (Cavazzana-Calvo et al. 1994), (Cavazzana-Calvo et al. 1990). The reasons why

CD69 was chosen over CD25 were already discussed in the previous chapter but CD25 alone has been selected by some groups, most commonly with the use of immunotoxins conjugated with a monoclonal antibody against CD25 (Cavazzana-Calvo et al. 1994), (Datta et al. 1994), (Mavroudis et al. 1998), (Harris et al. 1999). This were either ricin, pseudomonas or diphtheria toxin based. The level of specific depletion (7.6±1.4%) achieved with the pseudomonas based immunotoxin was comparable to the CD69 strategy although retention of 3rd party reactivity (64.2±5.0%) was not as well preserved (Mavroudis et al. 1996). Impressive results were obtained with the ricin based toxin (6% specific depletion and 92.7% 3rd party retention) (Cavazzana-Calvo et al. 1990) but this was not tested in an HLA-matched setting. The use of immunotoxins however, results in the inevitable loss of the activated alloreactive cells. Cellular recovery may be poor and it is difficult to estimate the actual level of depletion achieved. It also requires the manufacture and the approval of the immunotoxins for clinical use. The risk of non-specific toxicity may be a potential problem. The removal of CD25+ alloreactive cells by immunomagnetic separation seemed more attractive in view of the potential disadvantages of immunotoxins (Garderet et al. 1999), and an efficient selective depletion of anti-host reactivity was achieved. This however has again been achieved only in MHC-mismatched pairs and not in the matched setting. Another potential useful activation marker is OX40. The association of OX40 and GvHD has been highlighted and in EAE, selective depletion of OX40+ cells with a ricin conjugated immunotoxin was successful in ameliorating the disease although this same strategy has not been applied in the prevention of GvHD. However, restriction of its expression to CD4+ cells would severely reduce its applicability to GvHD prophylaxis. Other activation markers like HLA-DR and CD71 have not been used singly in allodepletion strategies and only in combination with CD25 and CD69. Fluorescent activated cell (FACS) sorting has been used to eliminate activated

alloreactive cells and this was done based on both cell size and a combination of activation markers (CD25, CD69 and HLA-DR). It is however a slow process and designed for positive rather than depletion selection. Cell recovery was also very poor, estimated at no more than 10% (Rencher et al. 1996). A combined strategy of removal of CD25+ and CD69+ cells by immunomagnetic separation was attempted with good results (75% depletion of the alloreactive response removed) (Fehse et al. 2000). Though this was only tested in mismatched pairs, its rationale and approach was complementary to that described in this chapter and would be discussed at greater length in chapter 8.

In terms of the stimulator cell population, recipient PBMCs have been uniformly used except in three instances. Recipient keratinocytes were chosen by one group (van Dijk et al. 1999) with the rationale being that they represented one of the theoretical key target tissues in GvHD. However, it has been shown that keratinocytes lack expression of costimulatory molecules important for efficient antigen presentation and hence the strategy also required the prior transfection of keratinocytes with B7.1 or B7.2. This meant having to obtain biopsies from patients pre-transplant and transfecting them with B7.1 or B7.2. The other stimulator cell population used was patient derived B lymphoblastoid cell lines (BLCL) in view of their high level of MHC antigen expression (Rencher et al. 1996). The prime concern envisaged with this approach however remains the issue of safety in using an EBV transformed cell line for clinical use. Moreover, it is a labour intensive and time consuming process. Retention of anti-EBV reactivity, one of the main aims of this strategy may also be lost due to the use of an EBV transformed cell as stimulator. PHA blasts have been used as stimulators for a CD25 immunotoxin strategy (Datta et al. 1994) but would seem unsuitable as any membrane bound PHA would non-specifically upregulate CD69 and CD25.

72-96 hours was chosen to be the optimal time for separation and this was in agreement with other groups using immunomagnetic separation with a combination of activation markers including CD69. 48 hours was the time point chosen by Fehse for the combined immunomagnetic separation of CD25 and CD69 but it was acknowledged from the kinetics of antigen expression that later time points (72 and 96 hours) for separation might be indicated (Fehse et al. 2000). If the depletion was delayed to 120 hours, the alloantigen specific depletion was found to be noticeably reduced (van Dijk et al. 1999). With immunotoxins conjugated to anti-CD25, depletion was performed at earlier time points (48-72 hours). The temporal dynamics of CD25 expression would suggest that this would not be the optimal time for depletion although it has been argued that the use of immunotoxins might be able to target very low expressing CD25+ cells. A 1:1 stimulator/responder (S/R) ratio was uniformly used and it was shown that with a S/R ratio of 1:2, specific depletion was incomplete while with a S/R ratio of 2:1, 3rd party response was less well preserved (Mavroudis et al. 1996).

This allodepletion strategy has largely been successful in mismatched or haploidentical pairs but the difficulty remains to translate this work into matched donor-recipient pairs. This has been reported by 2 other groups-one using keratinocytes as stimulator cells followed by depletion of the alloreactive lymphocytes with immunomagnetic separation and the other using PBMCs in a simple MLC with an immunotoxin for removal (Mavroudis et al. 1998), (van Dijk et al. 1999). The latter method was a little surprising in that a simple MLC involving matched pairs should result in no upregulation of CD25. The level of CD25 expression was not documented but the likeliest explanation remains that perhaps immunotoxins are able to target very low expressing CD25+ cells.

The cytokine based modified MLC was used by us as the in-vitro test of alloreactivity. Other assays used have included HTLp and CTLp (Mavroudis et al. 1998), (van Dijk et al. 1999). All have variously been shown to be predictive for GvHD. A recent study has highlighted the reliability and superiority of the skin explant test over the HTLp and CTLp assays and like the cytokine based modified MLC, the skin explant model is linked with the crucial role of cytokines in the pathogenesis of GvHD (Dickinson et al. 1998). Perhaps, this assay could be performed in parallel with the cytokine based modified MLC as further corroboration of our findings.

Preservation of 3rd party reactivity has been used as a surrogate marker for the retention of anti-viral and anti-leukaemic specificity. Specific anti-leukaemic activity has been measured indirectly with the retention of reactivity to patient PMBC containing leukaemic blasts (van Dijk et al. 1999), by limiting dilution assays (LDAs) (Montagna et al. 1999) and by the conservation of myeloid-specific anti leukaemic reactivity due to the restricted expression of some mHags when using lymphocytes as stimulators (Mavroudis et al. 1998). Retention of anti-viral activity against was demonstrated by showing that the allodepleted cells were able to proliferate against candida and CMV antigens. The same group extended this study further by measuring CTLp frequencies against CMV and EBV infected target cells (Valteau-Couanet et al. 1993), (Montagna et al. 1999). It remains crucial to ascertain if the allodepleted product contains anti CMV and EBV-specific lymphocytes and this forms the basis for the results described in chapter 6.

Attempts have been made to retrovirally insertion a suicide gene into a donor/recipient cell coculture (Herpes simplex type 1 thymidine kinase gene) together with ganciclovir. This is based on the finding that activated alloreactive cells are dividing and the

administration of ganciclovir would preferentially target these cells leading to an arrest of DNA synthesis and cell death. (Bonini et al. 1997), (Mavilio et al. 1994). This strategy however, requires that all alloreactive cells incorporate the retroviral vector.

Allodepletion with α-CD69 has several advantages over the protocols discussed above:

i) CD69 is upregulated early and on all cells of relevance in GvHD

ii) the MACS columns enable a good median depletion efficiency to be achieved (80.3%) as well as good cellular recovery. The depleted fraction demonstrated good cell purity (range 94-99.8%). The superiority in terms of depletion efficiency of the MACS separation columns over Dynal bead has been demonstrated and corroborated by another group (van Dijk et al. 1999)

iii) the use of immunomagnetic bead columns is already in clinical use for CD34 selection as a sterile procedure

iv) it is easy, reproducible and the degree of depletion can be reliably quantified unlike immunotoxin.

v) the alloreactive cells are easily recoverable and can be cryopreserved for ready immediate access. This would be useful if taken into the clinical arena as DLI if the patient relapses. This could be of particular value in the treatment of relapsed acute leukaemias, especially if donor availability and recall is a problem

vi) crucially, the protocol was effective in both mismatched and matched pairs.

5. Chapter 5. Cytokines and their effects on allorecognition by lymphocytes.

5.1 Introduction:

Dysregulation of complex cytokine networks is an essential component in the pathophysiology of the GvH reaction and disease. In view of this pivotal role, the use of cytokines was incorporated into the allodepletion strategy as described in chapter 4. This was in the form of the modified mixed lymphocyte reaction (mMLC), based on the principle of the simple MLC and first described by Bishara (Bishara et al. 1999). As shown in chapter 4, the value of this predictive test for GvHD was corroborated in a series of 9 HLA matched pairs. The other in vitro predictive test that has been demonstrated to be more sensitive and reliable than either the CTLp or HTLp assay is the skin explant model (Sviland & Dickinson 1999), (Dickinson et al. 1998) and that too is crucially dependent on the use of cytokines in the assay system.

It is known that much of the function of lymphocytes is mediated by a host of secreted cytokines; less work has been done on the effect of cytokines on antigen presentation and lymphocyte interaction in an allogeneic setting. The mMLC has demonstrated that allorecognition is enhanced in the presence of cytokines by unmasking previously undetectable fine antigenic disparities that may exist between HLA matched related and unrelated pairs including mHag disparities. The aim of the work in this chapter was to

try to elucidate how cytokines might affect this process of alloreactive cell recognition. Certain cytokines, TNF-α and γ-IFN being examples, can induce or upregulate the expression of cell surface molecules on target tissues such as fibroblasts and vascular endothelial cells. These include MHC, costimulatory and adhesion molecules. The hypothesis postulated was that cytokines might affect PBMCs in a similar fashion by affecting expression of those molecules important for antigen and alloreactive recognition. This could then increase the number of rare MHC/peptide combinations above the threshold necessary for T cell stimulation. Upregulation of adhesion and costimulatory molecules could also reduce the threshold for T cell activation in allorecognition of antigenic differences and thus account for the positive reactions seen in the mMLC. This investigation would also contribute greater insight into the understanding of the diverse role that cytokines play in the alloreactive process of GvHD.

The cytokines used in the mMLC assay were principally γ-IFN and TNF-α, with IL4 or low dose IL2 added to the culture. As allorecognition involves interaction between the TCR with the MHC-peptide of an APC, the effects of γ-IFN, TNF-α and IL4 on T lymphocytes, monocytes and B cells were investigated in this chapter.

All antigens are presented in the context of Class I or II with CD4 cells recognising peptides in association with the latter and CD8 cells with Class I while mHags are also presented in the context of MHC class I or II. Thus, one can easily appreciate that any increase in MHC expression would mean an increase in antigen presenting capacity. The level of MHC class I and II expression were therefore analysed on lymphocytes and monocytes.

Various adhesion molecules that have been found to be associated with GvHD include CD11a (LFA-1), CD54 (ICAM-1), CD49d (VLA-4) and vascular cell adhesion molecule-1 (VCAM). The ligand for CD11a is CD54 while CD49d binds to VCAM. Adhesion molecules are essential for cell-cell contact which is required for antigen presentation. In the two signal model for productive antigen recognition, signal 1 is provided by the MHC-peptide and T cell receptor engagement while signal 2 is generated from costimulatory molecules of which the most important molecules are CD80 (B7.1) and CD86 (B7.2). The CD11a-CD54 interaction has also been shown to provide an important costimulatory signal. It was hypothesised that changes in expression of any or all of these immunomodulatory molecules would enhance alloantigen recognition and levels of expression were examined.

As detailed in section 2.3.4, the two parameters analysed flow cytometrically were the median fluorescent intensity (MFI) which is a measure of the intensity of expression of the antigen on the cell surface, and the percentage of cells expressing the antigen (% positive). The mean FSC was also measured and the MFI of the antigen/FSC ratio provided the best estimate of the relative density of expression of the antigen on the cell surface.

5.2 Results of the effect of cytokines on CD3+ T cells:

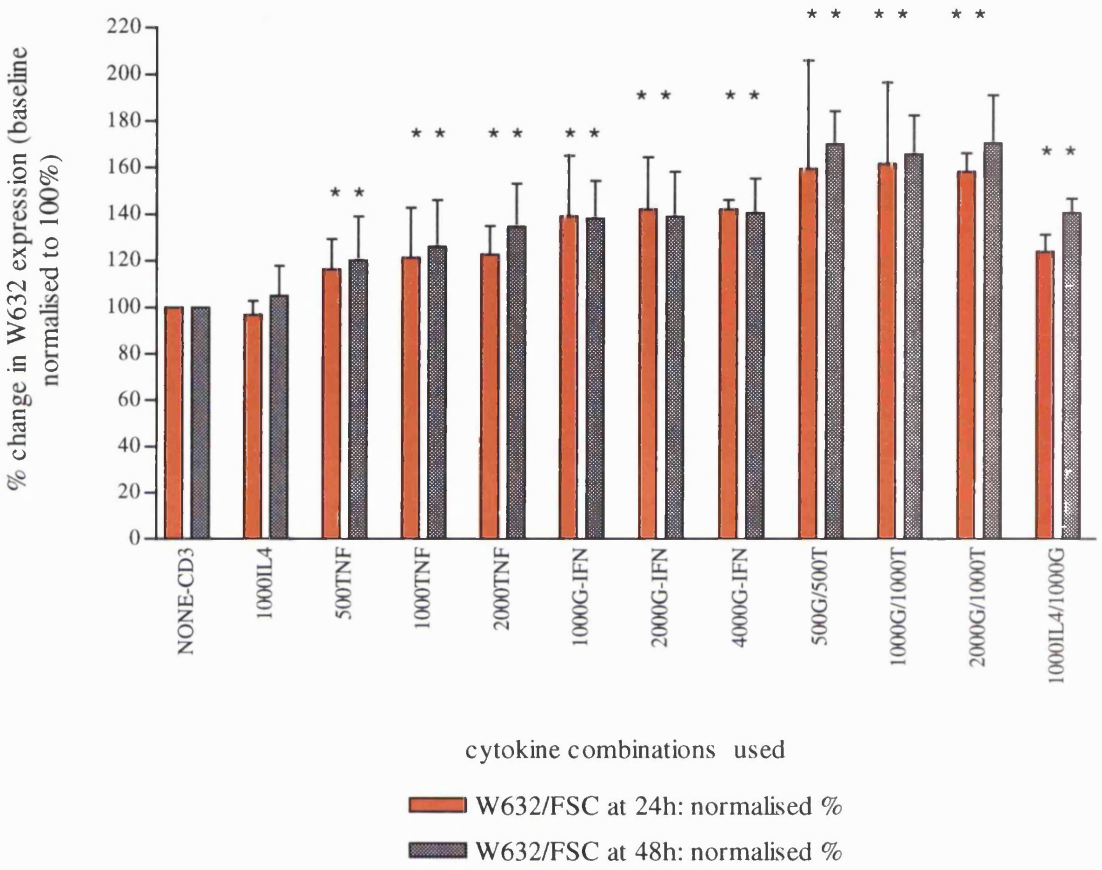
5.2.1 Cytokines and class I expression on CD3+ T cells:

As expected, all T cells expressed Class I as measured by W632. The Class I expression remained stable in untreated baseline cultures over the 48 hour period. Taking the density of expression (as measured by MFI of W632 divided by the mean FSC value) in baseline untreated cells and normalising to 100%, it could be seen from figure 5.1 that the addition of IL4 produced no change in W632 expression. TNF-α induced a moderate increase of 20% above baseline, γ-IFN an increase of 40% and the combination of γ-IFN+TNF an additive increase of 60% above baseline. All the increases were statistically significant by paired 2-tailed t-test ($p<0.05$). The lowest doses of cytokines used were able to produce these changes and increasing the concentrations of the respective cytokines did not cause any further significant rise in Class I expression. There was no statistically significant difference ($p>0.05$) between expression at 24 and 48 hours implying that the effect of cytokines was maximal at 24 hours and sustained for at least another 24 hours.

5.2.2 Cytokines and class II expression on CD3+ T cells:

At baseline, only about 7% of all T cells were DR positive. Neither the density of expression nor the percentage of DR expressing T cells were affected by any of the cytokine combinations tested ($p>0.05$, paired t-test).

Figure 5.1: Effect of cytokines on the density of expression of Class I on T cells. Baseline values normalised to 100%



PBMCs were incubated with the various cytokine combinations shown above. Baseline cultures were exposed to no cytokines (indicated none on x-axis). The relative density as measured by MFI of W632/ mean value of FSC was determined for each cytokine combination at 24 and 48 hours. The relative density of the baseline cultures was normalised to 100% and the relative density of the cytokine cultures were expressed as a % change above baseline. All results presented as mean $\pm$ s.d.

G or G-IFN: γ-IFN

T or TNF : TNF-α

* : statistically significant ($p<0.05$) : paired t-test

5.2.3 Cytokines and CD54 (ICAM-1) expression on CD3+ T cells:

At baseline, 38.0 ± 13.8% of T cells were CD54 positive as shown in Table 5.1. Only γ-IFN had a statistically significant effect on T cells increasing it to 69.6 ± 14.5%. As in its effect on class I expression, the lowest dose of γ-IFN (1000 U/ml) was sufficient to induce the rise and increasing the dose did not cause a further increase. Both TNF-α and IL4 had no significant effect on CD54 expression although when TNF-α was added to γ-IFN, the combination produced a synergistic increase to 80%. There was no synergism evident with γ-IFN + IL4. These increases were observed by 24 hours and although there was a further small rise at 48h, this was not found to be significant ($p>0.05$) and the increase was also seen in baseline cultures, indicating a probable response to in-vitro culture. Cytokines only altered the proportion of CD54 expressing cells. The level of expression of CD54 in T cells as reflected by the MFI remained unaltered as shown in Table 5.1 ($p>0.05$ by paired t-test). The density of expression as measured by MFI of CD54 divided by mean value of FSC was about 20 times less than Class I expression on the T cells.

5.2.4 Cytokines and CD11a (LFA-1) expression on CD3+ T cells:

All CD3+ cells expressed CD11a on their cell surface. No significant changes in CD11a expression in terms of density of expression were detected with any of the cytokine combinations tested.

Table 5.1 Effect of cytokines on CD54 (ICAM-1) expression on T cells as measured by median fluorescent intensity (MFI) and percentage of T cells that were positive.

Cytokines U/ml	MFI of CD54+ T cells at 24h	% of CD54+ T cells at 24h	MFI of CD54+ T cells at 48h	% of CD54+ T cells at 48h
NONE	16.1±4.9	38.0±13.8	15.5±4.7	43.6±15.6
1000 IL4	18.2±6.0	41.0±13.5	17.0±5.3	47.5±14.6
500 TNF-α	16.0±4.2	36.9±13.1	15.1±3.9	45.9±22.4
1000 TNF-α	16.5±4.4	38.8±14.5	14.8±3.4	44.2±23.0
2000 TNF-α	15.2±5.4	31.1±10.7	14.3±3.6	60.5±31.6
1000 γ-IFN	18.8±4.3	69.6±14.5 *	23.2±6.0	71.0±17.8 *
2000 γ-IFN	18.2±3.8	67.4±14.6 *	22.9±7.5	74.2±18.2 *
4000 γ-IFN	17.1±3.0	61.8±17.4 *	21.4±6.9	74.9±17.2 *
500 γ-IFN + 500 TNF-α	21.5±4.0	78.2±11.6 *	27.8±9.3	84.3±8.7 *
1000 γ-IFN + 1000 TNF-α	22.0±4.0	79.4±12.7 *	27.3±7.7	86.5±10.2 *
2000 γ-IFN + 1000 TNF-α	20.9±5.2	75.5±15.8 *	27.1±11.4	81.8±13.0 *
1000 γ-IFN + 1000 IL4	19.6±4.4	67.2±17.9 *	24.3±9.3	75.3±16.4 *

PBMCs were incubated with the various cytokine combinations shown above. Baseline cultures were exposed to no cytokines. The level of expression as measured by MFI and the percentage of T cells that were CD54+ were determined for each cytokine combination at 24 and 48 hours. All results are presented as mean ± s.d.

* : statistically significant ($p<0.05$) : paired t-test

5.2.5 Cytokines and CD80/86 (B7.1/2) expression on CD3+ T cells:

Only about 1-2% of T cells expressed the CD80 or CD86 costimulatory molecules and this was not affected by any of the cytokine combinations tested. This was not surprising as CD80 and CD86 have mainly been found to be expressed on antigen presenting cells (APCs).

5.2.6 Cytokines and CD49d (VLA-4) expression on CD3+ T cells:

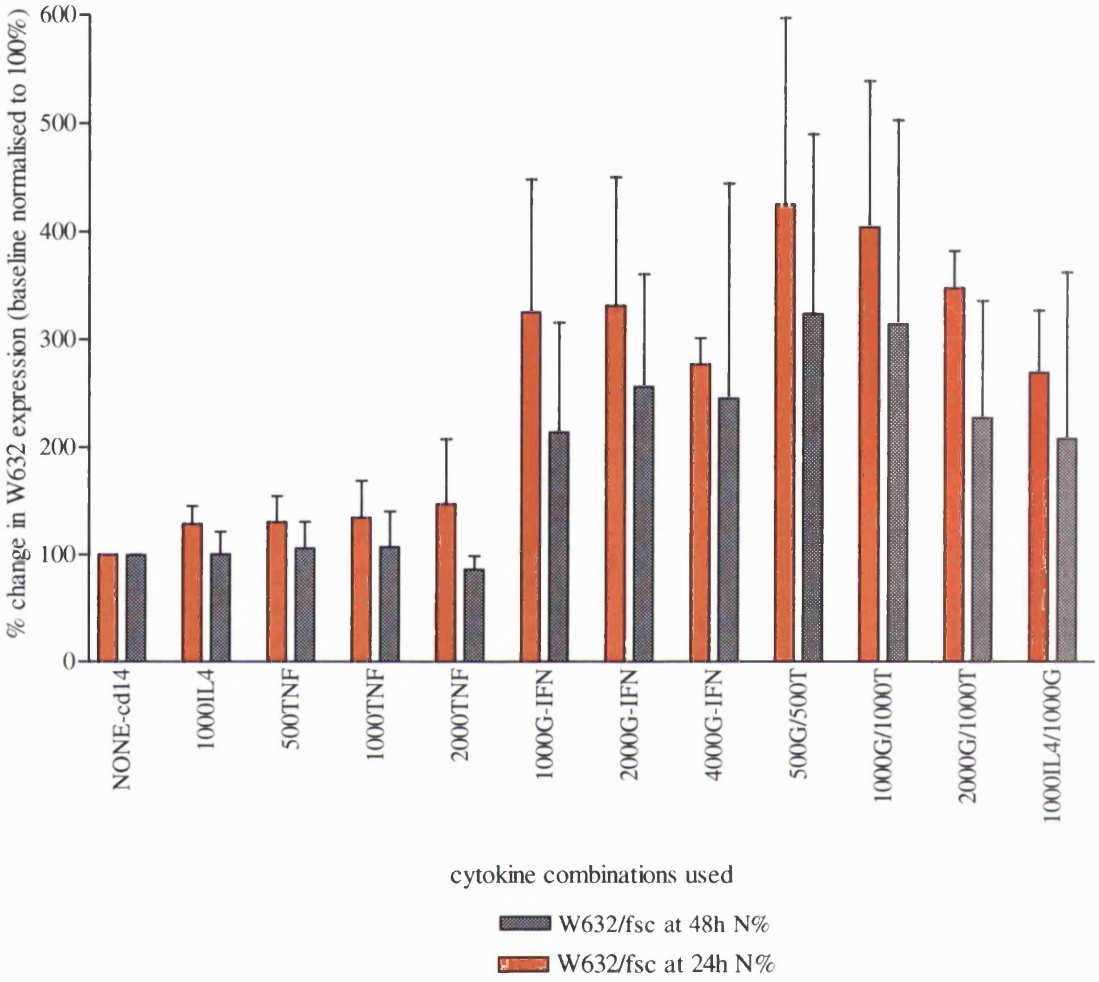
The majority of T cells (90%) expressed CD49d on their cell surface. This level of expression was not altered with any of the cytokines used, either singly or in combination.

5.3 Results of the effect of cytokines on monocytes:

5.3.1 Cytokines and class I expression on monocytes:

As shown in Figure 5.2, Class I expression in monocytes was found to be extremely sensitive to the effects of γ-IFN. While both IL4 and TNF-α induced a rise of about 30-40% above baseline, γ-IFN alone induced a three fold increase in the level of expression. Synergy between γ-IFN + TNF-α was evident with the cytokine combination inducing a four fold increase (all $p<0.05$) while this was not seen with γ-IFN + IL4. There was no statistical difference when the concentrations of the cytokines, either alone or in combination were increased and all effects were maximal at the lowest dose of cytokines used. The level of expression fell by a small margin at 48 hours but this was not found to be significant on paired t-testing ($p>0.05$).

Figure 5.2: Effect of cytokines on the density of expression of Class I on monocytes. Baseline values normalised to 100%



PBMCs were incubated with the various cytokine combinations shown above. Baseline cultures were exposed to no cytokines (indicated none on x-axis). The relative density as measured by MFI of W632 / mean value of FSC was determined for each cytokine combination at 24 and 48 hours. Baseline cultures were normalised to 100% and the changes in the relative density of the cultures exposed to the various cytokines were expressed as a % change above baseline. All results presented as mean $\pm$ s.d.

G or G-IFN: γ-IFN

T or TNF : TNF-α

All increases seen were statistically significant ($p<0.05$) - paired t-test

5.3.2 Cytokines and class II expression on monocytes:

Monocytes intrinsically express class II on their cell surface and this was confirmed in the assay system. There was an increase in the density of expression in baseline samples simply by overnight culture in complete medium: rising from a mean of 0.36 $\pm$ 0.21 to 2.9 $\pm$ 2.5 over a 24 hour period. This level of DR expression however could be further enhanced by the addition of cytokines, in contrast to T cells which showed no effect. γ-IFN was the most potent and induced a further 3.8 fold increase in the density of Class II expression while TNF-α or IL4 both increased it by up to 2-fold as shown in Table 5.2. There was no significant difference with respect to the various concentrations of cytokines used ($p>0.05$). Unlike Class I expression, there was no additive effect with the combination of γ-IFN + TNF-α or γ-IFN + IL4 and it appeared that γ-IFN alone was capable of inducing maximal Class II expression. All changes induced by the cytokines were seen at 24 hours with no further increase at 48 hours (data not shown).

5.3.3 Cytokines and CD54 (ICAM-1) expression on monocytes:

All monocytes were found to express CD54.

TNF-α and γ-IFN were both equally potent in inducing almost a doubling of the density of expression of CD54 whereas IL4 had a much smaller (20%) but still significant increase (Table 5.2). The combination of TNF-α + γ-IFN or IL4 + γ-IFN did not increase expression any further. The effect of the various cytokines remained sustained at 48 hours with no further significant rise (data not shown).

Table 5.2 Effect of cytokines on the density of expression on class II, CD54 and CD11a expression on monocytes as measured by median fluorescent intensity (MFI).

Cytokines All U/ml	MFI of DR/FSC at 24h	MFI of CD54/FSC at 24h	MFI of CD11a/FSC at 24h
NONE	2.9±2.5	1.5±1.3	0.50±0.26
1000 IL4	5.7±3.5 *	1.7±1.4 *	0.47±0.16
500 TNF-α	4.6±2.3 *	2.6±1.1 *	0.47±0.22
1000 TNF-α	4.8±2.7 *	2.7±1.2 *	0.45±0.20
2000 TNF-α	6.6±5.0 *	2.7±1.4 *	0.56±0.30
1000 γ-IFN	9.8±3.1 *	2.6±1.4 *	0.62±0.30
2000 γ-IFN	9.9±3.2 *	2.4±1.2 *	0.61±0.22
4000 γ-IFN	10.6±3.4 *	1.8±0.3 *	0.69±0.23
500 γ-IFN + 500 TNF-α	9.2±3.9 *	2.7±1.4 *	0.48±0.21
1000 γ-IFN + 1000 TNF-α	9.8±3.8 *	2.9±1.4 *	0.54±0.29
2000 γ-IFN + 1000 TNF-α	11.3±2.7 *	2.1±1.0 *	0.60±0.26
1000 γ-IFN + 1000 IL4	9.1±3.8 *	2.1±0.3 *	0.73±0.22

PBMCs were incubated with the various cytokine combinations shown above. Baseline cultures were exposed to no cytokines. The density of expression as measured by MFI of respective antigen/ median value of FSC was determined for each cytokine combination at 24 and 48 hours (data not shown).

All results are presented as mean ± s.d.

* : statistically significant change ($p<0.05$) - paired t-test

5.3.4 Cytokines and CD11a (LFA-1) expression on monocytes:

CD11a was expressed in all monocytes analysed. CD11a, as in T cells remained relatively resistant to the effect of cytokines and only γ-IFN demonstrated a consistent trend towards some degree of upregulation although it did not reach statistical significance. This did not alter over the 48 hour period.

5.3.5 Cytokines and CD80/86 (B7.1/7.2) expression on monocytes:

At baseline, all monocytes expressed CD86 but only a minority were (<3%) CD80 positive. In vitro culture over 24 hours without the addition of any cytokine induced a variable proportion of monocytes to express CD80 (mean 65.6 ± 26.9%).

Over and above this rise, γ-IFN induced the most potent changes:

i) an increase in the density of expression of CD80 positive cells, which however did not approach significance (p=0.08) as shown in Table 5.3

ii) an increase in the number of CD80 positive monocytes to over 90% ($p<0.05$)

iii) a 6 to 8 fold increase in the density of CD86 expressing cells.(p=0.009)

Although there was marked variability in the density of expression of both costimulatory molecules CD80 and CD86 between individuals, the effect seen with γ-IFN was consistent, but it was less so for TNF-α. There was no significant effect seen with either TNF-α or IL4 and the regulation of CD80/86 expression on monocytes. Although TNF-α appeared to upregulate CD86 expression (Table 5.3), this was seen in some samples only and not in others and was therefore not statistically significant (paired t-test). Adding IL4 or TNF-α to γ-IFN in combination did not produce any additional effect, with γ-IFN alone producing a maximal rise except with the density of

expression of CD80 where the combination of γ-IFN + TNF-α induced a further rise above that of γ-IFN alone. All levels remained sustained at 48 hours (data not shown).

5.3.6 Cytokines and CD49d (VLA-4) expression on monocytes:

All monocytes, in common with T cells were found to express CD49d on their cell surface. Unlike T cells where CD49d expression was not affected by any of the cytokines used, expression in monocytes could be regulated by TNF-α but not γ-IFN or IL4 as shown in Figure 5.3. TNF-α in the lowest concentrations used (500 U/ml) downregulated the level of expression of CD49d on monocytes by 50% ($p=0.03$). There was no further downregulation seen with increasingly doses of the cytokine. IL4 also downregulated CD49d expression but this did not attain statistical significance. γ-IFN had the opposite effect but this was not statistically significant. The opposing effects of γ-IFN and TNF-α was seen clearly when used in combination. There was downregulation of CD49d expression but not as much seen as with TNF-α alone.

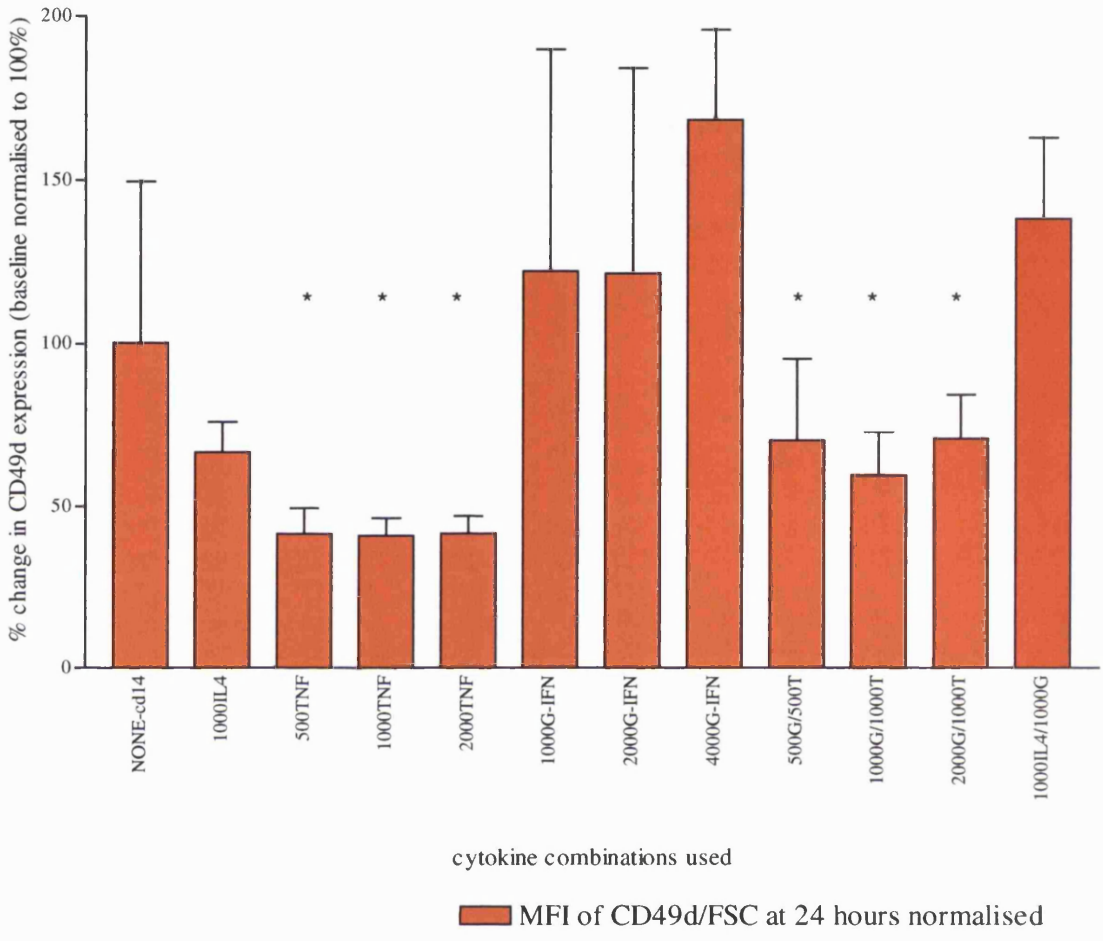
Table 5.3 Cytokines and the regulation of CD80 and CD86 expression on monocytes.

Cytokines All U/ml	Density of expression of CD80 at 24h $X10^{-2}$	% of CD80+ monocytes at 24h	Density of expression of CD86 at 24h $X10^{-2}$	% of CD86+ monocytes at 24h
NONE	3.6 ± 1.4	71.3 ± 27.8	12.7 ± 7.5	100
1000 IL4	4.5 ± 1.5	81.9 ± 19.3	14.8 ± 5.8	100
500 TNF-α	4.1 ± 1.3	77.9 ± 20.7	38.7 ± 44.8	100
1000 TNF-α	4.2 ± 1.3	77.1 ± 19.9	42.1 ± 41.4	100
2000 TNF-α	6.1 ± 4.3	77.9 ± 21.9	67.3 ± 47.5	100
1000 γ-IFN	11.5 ± 9.3 *	91.9 ± 6.7 *	70.7 ± 47.4 *	100
2000 γ-IFN	11.8 ± 9.1 *	91.7 ± 7.1 *	70.7 ± 42.7 *	100
4000 γ-IFN	12.2 ± 9.1 *	91.9 ± 6.4 *	104 ± 34.4 *	100
500 γ-IFN + 500 TNF-α	11.7 ± 5.3 *	94.4 ± 4.2 *	62.4 ± 49.7 *	100
1000 γ-IFN + 1000 TNF-α	16.9 ± 12.3 *	91.6 ± 6.3 *	60.5 ± 39.8 *	100
2000 γ-IFN + 1000 TNF-α	17.2 ± 15.0 *	92.6 ± 5.0 *	83.3 ± 34.6 *	100
1000 γ-IFN + 1000 IL4	7.8 ± 6.9 *	90.2 ± 8.7 *	74.2 ± 41.5 *	100

PBMCs were incubated with the various cytokine combinations shown above and the effect on monocytes analysed. Baseline cultures were exposed to no cytokines. The density of expression as determined by the ratio of MFI of CD80 or CD86 over the mean value of FSC was calculated for each cytokine combination as well as the proportion of monocytes that were CD80 or CD86 positive expressed as a percentage. All results are presented as mean ± s.d.

*: statistically significant change ($p<0.05$) - paired t-test

Figure 5.3: Effect of cytokines on the density of expression of CD49d on monocytes. Baseline values normalised to 100%



PBMCs were incubated with the various cytokine combinations shown above. Baseline cultures were exposed to no cytokines (indicated none on x-axis). The relative density as measured by MFI of CD49d / mean value of FSC was determined for each cytokine combination at 24 and 48 hours. Baseline cultures were normalised to 100% and the changes in the relative density of the cultures exposed to the various cytokines were expressed as a % change above baseline. All results presented as mean $\pm$ s.d.

G or G-IFN: γ-IFN

T or TNF : TNF-α

* : statistically significant change ($p<0.05$) - paired t-test

5.4 Results of the effect of cytokines on B cells:

5.4.1 Cytokines and the regulation of MHC, costimulatory and adhesion molecules on B cells:

All B cells expressed both MHC Class I and II on their cell surface. The expression of Class II was not altered by any of the cytokines used. Class I expression was significantly increased by 50% with γ-IFN ($p=0.002$), and 20% with both TNF-α ($p=0.009$) and IL4 ($p=0.02$) as shown in Table 5.4. There was a further additive increase of up to 90% when cytokine combinations of γ-IFN and TNF-α or γ-IFN and IL4 were used.

All B cells were found to express CD54. The density of expression was most affected by IL4 which increased it by 55% ($p=0.002$) as shown in Table 5.4. γ-IFN also upregulated expression but this was only significant at higher doses (4000 U/ml). TNF-α had no effect on CD54 but the combination of TNF-α and γ-IFN did significantly increase the level of expression even at the lowest doses. γ-IFN and IL4 in combination nearly doubled the density of expression ($p<0.001$) and was more effective then either cytokine alone.

CD11a, CD80, CD86 and CD49d expression were unaffected by any of the cytokine combinations used except for IL4 which increased CD80 expression by 25%.

Table 5.4 Cytokines and its effect on Class I and CD54 expression in B cells

Cytokines All U/ml	Density of expression of Class I at 24h normalised %	Density of expression of CD54 at 24h $X10^{-2}$
NONE-CD3	100	8.3 ± 3.6
1000 IL4	118.7 ± 12.0 *	13.0 ± 6.1 *
500 TNF-α	115.9 ± 11.1 *	7.4 ± 3.5
1000 TNF-α	118.8 ± 18.8 *	7.6 ± 4.5
2000 TNF-α	122.9±19.6 *	9.1 ± 4.5
1000 γ-IFN	161.9 ± 23.7 *	10.9 ± 4.3
2000 γ-IFN	165.6 ± 24.4 *	10.9 ± 4.6
4000 γ-IFN	169.1 ± 6.7 *	12.0 ± 4.9 *
500 γ-IFN + 500 TNF-α	188.7 ± 40.2 *	12.5 ± 6.0 *
1000 γ-IFN + 1000 TNF-α	188.7 ± 33.3 *	11.6 ± 5.9 *
2000 γ-IFN + 1000 TNF-α	193.0 ± 11.6 *	12.3 ± 6.8 *
1000 γ-IFN + 1000 IL4	172.5 ± 13.4 *	15.7 ± 4.9 *

PBMCs were incubated with the various cytokine combinations shown above and the effect on B cells analysed. Baseline cultures were exposed to no cytokines. The density of expression as determined by the ratio of MFI of W632 (Class I) or CD54 over the mean value of FSC was calculated for each cytokine combination. The baseline values were normalised to 100% and the changes calculated accordingly.

All results are presented as mean ± s.d.

* : statistically significant change ($p<0.05$) - paired t-test

5.4.3 A comparison of the relative densities of expression of MHC molecules on lymphocytes and monocytes.

Table 5.5 compares the relative density of expression in T cells, B cells and monocytes. At baseline without the addition of cytokines, the density of expression of Class I was fairly comparable among T cells, B cells and monocytes. The marked differential sensitivity to cytokines however was evident as 24 hour exposure to 2000 U/ml γ-IFN + 1000U/ml TNF-α produced a 73% increase in Class I expression in T, 93% in B cells and an almost 4 fold increase in monocytes. In comparison, the density of Class II expression on the minority of DR positive T cells was 10x times less (0.09) than its class I density (0.82) and 30 times less than the corresponding class II expression on monocytes at 24h. B cells expressed the highest level of class II on its surface, 80 times the level of T cells and 2.5 times that of monocytes. This disparity became most marked after exposure to γ-IFN: monocytes had a 2 log greater density of expression compared to DR+ve T cells.

Table 5.5 A comparison of the density of expression of Class I and II molecules in T cells, B cells and monocytes with and without cytokines.

Density of expression of Class I and II	**Baseline(no cytokines)**	**2000 U/ml γ-IFN + 1000U/ml TNF-α**
Class I density (W632/FSC) CD3+ cells at 24h	0.82	1.42 *
Class I density (W632/FSC) CD14+ cells at 24h	0.96	3.67 *
Class I density (W632/FSC) CD19+ cells at 24h	0.82	1.58 *
Class II density (DR/FSC) CD3+ cells at 24h	0.09	0.101
Class II density (DR/FSC) CD14+ cells at 24h	2.92	11.34 *
Class II density (DR/FSC) CD19+ cells at 24h	7.24	7.32

* : statistically significant change ($p<0.05$) - paired t-test

5.5 Discussion:

Cytokines are soluble proteins or glycoproteins made by cells which affect the behaviour of other cells. Regulatory mechanisms have evolved to keep cytokines under tight control and these occur through various feedback loops to form a complex cytokine network. T cells interact within this network and much of their development and function are mediated by cytokines. In turn, the various T cell subsets (Th1, Th2, Tc1, Tc2) are responsible for the expression and secretion of various cytokines as a result of productive antigen stimulation.

The results in this chapter demonstrate the profound effect cytokines such as TNF-α, γ-IFN as well as IL4 have on lymphocytes and monocytes. These changes involved molecules that are known to be intimately involved in antigen recognition and cell-cell contact and support the association that exists between the pro-inflammatory "cytokine storm" and subsequent development of GvHD. T cells were found to be most sensitive to γ-IFN and to a lesser extent, to TNF-α. IL4 had little if any effect on T cells. The changes seen on T cells were firstly, an increased density of class I allowing for more MHC-peptides to be displayed on the cell surface and secondly, more T cells expressing CD54, important both as an adhesion and costimulatory molecule in productive antigen presentation. Monocytes displayed exquisite sensitivity with exposure to cytokines and changes were seen in all molecules tested except for CD11a. Like T cells, monocytes were most responsive to γ-IFN but TNF-α and notably, IL4 also exerted potent effects. Besides changes in class I, class II and CD54, it would be envisaged that the increased levels of CD80 and CD86 would enhance their capacity as antigen presenting cells by providing adequate and essential co-stimulation. The only cytokine that induced any significant change in CD49d (VLA-4) expression was TNF-

α and only in monocytes. Surprisingly, it caused a downregulation of expression although γ-IFN did exhibit a trend for upregulation which was not statistically significant. The natural ligands for CD49d include VCAM on APCs, MadCam-1 and extracellular matrix proteins fibronectin and thrombospondin. It has been said that the CD49d and VCAM system may be more important in the trafficking of leukocytes across endothelial surfaces rather than in APC-T cell interactions (Schlegel 1997), (Itoh et al. 2000), (Springer 1990) .

B cells, like monocytes are important antigen presenting cells. Unlike T cells and monocytes, they were found to be most responsive to IL4 and to a lesser extent γ-IFN. Class I, CD54 and CD80 were the cell-surface molecules upregulated after exposure to cytokines. Class II was already densely expressed on the cell surface at baseline and cytokine exposure did not alter it further. The advantage of the flow cytometric method employed in this study was that a variety of cell surface molecules on different mononuclear cell subsets could be analysed simultaneously in one experiment.

The importance of adhesion molecules in GvHD was highlighted by studies which demonstrated elevated levels of adhesion molecules in target tissues and a correlation with the occurrence of GvHD: increased levels of VCAM-1 expression in the skin and increased ICAM-1 (CD54) in the skin, gut, liver and endothelium (Norton et al 1992a), (Norton et al. 1992b), (Norton et al. 1991b). Blockade of important adhesion molecule interaction (CD11a-CD54, CD49d-VCAM) or costimulatory pathways (CD80:CD28) using either monoclonal antibodies anti-LFA-1 and anti-VCAM antibodies or negative signalling molecules (CTLA-4) have also been shown to reduce the severity of GvHD (Tanaka et al. 1995), (Schlegel et al. 1995), (Harning et al. 1991), (Blazar B.R. et al. 1995a).

The results of this chapter have emphasised several important points regarding the interaction of cytokines with lymphocytes and monocytes. For each cell surface marker examined (MHC, costimulatory or adhesion), there was differential sensitivity among the 3 cell types (monocytes, T cells and B cells) in their response to a particular cytokine. As shown in Table 5.5, there was a similar baseline expression of Class I in all 3 cell types but exposure to γ-IFN highlighted the increased sensitivity of monocytes compared to T or B cells. This differential sensitivity was even more obvious with class II expression where all 3 cytokines, especially γ-IFN induced a substantial upregulation on monocytes while having no significant effect on T or B cells. In line with this finding, it has previously been reported that Class II expression on human T cell lines is regulated by other factors other than γ-IFN (Gerrard et al. 1988) Conversely, for a particular adhesion molecule, each cell type showed different sensitivities to different cytokines. For example, in CD54 expression, TNF-α was the most potent stimulator of increased expression in monocytes whereas in T cells, it was γ-IFN and in B cells, IL4 that exerted the greatest effect.

For each cell surface marker, the density of expression and the proportion of positively expressing cells were the two parameters measured. It was found that cytokines could alter both: a greater percentage of cells expressing the adhesion molecule without altering its density (CD54 in T cells), a higher density of expression in already positive cells (Class I in T cells) or both (CD80 in monocytes). An important point to note however, was that in this study, the antibody staining protocol used detected either the presence or absence of the cell surface marker. It could be possible that cytokines might affect a 3rd property of these adhesion or costimulatory molecule which is a conformational switch from low to high affinity (Cabanas & Hogg 1993). This might

be operative for CD11a, expressed on all mononuclear cells and which somewhat surprisingly, demonstrated no sensitivity to any of the cytokines tested in contrast to its ligand CD54. It is however well documented that CD11a exists in more than one conformational form and it may be that cytokines induced a transformation from a low to a high affinity state (Cabanas & Hogg 1993). Unfortunately, this could not be tested in the antibody and assay system used.

Increasing the dose of cytokines in nearly all the experiments did not increase the effect of the cytokine, that is, 1000U/ml γ-IFN was as effective as 4000U/ml γ-IFN. This was not surprising as the effect of cytokines is often exerted within the local milieu of the secreting cell. Hence systemic concentrations are less vital than the local presence of these cytokines in influencing cellular contact or antigen presentation.

The effects seen with the cytokines were evident and maximal at 24 hours. The 48 hour time point confirmed that these effects were sustained up to 48 hours. It remained possible however that certain changes induced might only appear later on and therefore not be detected by this study. How sustained this cytokine induced changes were beyond 48 hours was also not addressed. The initial changes seen during these time points were of greatest interest to us as the allodepletion strategy was based on CD69 upregulation at 72-96 hours.

The cytokine cascade in GvHD highlight the intimate interactions between cytokines and this complex network was illustrated by results in this chapter showing that γ-IFN and TNF-α together often exerted an additive (class I in T cells) or even synergistic (CD54 in T cells) effect although in one instance (CD49d in monocytes), their actions were opposing. γ-IFN and IL4 exhibited this interaction with B cells (CD54). This is

important clinically because in many inflammatory situations including GvHD, γ-IFN and TNF-α are produced and released in large quantities. Different T cell subsets produce distinct cytokines with γ-IFN secreted by Th1 cells and IL4 by Th2 cells. The more dramatic changes seen with γ-IFN in this study lends support to the idea that acute GvHD is associated with a dramatic skewing towards a predominantly Th1 repertoire. Besides having a central role in cell-mediated immunity, γ-IFN exerts profound effects on leukocyte-endothelium interactions and is involved in the activation of granulocytes, macrophages and natural killer cells (Boehm et al. 1997). TNF-α is the prototype pro-inflammatory cytokine and has been implicated as one of the prime effectors in producing the deleterious effects seen during acute GvHD (Hill et al 1997b). The data presented here suggest that it also influences the initiation of antigen recognition, singly or more significantly, in association with γ-IFN.

The interactions described here between cytokines and the changes in the MHC, costimulatory and adhesion molecules seen on monocytes and lymphocytes has provided the basis for understanding the mechanism of the modified MLC assay used in the allodepletion strategy. It has also further elucidated the complex role that the "cytokine storm" plays in the initiation of the GvH reaction including a vital role in antigen and allogeneic recognition. In terms of tumour immunology and the GvL response, it has been shown that cytokine treatment can lead to the induction of costimulatory and adhesion molecules on leukaemia blasts. This then allowed for productive anti-tumour T cell responses, confirming the role of cytokines in influencing antigen or allorecognition (Lim et al 1998).

6. Chapter 6. Retention of anti-CMV and anti-EBV reactivity in the allodepleted graft

6.1 Introduction:

One of the central aims in the development of the CD69 allodepletion strategy was the preservation of anti-viral activity in the engineered graft. In vitro studies demonstrated that 3rd party reactivity was largely preserved and close to 90% of the initial T cell pool in the graft retained. The aim of the work presented in this chapter was to examine if this translated into a potent anti-viral response. This would be crucial as CMV and EBV infections remain major complications post allogeneic transplantation and the CTL response is thought to play an important role in defence against both viruses. Current GvHD preventive approaches of rigorous T cell depletion or steroids/cyclosporin/methotrexate result in prolonged lymphopaenia and global immunosuppression which predisposes the recipient to both infections. Moreover, drugs used in the treatment of CMV and EBV infections are limited and toxic compared to the range of effective therapy in bacterial infections.

Prompt recovery of T cell immunity confers the best protection against both CMV and EBV associated disease. A strong association exists between rigorous T cell depletion and the incidence of infections. There is an urgent need to address this problem of delayed immune reconstitution as T cell depletion becomes more common with the trend to CD34 selected and mismatched transplants. The assumption that this selective

depletion strategy would not sacrifice anti-viral immunity was tested by looking at the specific anti-CMV and anti-EBV response of the allodepleted T cell population compared to the unmanipulated graft. This detection of specific anti-CMV and anti-EBV responses was greatly facilitated by the developments in tetramer technology, ELISPOT assays and isolation of immunogenic viral derived peptides.

Analysis of the antigen specificity of the CMV-specific CTL response has demonstrated that it is directed almost entirely to a single tegument protein, pp65 (McLaughlin-Taylor et al. 1994), (Riddell et al. 1994). As a component of the virion tegument, pp65 is introduced into host cells during viral entry. In a comparative analysis of fourteen CMV proteins, pp65 represented the dominant antigen recognised by T cells (Beninga et al. 1995). In view of this, peptides derived from pp65 were used in this study to quantify the level of anti-CMV activity.

The EBV-specific CTL response is more diverse with all nine latent proteins and a few of the immediate early and early lytic gene products identified as targets for the CTL (Haque & Crawford 1999). However, some are more dominant than others in eliciting a response. One such dominant peptide is SVRDRLARL derived from the latent antigen EBNA3A and presented in an HLA-A2 restricted manner. This peptide was used in this study to quantify the level of anti-EBV activity.

6.2 MATERIALS AND METHODS:

6.2.1 Samples and subjects:

A panel of 11 healthy volunteers was chosen and 20mls of peripheral blood were taken from each in preservative free heparin. These were sent to the Anthony Nolan Research Institute for HLA tissue typing and to the Clinical Virology Department, Royal Free Hospital for determination of their CMV and EBV status. Tissue typing was performed using serology for Class I and molecular typing for Class II. IgG antibodies were tested to look for past exposure to the CMV and EBV viruses with positivity defined by being IgG +ve.

6.2.2 CMV proteins and peptides:

Work was done at the Anthony Nolan Research Institute where screening of the protein sequence of pp65 revealed 17 peptides which fulfilled the anchor binding motif requirements for HLA-A*0201. The binding affinity of these peptides to HLA-A*0201 was confirmed in a T2 stabilisation assay and also their ability to induce an HLA-A*0201 restricted CTL response in vitro (Solache et al. 1999). Three peptides were identified of which peptide AE42 was deemed immunodominant. Crucially, the AE42 peptide specific CTLs were shown to be capable of recognising naturally processed pp65 in an HLA-A*0201 restricted fashion. Its amino acid sequence is NLVPMVATV. HLA-A2 was chosen as this is the most common HLA type within a Caucasoid population (50%). In all experiments done here, this peptide AE42 was used to examine responses from HLA-A2 +ve, CMV +ve individuals.

6.2.3 Protocol for testing anti-viral activity:

HLA-A2 +ve, CMV +ve (as denoted by CMV IgG positivity) responders were selected. PBMCs were obtained using discontinuous density gradient centrifugation and a one way MLC set up with completely mismatched irradiated stimulator cells. Autologous controls were also set up in parallel consisting of responder cells alone and responder cells with irradiated autologous stimulators. All cells were resuspended at $1x10^6$ cells/ml. At t=72-96 hours, in keeping with the in-vitro allodepletion strategy, activated alloreactive responder cells were identified by presence of CD69 and depleted via a MACS 'AS' column. This was done using indirect labelling with anti CD69-PE (Becton Dickinson) and anti-PE microbeads as described in section 2.2.4.

After immunomagnetic separation, various cellular fractions were obtained, ready for analysis by tetrameric-peptide complexes and the ELISPOT assay for specific anti-CMV activity. The fractions obtained were:

i) fresh unmanipulated PBMCs from the donor alone

ii) allodepleted CD69 negative fraction

iii) the alloreactive CD69 positive fraction

iv) donor cells cultured for 72h (same time period as cells in the MLR).

The aim was to measure the specific anti-CMV CD8 activity of the fresh unmanipulated cells of the CMV +ve donor and to see how much of this activity was retained in the allodepleted fraction after removal of CD69 +ve alloreactive cells. This could be expressed as a percentage:

$$\frac{\%\text{ of CD8 cells responding to the CMV-peptide in the allodepleted fraction}}{\%\text{ of CD8 cells responding to the CMV-peptide in the unmanipulated fraction}} \times 100$$

To exclude any possible changes in T cell subsets and function due to time left in culture or due to the foetal calf serum (FCS), the specific anti-CMV activity of donor cells left in culture (fraction 4) was also assessed.

The same protocol was employed to look for anti-EBV reactivity except that an HLA-A2 +ve and EBV IgG +ve donor was chosen. In instances where the donor was CMV IgG +ve as well, the same donor was used for both protocols.

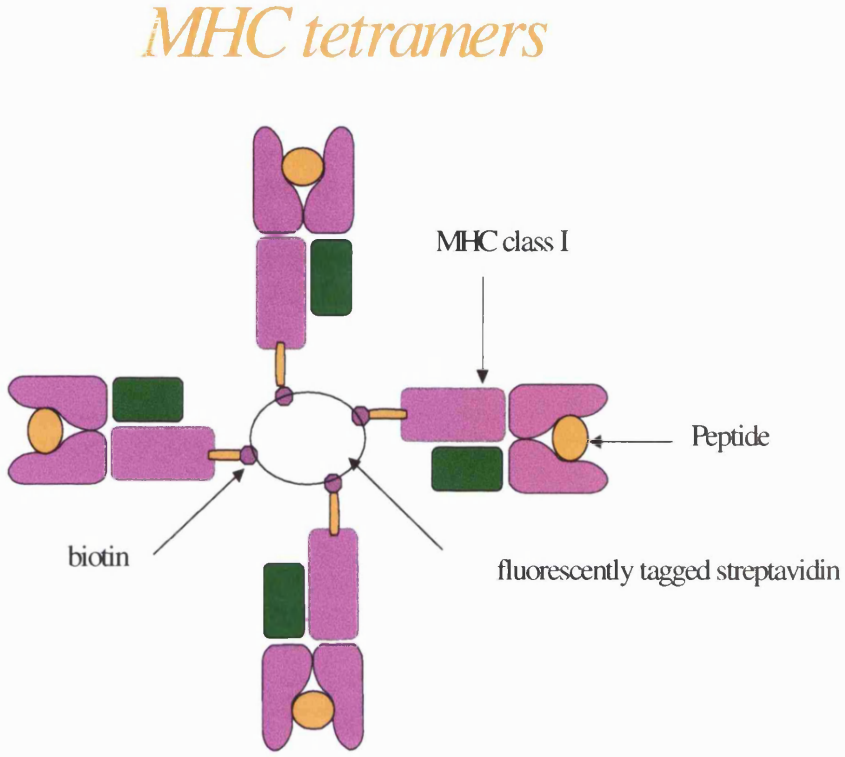
6.2.4 EBV proteins and peptides:

Several peptides reflecting MHC class I restricted epitopes for CD8+ve cells have been synthesised. These represented both lytic and latent EBV derived proteins. For this study, only HLA-A2+ve individuals were studied. The peptide used was SVRDRLARL derived from the latent cycle protein EBNA3A and presented in an HLA-A2 restricted manner (Tan et al. 1999). This was used to examine responses from HLA-A2 +ve, EBV +ve individuals.

6.2.5 CMV tetrameric MHC-peptide complexes:

MHC class I tetramers were synthesised according to Altman et al, (Altman et al. 1996) and folded with the CMV pp65 (495-503:NLVPMVATV) HLA-A2 restricted peptide. These soluble CMV tetramers were produced at the Anthony Nolan Research Institute. Figure 6.1 illustrates the configuration of a MHC tetrameric-peptide complex consisting of 4 biotinylated MHC class I molecules bound together by a fluorescently tagged (phycoerythrin) streptavidin molecule.

Figure 6.1 Schematic representation of a MHC-peptide tetrameric complex



Briefly the principle was as follows: Recombinant MHC class I heavy chain or β_2m protein was produced in Escherichia coli cells transformed with the relevant expression plasmids. Expression of the heavy chain was limited to the extracellular domain, and the C-terminus of this domain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. HLA-A2 complexes were folded in vitro using 30mg of heavy chain protein, 25mg of β_2m and 10 mg of the CMV derived HLA-A2 binding peptide AE42 . Protease inhibitors (2 ug/ml pepstatin, 2 ug/ml leupeptin and 0.2mM PMSF) were used to preserve the protein. The MHC complexes were biotinylated using purified recombinant BirA enzyme at final concentration of 10uM, with 0.5mM biotin and 5mM ATP. The reaction was incubated at room temperature for 16 hours to achieve a biotinylation level of 80%. The biotinylated MHC-peptide complexes were purified by gel filtration chromatography (using buffer containing 20mM Tris (pH 8.0) and 50mM NaCl) and anion exchange chromatography (0-0.5 M NaCl gradient). Tetramers were made by mixing the biotinylated protein complex with streptavidin-phycoerythrin at a molar ratio of 4:1.

6.2.6 Detection of CTLs bound to tetramers using flow cytometric analysis:

Simple flow cytometric staining of antigen-specific T cells is difficult as soluble peptide-MHC complexes have an inherently fast dissociation rate from TCR. Tetrameric peptide-MHC complexes binds more than one TCR on a specific T cell leading to correspondingly slower dissociation rates, making the complexes more amenable to flow cytometric analysis.

Staining of cells was performed in a 96-well microtitre plate. Cells were washed with RPMI + 5%FCS + 5mM NaN_3 (abbreviated as solution 1) and resuspended in 100*u*l of solution 1. 1 *u*l of the tetramer solution was added to the cells and left to incubate for 30 minutes at 37^0C. Excess tetrameric complexes were washed off and cells resuspended in solution 1. Anti-CD3 FITC and anti-CD8 PerCp were added and incubated for another 30 minutes on ice. The cells were then washed twice with solution 1 and fixed in 100*u*l of RPMI and 1% formaldehyde. The cells were now ready for flow cytometric analysis.

The acquisition-analysis method was as follows and illustrated in Figure 6.5: 100000 events were acquired within the lymphocyte gate (R1) as defined on a FSC vs SSC dot-plot. Lymphocytes in R1 were then analysed on a CD8-FITC vs CD3-PerCp dot-plot. Only cells that fell within the upper right quadrant were considered in the tetramer analysis, that is cells which were CD3+CD8+ CTLs. This 2^{nd} gate was termed R2. Taking only these CD8+ T cells into consideration (R2), a dot-plot of tetramers (PE stained) vs CD8-PerCp was analysed. Only CMV-specific CD8+ T cells that recognised AE42 peptide bound to the tetramers would appear in the upper right

quadrant, being both CD8PerCp positive and tetramer staining postive. They were then presented as a percentage of total CD8+ve cell population.

6.2.7 ELISPOT assay for detection of specific anti-CMV and anti-EBV activity:

The immunogenic CMV "peptide 42" was fed to the PBMCs of an HLA-A2 +ve, CMV +ve individual. CMV-specific CD8+ve cells respond by prompt secretion of γ-IFN. An ELISA technique called the ELISPOT or enzyme linked immunospot assay was used to detect these γ-IFN secreting clones on a 96 well plate and the frequency of responding CD8+ve cells calculated.

For each individual, there were three cell populations: unmanipulated responder cells, allodepleted responder cells and responder cells left in culture. The assay was a three day procedure (McCutcheon et al. 1997). Day 1 involved preparation of the "capture plate". Mouse anti-human γ-IFN (Endogen M700A) was diluted to 10ug/ml in sterile 0.1M $NaHCO_3$ pH 8.2 buffer. 50ul of this was then added to flat bottomed 96 well sterile microtitre plates (Nunc Maxisorp 44204) and left to incubate at 4°C overnight. At the same time, an aliquot of each responding cell population was stimulated with the "peptide 42", another with PHA as a positive control and one with either no peptide or a mock peptide as a negative control. All cells were incubated overnight at 37°C / 5%CO_2.

On Day 2, the "capture plate" was blocked with 250ul/well PBS and 5% bovine serum albumin (BSA) at room temperature for one hour. The capture plate was then washed with PBS and a second wash with complete medium (RPMI1640 with 10%FCS and 5% penicillin/streptomycin). The responding cells (9 different sets-3 sets of responder

cells, 3 sets of stimuli) were then transferred from the "stimulation plate" to the "capture plate" at a density of $2x10^5$ cells/well in a volume of 100ul/well. Each was done at least in triplicates The "capture plate" was left undisturbed and incubated at 37°C / 5%CO_2 for 20 hours.

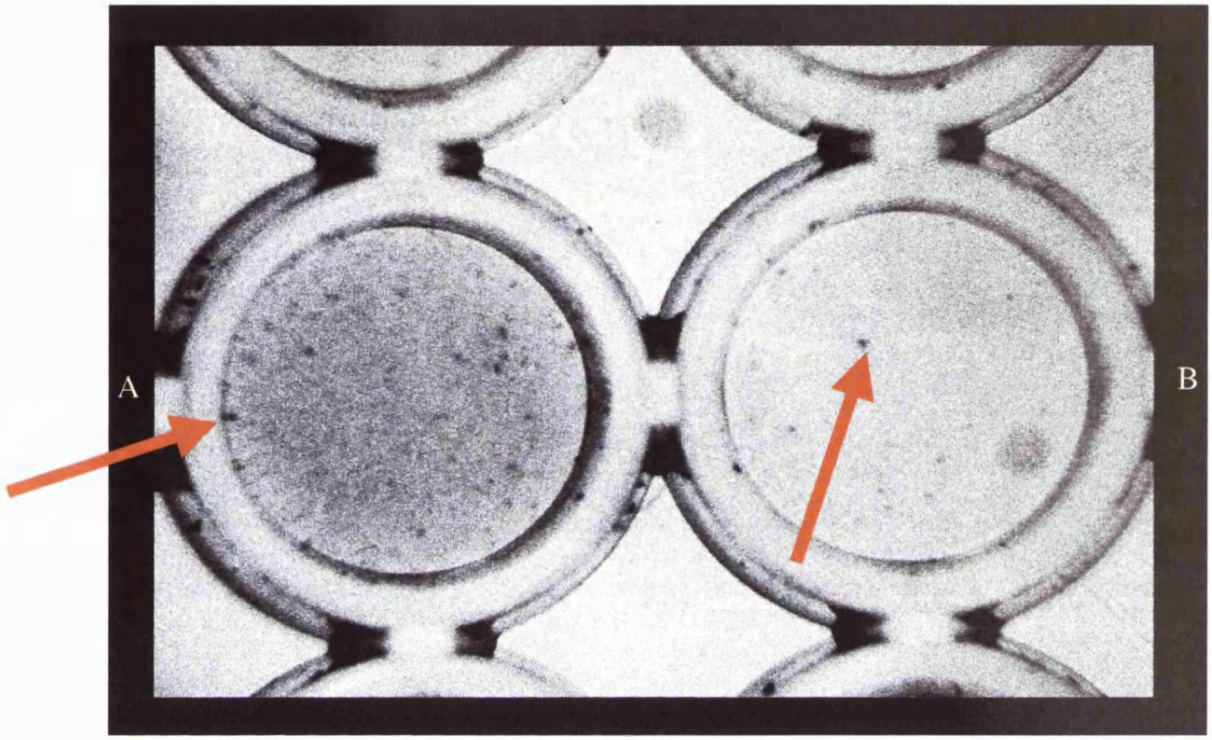
On Day 3, the cells were discarded and the plate washed 3 times with PBS + 0.1% Tween20 (PBST). To each well, 100ul of rabbit anti-human γ-IFN polyclonal antibody (Endogen P700) was added which had been diluted 1:500 in PBST + 1% BSA. The wells were left to incubate for 2 hours at room temperature and washed 3 times with PBST. After this, another wash with TBS + 0.05% Tween20 (TBST) was performed. To each well was now added 100ul of alkaline phosphatase-conjugated goat anti-rabbit polyclonal antibody (Sigma A9919) diluted 1:2000 in TBST + 1% BSA. This was left to incubate for 90 minutes at room temperature and after that, washed 3 times with TBST. A final wash with alkaline phosphate buffer (APB) was performed. The alkaline phosphatase substrate solution was prepared by mixing 10mls APB with 32ul of BCIP (Gibco 18280-016) and 44 ul NBT (Gibco 18280-016). 50 ul of this substrate solution was added to each well and incubated at room temperature until a colour reaction was visible (15-45 minutes). The reaction was terminated by washing twice in water and the wells dried. The spots representing γ-IFN production by single cells were then counted and the frequency of specific T cell responders calculated after subtracting negative control values. This was done by examining the plates using video capture and the NIH Image 1.61/ppc software. The number of spots in the negative control and in the test plate were counted and the mean from each of the triplicates calculated. As $2x10^5$ cells were added initially to each well, the frequency of CMV-specific responders was calculated using the following formula:

$$\frac{\text{(number of spots in the test plate) - (number of spots in the negative control plate)}}{2\text{x}10^5 \text{ cells}}$$

The EBV ELISPOT assay was performed in the same way except that SVRDRLARL was used as the stimulating peptide.

Figure 6.2 illustrates an ELISPOT assay to look at the frequency of T cells responding specifically to the CMV AE42 peptide.

Figure 6.2 ELISPOT assay-counting the spots using the NIH Image software:



An example of an ELISPOT assay performed in an HLA-A2 individual on 96-well plates. Each spot represents γ-IFN production by a single cell specific for the fed peptide, in this instance CMV AE42. The plates were examined using camera capture and the NIH Image 1.61/ppc software. "A" refers to one of a set of triplicate plates set up for the test culture (stimulated with AE42) while "B" is one of a set of triplicate plates for the negative control (stimulated with a non HLA-A2 binding flu peptide). The number of spots in the test plate and the negative control were counted and the mean from each of the triplicates calculated. The frequency of CMV-specific responders was calculated using the formula listed above in section 6.2.7.

6.3 Results:

6.3.1 Donor selection:

Table 6.1 lists the panel of healthy volunteers that had been HLA-typed as well as their CMV and EBV status. For the study, only HLA-A2 individuals were selected due to the MHC restriction of the CMV and EBV peptides used. From the 14 volunteers, 7 were HLA-A2 positive (50%) which is roughly in keeping with the frequency in a Caucasian population. 5 out of 14 were CMV+ and all except one were EBV+, frequencies which mirror that of the general population. 3 individuals: YM, DJ and LF were suitable as subjects and form the results of this chapter.

6.3.2 Results for baseline anti- CMV reactivity:

Initially, the baseline anti-CMV activity was assessed for all three donors and the results are shown in table 6.2. Flow cytometric analysis showed that the percentage of CD3+ cells that were tetramer-reactive ranged from 0.11-1.75% and when calculated as a fraction of the total CTL population (CD3+CD8+), the values were 0.30-5.0%. The specificity of the binding was confirmed by the fact that with a non-A2, CMV+ve individual acting as a control, only 0.01% of CD3+ cells were bound to tetramers. The ELISPOT assay corroborated this finding with the same trend shown for the three individuals. Table 6.2 shows that the magnitude of the anti-CMV response as measured by the ELISPOT assay ranged from 25-320 per $2x10^5$ PBMCs or 0.018-0.27% of the total CD3+ cells. The results of the ELISPOT assay represented were a mean 6 fold lower than the corresponding tetramer binding.

Table 6.1 Donor selection for CMV+ or EBV+ HLA-A2 individuals for the study

Individual	Class I type	Class II type	CMV status	EBV status
YM	A2, 74(19); B44, 39; Bw4, 6	DRB1*0405/10, 07; DRB4*01	positive	positive
PT	A2403(9), 31(19); B18, 49(21); Bw4, 6	DRB1*0403/06/07/20, 1101/03/04/08/11; DRB3*02, DRB4*01	negative	positive
DJ	A2, 25(10); B14, 58(17); Bw4,6	DRB1*1303/12/21, *0701; DRB3*0101, DRB4*01	positive	positive
LF	A1, 2; B63(15), 38(16); Bw4	DRB1*03011/012/04/05/06/07; DRB3*0101, *02	positive	positive
VD	A2, 3; B35, 41; Bw6	DRB1*0404/08/19; 1201/02; DRB3*02, DRB4*01	negative	positive
CG	A11; b18, 35; Bw6	DRB1*0405/10, *0901; *1201/02; DRB4*01	negative	positive
ML	A2, 24(9); b51(5), 44; Bw4	DRB1*0403/06/07/20; *1101/03/04/08/11/15/; DRB3*02, DRB4*01	negative	positive
JN	A1, 30(19); 8,41; Bw6	DRB1*0301/05/06/07; 0701; DRB3*0101, DRB4*01	negative	positive
JY	A1, 24(9); B27, 62(15); Bw4, 6	DRB1*1301/02/16, 1301/02/05/06/07/09/10/11/14; DRB3*0101, *02	positive	positive
AW	A3, 24(9); B7, 55(22); Bw5; DRB1*1401/07/08/22, *1501/03/04/05	DRB3*02, DRB5*0101	negative	negative
BH	A1, 68(28); B14, 49(21); Bw4, w6	DRB1*0101/04, *1301/02/16; DRB3*0101	negative	positive
RC	A2, B44, 47, Bw4	Not done	negative	positive
FOB	A2, 3; B14, 62; Bw6	Not done	negative	positive
PS	A1, 11; B7, 62; Bw6	Not done	negative	positive

14 random healthy volunteers were chosen and HLA-typed for both Class I and II. They were also defined in terms of their CMV and EBV status by serological testing for presence of IgG antibodies.

Table 6.2 Specific anti-CMV reactivity as measured by tetramer binding and the ELISPOT assay

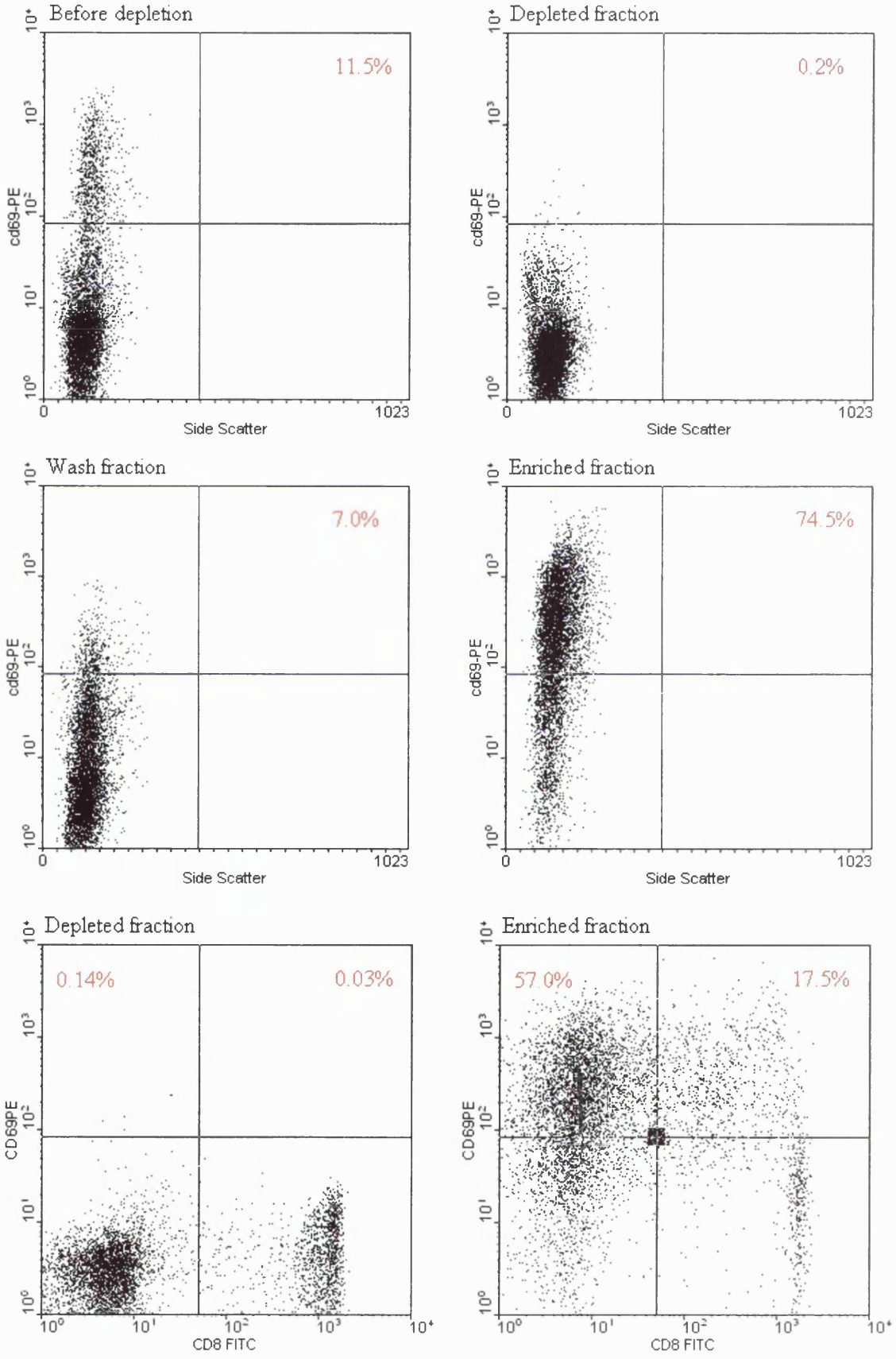
CMV assay	DJ	YM	LF
Tetramer: % of CD3+ cells	1.75	0.35	0.11
Tetramer: % of CD8+ cells	5	0.77	0.30
ELISPOT: no/2x10^5 PBMCs	320	90	25
% of CD3+ cells in PBMCs	60	75	70
ELISPOT: % of CD3+ cells	0.27	0.06	0.018

1x10^5 cells within the lymphoid gate were acquired flow cytometrically for the tetramer binding assay. For the ELISPOT assay, 2x10^5 PBMCs were placed into each 96-well plate and the no. of γ-IFN secreting cells calculated. Results were presented as the mean of 3 triplicate wells.

6.3.3 Results for the retention of anti-CMV reactivity

The result of allodepletion of a representative experiment is shown in Figure 6.3. The first four dot-plots are SSC vs CD69PE and the last two CD8-FITC vs CD69PE. It illustrates the efficiency of allodepletion with only 0.03% CD8+CD69+ alloreactive cells left in the depleted fraction. The efficiency of allodepletion was 97% from 11.5% CD69+ prior to sorting and 0.2% post depletion. As can be seen from the enriched fraction, the alloreactive cells removed included CD8+ cells

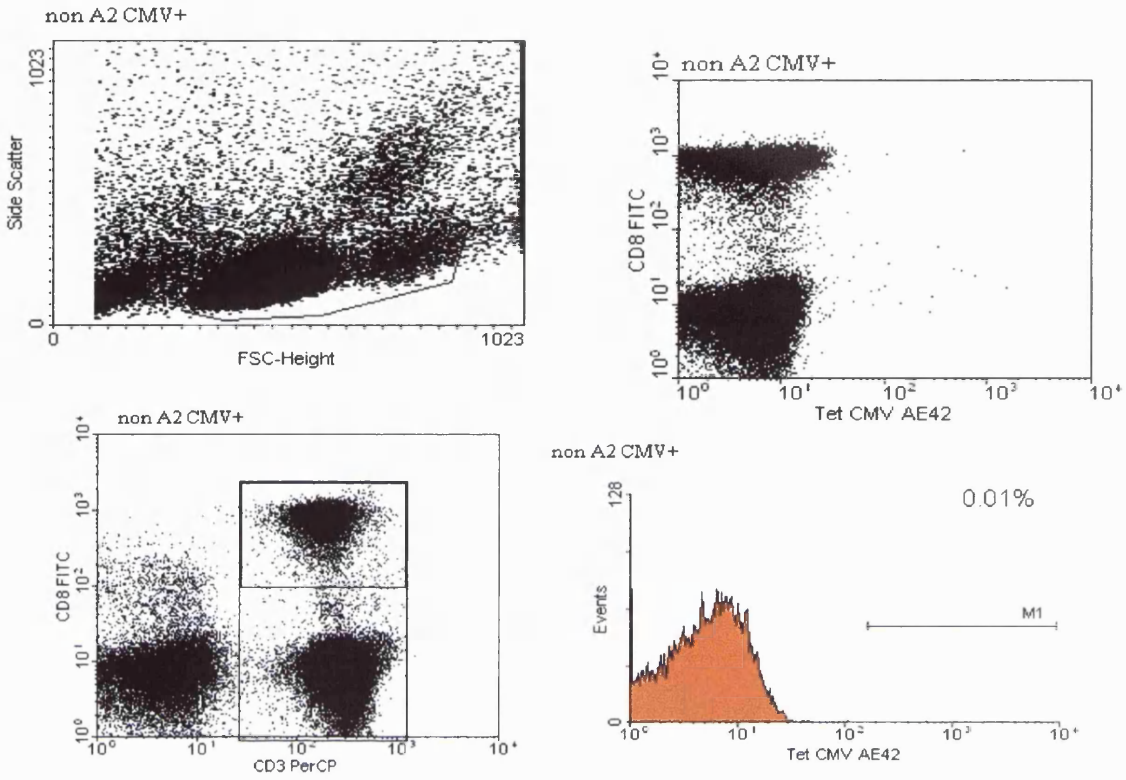
Figure 6.3 Efficiency of depletion of alloreactive cells using the MACS device



Figures 6.4 to 6.6 illustrates the finding that selective removal of alloreactive cells does not impair the specific anti-CMV response. Figure 6.4 demonstrates the specificity of tetramer binding in an HLA-restricted fashion. CMV+, T cells from a non HLA-A2 individual did not bind to the CMV-peptide 42 as indicated by only 0.01% being tetramer positive on the histogram. In contrast, fresh PBMCs from donor DJ who was CMV+ and HLA-A2+ demonstrated clear binding of the tetrameric-peptide complex as shown in Figure 6.5. A discrete cluster of CD3+8+ T cells was bound to the tetrameric-peptide complex appearing on the upper right quadrant on a tetramer vs CD8-FITC dot-plot (Figure 6.5). This population constituted the CMV peptide 42-specific T cells and was 1.61% of the entire CD3+CD8+ repertoire. Figure 6.6 shows the results of one representative experiment following selective depletion with CD69. The discrete population of CMV peptide AE42-specific T cells were still present following depletion. In vitro culture of donor cells in an MLC did not alter the capacity to bind to the tetramers and would not be a confounding factor in this set of experiments. When expressed as a percentage on a histogram, 1.46% of the total CD3+CD8+ cells were CMV-peptide 42 specific in the depleted fraction. Given the intraassay c.v. of this test is about 12%, this value indicates no loss in reactivity after allodepletion (1.61% prior to depletion). Similar results were obtained with other completely mismatched pairs in this series of experiments. A mean of 82.0 $\pm$ 12.2% was achieved in the preservation of anti-CMV response.

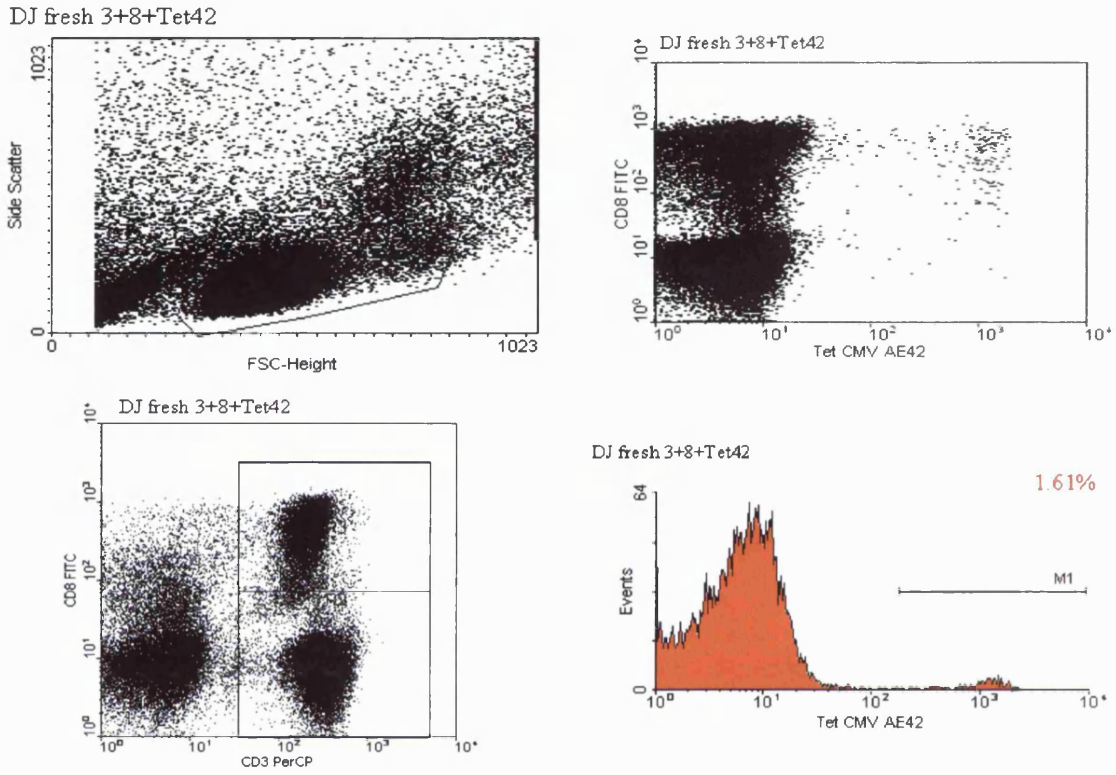
The same protocol and cell fractions obtained were analysed using the ELISPOT assay. The results of one representative experiment are shown in Figure 6.7. As can be seen, a non-A2, CMV+ individual (row B) showed no γ-IFN production regardless of whether the CMV AE42 peptide was fed to the wells. In contrast, the "enriched fraction" (row F) spontaneously produced γ-IFN irrespective of the presence of peptide. This could

Figure 6.4 Specificity of the tetramer assay: non-binding negative control



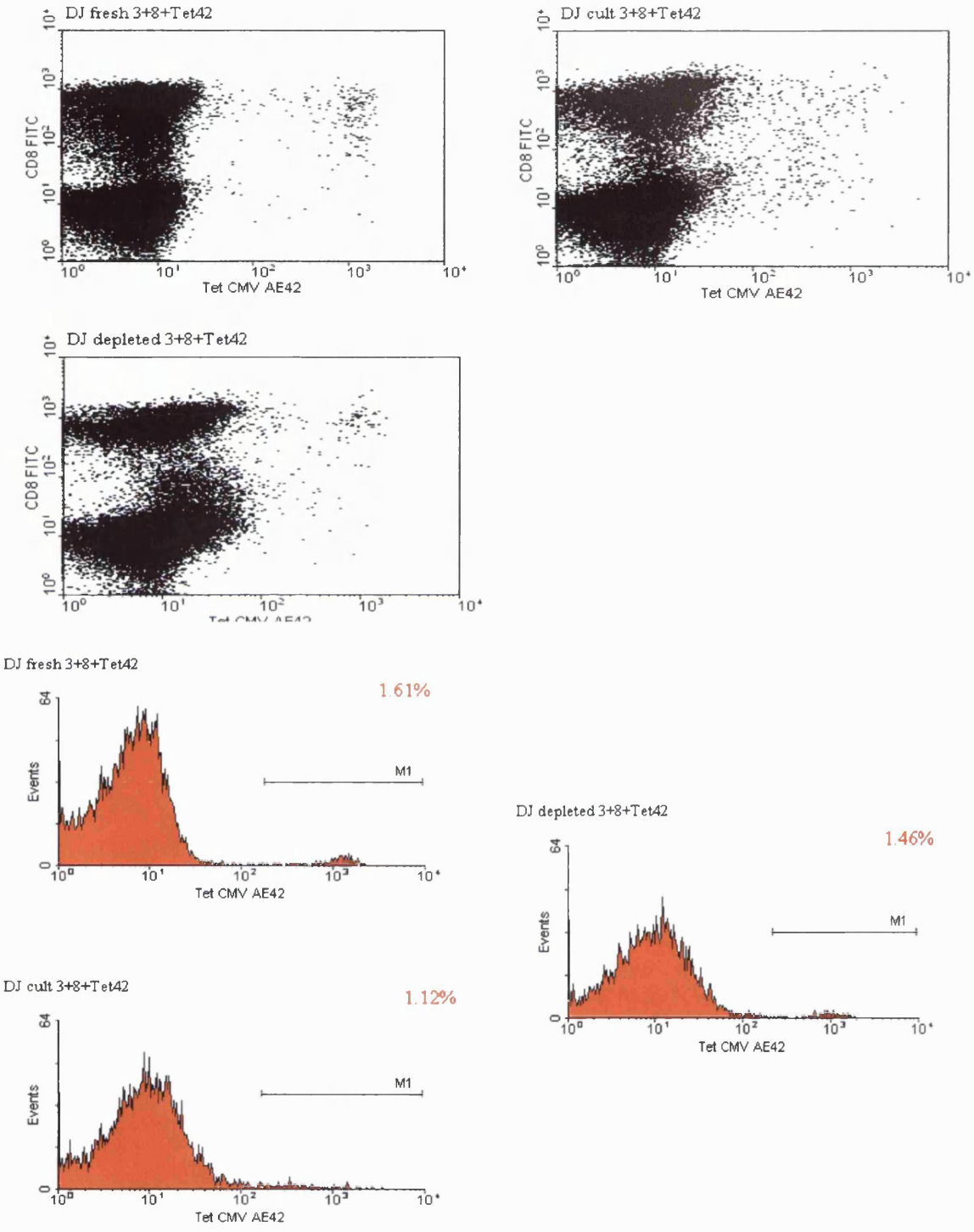
The acquisition-analysis method was as follows: 100000 events were acquired within the lymphocyte gate (R1) as defined on a FSC vs SSC dot-plot. Lymphocytes in R1 were then analysed on a CD8-FITC vs CD3-PerCp dot-plot. Only cells that fell within the upper right quadrant were considered in the tetramer analysis, that is cells which were CD3+CD8+ CTLs. This 2nd gate was termed R2. Taking only these CD8+ T cells into consideration (R2), a dot-plot of tetramers (PE stained) vs CD8-PerCp was analysed. Only CMV-specific CD8+ T cells that recognised AE42 peptide bound to the tetramers would appear in the upper right quadrant, being both CD8PerCp positive and tetramer staining positive. They were then presented as a percentage of total CD8+ve cell population.

Figure 6.5 Flow cytometric analysis of tetramer binding with fresh PBMCs from an HLA-A2+ CMV+ individual



The acquisition-analysis method was as follows: 100000 events were acquired within the lymphocyte gate (R1) as defined on a FSC vs SSC dot-plot. Lymphocytes in R1 were then analysed on a CD8-FITC vs CD3-PerCp dot-plot. Only cells that fell within the upper right quadrant were considered in the tetramer analysis, that is cells which were CD3+CD8+ CTLs. This 2nd gate was termed R2. Taking only these CD8+ T cells into consideration (R2), a dot-plot of tetramers (PE stained) vs CD8-PerCp was analysed. Only CMV-specific CD8+ T cells that recognised AE42 peptide bound to the tetramers would appear in the upper right quadrant, being both CD8PerCp positive and tetramer staining positive. They were then presented as a percentage of total CD8+ve cell population.

Figure 6.6 Retention of anti-CMV activity after CD69 allodepletion:



The upper three dot-plots of tetramer (PE) vs CD8-FITC show a discrete population of CMV AE42 peptide reactive CTLs in the upper right quadrant with fresh PBMCs from the donor. This discrete population was preserved after allodepletion and percentages are given in the lower 3 histograms. In-vitro culture did not affect the assay.

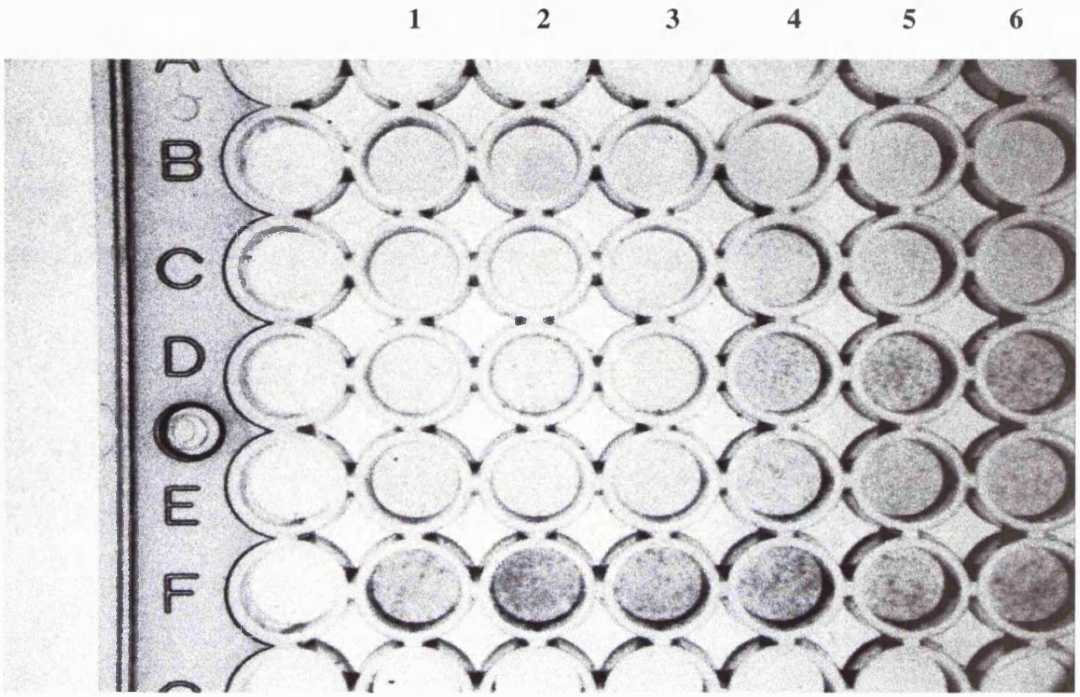
be explained by the fact that these were the alloreactive cells that had been subjected to alloantigen stimulation and were already producing γ-IFN as a result. Hence, all wells were positive. For "cultured cells" (row C), "fresh unmanipulated cells" (row D) and the "depleted fraction" (row E), there was only γ-IFN production when peptide was fed to the wells indicating a specific CMV response. Table 6.3 shows the results obtained with two sets of experiments. It can be seen that the frequency of CMV AE42 peptide reactive cells as measured by the ELISPOT assay was 5.5-7.2 fold less than the corresponding tetramer assay. It was also consistent in that the trend paralleled that of the tetramer binding cells. As measured by the ELISPOT assay, the mean preservation of anti-CMV response was 92.1 $\pm$ 6.4%.

Table 6.3 Results of ELISPOT assay to determine retention of anti-CMV activity

Cell. fraction	**No. of spots/2x10^5 PBMCs**	**Freq. of CMV AE42 specific CD8+ cells expressed as a %**
Fresh cells (1)	68	0.097
Depleted cells (1)	67	0.085
Cultured cells (1)	48	0.069
Fresh cells (2)	150	0.29
Depleted cells (2)	112	0.28
Cultured cells (2)	88	0.20

This table shows the results of two independent experiments (1) and (2). All results were initially expressed as no. of spots/2x10^5 PBMCs. As the proportion of CD8+ cells in the PBMCs is known, the result was also expressed as a % of the CD8+ cells for easy comparison with the tetramer assay

Figure 6.7 Using the ELISPOT assay to assess retention of specific anti-CMV activity



The ELISPOT assay was set up as described in Section 6.2.7. All cell fractions were set up in triplicate. Columns 1-3 were wells where no peptide was added while columns 4-6 were wells fed with CMV AE42 peptide.

Row B: the negative control (non-A2, CMV+ individual).

Row C: unmanipulated cells left in culture

Row D: fresh unmanipulated cells

Row E: "depleted fraction"

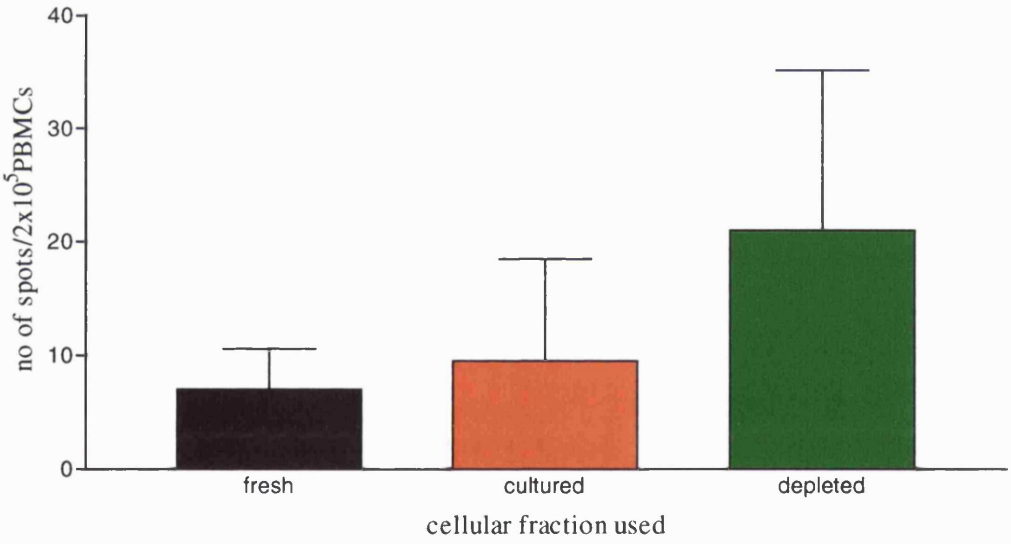
Row F: "enriched fraction"

All values were expressed as the mean of triplicate values.

6.3.4 Results for the retention of anti-EBV reactivity:

Figure 6.8 shows the result obtained with the ELISPOT assay for the retention of anti-EBV reactivity. The baseline frequency of CTLs reactive against the EBNA3A derived peptide SVRDRLARL was almost 10 fold less when compared against the corresponding results for the CMV AE42 peptide. There was also a doubling in the frequency of reactive CTLs after depletion.

Figure 6.8 ELISPOT assay for the frequency of EBV-reactive CTLS to EBNA3A derived peptide SVRDRLARL



The ELISPOT assay was set up as described in Section 6.2.7. All cell fractions were set up in triplicate. The cellular fractions used:

fresh: fresh unmanipulated cells

cultured: unmanipulated cells left in culture

depleted: depleted fraction post sorting

All values expressed as mean ± s.d.

6.4 Discussion:

The work presented in this chapter has demonstrated the retention of anti-CMV and anti-EBV activity after CD69 allodepletion. This implies that the lymphocytes mediating anti-viral immunity can be effectively distinguished from those mediating GvHD. The relatively high frequency of responding CD8+ve cells (0.3-5%) to CMV was in keeping with the results reported by other groups for CMV (4.4%) and EBV (5.5%) (Tan et al. 1999), (Jin et al. 2000). In fact, there was a predominance of CMV-pp65- specific CTL even when co-infection with HIV and EBV was present in a cohort of individuals studied (Jin et al. 2000). This was felt to be related to the fact that only CMV infected monocytes and macrophages, thus allowing for more efficient presentation and greater CTL generation. The frequency of responding cells to EBV reported in this chapter as measured by the ELISPOT assay was almost ten-fold less than that found in CMV. Unlike CMV where the major response is to the pp65 protein, both latent and lytic proteins for EBV are known to elicit specific CTL responses leading to a broader antigenic repertoire. Moreover SVRDRLARL used may not be the dominant peptide and this was also the finding elsewhere (Tan et al. 1999) where SVRDRLARL elicited a weaker response than the lytic protein derived HLA-A2 restricted peptide GLCTLVAML. The observed frequency was similar to that described here.

Limiting dilution assays (LDA) used to be the standard for quantifying the frequency of responding cells as in the CTLp assay. They however require relatively large quantities of PBMCs and relie on 2 rounds of in-vitro stimulation to detect T cell responses (Sharrock et al. 1990). The advent of tetramer technology has not only allowed direct visualisation of CTL effector cells by means of flow cytometry but it can quantify the

frequency of these CTLs without use of in vitro culture, a necessary and laborious step in LDAs. Tetramer staining is also a quick and reproducible process, with results obtainable within a day, if not hours, providing a stock of tetramers that has been batch tested is ready. The method easily stains large numbers and can also detect rare cells. It is specific and most investigators have thus far reported no cross-reactions (McMichael & Kelleher 1999). Using multi-parametric flow cytometry, it is also possible to further characterise these CTL effector cells in terms of other cell surface markers. One possible drawback may be that tetramers measure the frequency of CTLs to only one particular epitope and a negative binding may not necessarily indicate an absent response. Hence, individuals who show an absence of tetramer staining may in fact possess CTLs capable of recognising other epitopes or proteins not tested in the assay. In EBV for instance, HLA-A*0201 may not be a major presentation allele if HLA-A11 is also present as has been demonstrated at a population level (Rickinson & Moss 1997). Another disadvantage is the necessity to manufacture specific tetramers for every HLA molecule and its corresponding peptide studied whereas in a LDA, the entire virus (CMV infected fibroblasts) could be used as stimulators without knowing what the dominant epitopes was. A third drawback is that recently activated CTLs can downregulate their TCRs and therefore not bind to the tetramers. MHC tetramers may therefore be more useful in quantifying epitope-specific CTLs in subjects with known HLA types like BMT patients while the traditional LDA method is more useful to examine the breadth of CTL to a large number of antigens.

The ELISPOT assay used here measures γ-IFN production as a functional readout for T cell activity. This is not the case with tetramers and it has been reported that some tetramer-staining CTLs appeared functionless (Zajac et al. 1998). All three methods are therefore complementary. The ease and reproducibility of the ELISPOT and

tetramer assays opens up the possibility of being able to rapidly screen individuals and donors for their levels of CMV and EBV-specific T cell immunity and to test the leukocyte product prior to infusion.

A higher frequency for tetramer staining compared to the ELISPOT assay (4.4 times) and to the LDA (22 times higher) has been the finding of most studies (Jin et al. 2000), (Tan et al. 1999). This was in keeping with our results. LDAs measure T cells which have to both proliferate and exhibit cytolytic activity in the killing assay. The higher frequencies seen with the ELISPOT assay is because this detects all T cells that produce γ-IFN and not all of these would necessarily go on to proliferate and demonstrate cytotoxic function. The highest frequencies are seen with tetrameric staining. The only requirement here is that the CTLs possess and express the correct TCR for binding, Not all of these CTLs that bind to the antigenic peptide would go on to produce γ-IFN (as measured by the ELISPOT) or proliferate (LDA). Nonetheless, there exists a good and firm correlation between tetrameric complexes, the ELISPOT assay and the LDA (Tan et al. 1999).

Interestingly, when tetrameric HLA-mHag complexes were examined and their relationship with GvHD studied (Mutis et al. 1999a), the frequencies of mHag-specific CTLs were also found to be much higher than previously reported (10.4% at the time of GvHD).

The relationship between CMV and GvHD has already been mentioned. It can be envisaged that some of these CTLs may be cross reactive with allo-HLA as was demonstrated by Burrows et al with a CTL that recognised an HLA B8 restricted EBV derived epitope and an alloantigen B*4402 (Burrows et al. 1994). If this was translated

into a clinical scenario with a B8, CMV+ donor and a B*4402 recipient, a CMV reactivation post transplant would result in expansion of these CTLs that would also demonstrate potent alloreactivity towards host tissue and thus resulting in GvHD. That was why it was important to establish whether removal of allospecific cells would also deplete lymphocytes that may possess cross reactive anti-viral activity (Burrows et al. 1994). The results demonstrate that nearly all anti-viral activity is retained and if any cross reactive epitope CTLs exist, it would form only a very small proportion (<5%).

DLIs have been used to treat PTLDs with success although the risk of GvHD remains substantial. This has led to the generation of virus-specific CTLs. This antigen-specific adoptive immunotherapy approach has been (Riddell & Greenberg 1995) translated into clinical trials with some success. It involved the isolation and ex-vivo expansion of CD8+ CMV-specific T cells from a CMV+ve donor using CMV (AD169 strain) infected autologous fibroblasts as stimulator cells (Li et al. 1994), (Walter et al. 1995). 4 escalating doses ($0.3\text{-}10X10^8/m^2$) were administered at weekly intervals with no major toxic effects and grade II GvHD developed only in 3/14 patients after therapy. Reconstitution of CMV-specific activity was documented. The main drawback was that large numbers of CMV-specific lymphocytes (10^8 to 10^9) had to be expanded in vitro, the time scale involved (at least 4 weeks) and the need for autologous CMV infected skin fibroblasts as this is the only cell type to support CMV replication in vitro.

This antigen-specific immunotherapy strategy has been applied successfully to prevent EBV-associated disease as well as treat some established cases of PTLDs with minimal GvHD (Rooney et al. 1995), (Heslop et al. 1996). The CTLs were generated from donor-derived T cells sensitised against autologous B cells transformed with the EBV

strain from the patient. As both PTLDs and CMV disease have a rapid, lethal course, this approach necessitates the establishment of these lines prior to transplant. This can be an expensive and logistically difficult approach, particularly as only a small proportion of transplant recipients develop EBV lymphomas.

Thus far, using CMV infected fibroblasts has meant that CTLs can only be generated from CMV+ individuals; work is currently being done to determine whether APCs fed with whole CMV, pp65 or peptides can generate responses from CMV-ve donors. One could also vaccinate the donor with CMV vaccines to generate a cytotoxic CMV specific response. Alternatively, the possibility of generating CTLs from a universal donor or other donors with similar HLA type. But the logistics of finding another donor with the same HLA type is difficult and a universal cell pool seems unlikely to retain only CMV specificity. EBV would pose less of a problem because the infection is ubiquitous and almost all the donors would be EBV+ve.

These strategies involving infusions of virus specific cells however would not address the more general problem of impaired immune reconstitution leading to other infections (besides CMV and EBV) including HHV6 and adenovirus. The predominant CD8 population also means a lack of CD4 T cell help. The advantage of the CD69 allodepletion strategy is that the non alloreactive lymphocyte fraction contains CD4 cells and most likely, T cells capable of recognising other viruses.

The nagging question remains however as to whether these donor derived HLA-restricted CMV and EBV specific CTLs would be capable of recognising viral antigens in the context of HLA-disparity in a mismatched transplant. Lymphoid progenitors developing within the host's thymus might initially be restricted by host-unique HLA

determinants and relatively incapable of recognising EBV antigens presented on donor-derived EBV-transformed lymphocytes. The allodepletion strategy would also bypass this potential problem because there would be a pool of mature donor-derived EBV reactive T cells restricted to donor-unique HLA determinants available and capable of mounting a response.

The results presented in this chapter confirm that antiviral immunity is preserved after specific removal of alloreactive cells and encourages further development of the technique

7. Chapter 7. Testing the CD69 allodepletion strategy in a NOD/SCID murine GvHD model

7.1 Introduction:

In order to translate the CD69 allodepletion strategy into a clinical trial, it was appropriate to test this in an animal system to address any possible concerns regarding safety and in-vivo efficacy. Mice, rats and dogs are the most often used animals in bone marrow transplantation studies. Of these, mice have been the experimental animals of choice. They are easy to handle, have a rapid breeding cycle and are genetically well characterised with their MHC and mHags extensively studied.

7.1.1 The SCID mouse:

An autosomal recessive mutation resulting in severe combined immunodeficiency disease (SCID) developed spontaneously in a strain of CB-17 ($H\text{-}2^d$) mice (Bosma et al. 1983) and they were henceforth labelled as SCID mice (also known as C.B-17-scid or C.B-17Sz-scid/scid.) This spontaneous mutation is in a gene encoding DNA protein kinase catalytic subunit (NA-PKcs) which is on the centromeric end of chromosome 16. These CB-17 SCID mice fail to develop mature T and B cells and consequently are severely immunocompromised. They are able to initiate the recombination process of VDJ rearrangement: synapsis occurs between the DJ segments and double stranded

breaks are properly introduced at the juncture of the recombination signal sequences. Joining of the coding sequences, however, occurs some distance from the D and J segments resulting in deletion of one or both of the coding sequences. SCID mice have functional RAG-1 and 2 enzymes, their defect is instead in one or more double-strand break repair (DSBR) enzymes that rejoin cut DNA, resulting in an inability to resolve immunoglobulin and normal TCR gene rearrangement as well as an increased cellular sensitivity to ionising radiation (Bosma & Carroll 1991).

As they are severely immunocompromised, they have to be housed in a sterile, germ-free environment. The advantage however, is that these mice readily accept foreign grafts and cells from other strains or even other species and these cells are able to develop normally and survive in the SCID mouse. Although the T and B cell compartments are severely impaired, NK activity in SCID mice remains intact which can prove to be a barrier for successful engraftment (Barry et al. 1991). They have little or no serum Ig; the lymph nodes, spleen and thymus are abnormally small and virtually devoid of lymphocytes. All of the lymphoid organs consist primarily of vascularised supportive tissue and variable numbers of fibroblasts, histiocytes and macrophages. The *scid* mutation does not affect the differentiation of myeloid cells and macrophage activation and antigen presentation are unimpaired. However, it must be remembered that only mature T and B cells are absent: SCID and NOD/SCID mice contain normal numbers of double negative thymocytes but no single positive cells or CD3+ cells which would indicate functional rearrangement. The B cell maturation is arrested at the pro-B cell stage because SCID mice can still be transformed by the Abelson murine leukaemia virus which predominantly infects and transforms pro-B cells (Phillips & Spaner 1991).

The drawbacks in using the SCID mice are first, they retain NK cell function which can serve as a barrier to engraftment and second, the leaky phenotype which develops in between 2-20% of the mice which is not inherited and may reflect a low rate of somatic reversion at the *scid* allele. If maintained under germ-free conditions, the homozygous SCID mouse shows no abnormal external characteristics.

7.1.2 The NOD/SCID mouse:

The NOD/SCID mouse arose out of attempts to bypass the drawbacks of the SCID mouse. The inbred non obese diabetic (NOD) strain, first developed by S. Makino is characterised by a deficiency of complement, molecularly undefined defects of macrophage function (defective regulation of CSF-1 and γ-IFN and reduced secretion of IL1), decreased numbers of high affinity IL2 receptors, depressed syngeneic mixed lymphocyte reactions and impaired NK function (Shultz et al. 1995). The susceptibility to diabetes lies in chromosome 17 which is also where the MHC resides and has been provisionally designated as Idd-1. In addition, the NOD mouse has an increased number of T lymphocytes under the control of a separate gene not linked to the MHC locus leading to the abnormal accumulation of T cells in peripheral lymphoid tissue.

The NOD/SCID mouse (NOD/LtSz-scid) was developed by backcrossing the *scid* mutation (C.B-17/Sz-scid) onto the NOD/Lt strain background (NOD/Lt substrain of NOD/shi) and therefore incorporating these defects in innate immunity to those of the SCID mouse mentioned above. It is therefore congenic to the NOD strain in being similar to the NOD mouse in all respects except for the *scid* mutation. As such, both NOD and NOD/SCID mice have a unique H-2^{g7} haplotype. The NOD-SCID mouse like the NOD exhibits a whole spectrum of immunoregulatory defects. There is impaired

antigen presentation due to the instability of the Class II I-A^{g7} molecule and poor peptide binding capacity. It is also substantially lacking in NK cell activity although not completely absent (Steele et al. 1997), (Goldman et al. 1998) and there are suggestions of impaired cytokine regulation (Serreze & Leiter 1988). The NOD/SCID mouse has proved superior to the SCID mouse in terms of leukaemia engraftment (Steele et al. 1997). Unlike the NOD strain which spontaneously develops insulin dependent diabetes mellitus in 80% of females and 20% of males, the NOD/SCID mouse shows no such predilection and is as robust as the SCID mouse. It also does not exhibit the "leaky" phenomenon associated with the SCID mouse although it does develop thymomas (Shultz et al. 1995) (Goldman et al. 1998). These thymomas are associated with the expression of a NOD mouse unique endogenous ecotropic murine leukaemia provirus locus (EMV30) not expressed in the standard substrain NOD/Lt thymus. It is proposed that the *scid*-imparted block in thymocyte development synergises with the unusual features of T cell ontogeny and function (Prochazka et al. 1992) of the NOD strain and leads to the activation of the NOD unique EMV30.

7.1.3 Reports of GvHD in SCID and NOD/SCID mice:

The NOD/SCID mouse was chosen for the development of a model of allogeneic GvHD. This was because both the NOD/SCID and the SCID mouse have proved to be excellent tools for the study of human haematopoiesis and in human leukaemia models to study the process of leukaemogenesis and spread (Uckun 1996), (Bhatia et al. 1998), (Steele et al. 1997). Reports in the literature regarding allogeneic GvHD are sparse in the SCID mouse and non-existent in the NOD/SCID mouse. The initial aim of this chapter was therefore to investigate the factors necessary for the setting up of such a murine model.

It is interesting to note that in the context of the SCID and NOD/SCID models, investigators have reported a surprisingly low incidence of xenogeneic GvHD (Hoffmann et al. 1993), (Mosier 1990) even in the instance of intra-peritoneal injections (i/p) of human peripheral blood lymphocytes of up to $50x10^6$ splenocytes. The question posed was whether fundamental immunological differences inhibit the development of GvHD between such distant species. It has, however been suggested that the seemingly low incidence of xenogeneic GvHD could in part be explained by the failure of the human lymphocytes to engraft (Hoffmann et al. 1993). In SCID mice that do demonstrate good engraftment, fatal GvHD did develop. This was contrasted by a paper reporting on the occurrence of a milder non-fatal xenogeneic GvHD which was delayed in onset (Goldman et al. 1998). The characteristics of xenogeneic GvHD are also different histologically (van Bekkum 1993).

Reports of allogeneic GvHD in the SCID mouse have been more consistent. 5 or $10x10^6$ cells injected intraperitoneally into unconditioned mice from a major MHC mismatched donor (C57B6; H-2^b) resulted in the development of GvHD at day 5 with the liver, spleen, intestines, skin and tongue as target organs (Hoffmann et al. 1993). It was therefore speculated that in contrast to xenogeneic cells, allogeneic lymphocytes might migrate better, growth might be better sustained and antigen presentation more matched. The variability in the reports on allogeneic GvHD in the SCID mouse have been related more to differences in the cell dose administered, role of irradiation and mode of administration of donor cells; issues explored in the setting up of a murine GvHD model in this chapter.

When allogeneic GvHD in the SCID mouse was studied in greater detail (Phillips & Spaner 1991), it was found that both CD4+ and CD8+ cells were required to cause the characteristic pathology: CD4+ cells alone delayed the onset while CD8 cells alone produced no GvHD. In a xenogeneic GvHD and GvL system (Xun et al. 1995), aGvHD, cGvHD and GvL were found to be separable phenomena and mediated by different effector cells. NK cells produced only a very mild aGvHD but a marked GvL effect, T cells alone resulted in cGvHD, no aGvHD but marked GvL while a mixture of NK and T cells induced severe GvHD.

7.1.4 Role of irradiation in GvHD in SCID and NOD/SCID mice:

While in other murine models, sublethal doses of 600-900 cGy are regularly given as part of the transplant procedure, the SCID mouse is much more sensitive. Allogeneic GvHD has been described in unconditioned SCID mice (Hoffmann et al. 1993) and R Sakai investigated the effect of sublethal irradiation (200 cGy) on the development and tempo of GvHD (Sakai et al. 1997). They found that intravenous injection (i/v) of mismatched splenocytes after sublethal irradiation induced a fatal GvHD. Without irradiation, $25x10^6$ splenocytes induced only a mild GvHD with no fatality. This was corroborated by Xun who reported that transplantation of $50x10^6$ splenocytes intravenously immediately after sublethal irradiation of 400cGy was essential for the development of lethal acute GvHD (mice developed diarrhoea) (Xun et al. 1994). If the infusion of cells was delayed to day 5 or TBI omitted, they developed GvHD which was not fatal. This is probably because, TBI increases serum levels of TNF-α, IL1 and IL6 and this 'cytokine storm' interacts with effector cells to produce lethal GvHD. Moreover, although both the SCID and NOD/SCID mice are severely immunodeficient, they nonetheless possess a certain amount of natural immunity as

well as a certain degree, albeit impaired, of non-lymphoid resistance systems such as macrophages and NK activity. Sublethal irradiation may therefore be important to suppress these non-specific rejection mechanisms and permit full engraftment of the allogeneic lymphocytes (Fulop & Phillips 1986), (Shpitz et al. 1994).

With regard to the dose of sublethal irradiation, Goldman et al administered i/p injections of xenogeneic lymphocytes into NOD/SCID recipients irradiated with 350cGy (Goldman et al. 1998) . Van der Loo explored this issue more thoroughly and found that all animals died between 1-2 weeks after a dose of 450cGy or higher (van der Loo et al. 1998). At 350-400cGy, a late mortality between 35-50 days was observed in 20% of the animals whereas if the dose was reduced to 300cGy, all animals survived for at least 8 weeks. An intriguing result suggested that irradiation converts the form of GvHD from a chronic to an acute type (Phillips & Spaner 1991). In a completely mismatched setting, transplantation of large numbers of lymph node cells led to fatal chronic GvHD with death between 40-60 days but the addition of 400cGy irradiation to the protocol converted this to an acute fatal GvHD with death between 12-14 days. In both models, transfer of syngeneic lymphocytes resulted in 100% survival. When they analysed the GvHD here compared to normal mice, they found that both CD4+ and CD8+ donor lymphocytes were required to induce fatal GvHD: a mixture of purified CD4+ and CD8+ produced the same clinical picture as unpurified lymph node cells. If CD4+ cells were given alone, the onset of GvHD was delayed to 20 days while CD8+ cells alone produced no detectable GvHD. This was in contrast to normal mice where in a complete mismatched setting, either CD4+ or 8+ cells were sufficient in themselves to cause fatal GvHD (Sprent et al. 1990), (Korngold & Sprent 1985). Perhaps the inability of CD8+ cells alone to induce GvHD was due to the absence of host CD4+ cells to provide help.

7.1.5 Effect of the route of administration of donor cells on GvHD in SCID and NOD/SCID mice:

The route of administration of donor cells seemed to be an important variable in the development of GvHD in the SCID and NOD/SCID mouse. Fatal GvHD has been observed with both an i/p or an i/v route (Hoffmann et al. 1993), (Sakai et al. 1997), (Xun et al. 1994) although Tscherning (Tscherning et al. 1991) described the absence of GvHD following i/v injection of allogeneic mismatched cells (C57B6) into SCID mice despite clear evidence of engraftment. In contrast i/p injection of $20x10^6$ splenocytes induced fatal GvHD in all recipients within 3 weeks (Claesson et al. 1991). The allogeneic cells also demonstrated stable engraftment even at 8 weeks post transfer. The liver showed extensive mononuclear infiltration in the portal area with hepatocyte necrosis in the surrounding parenchyma while the lungs showed leukocyte infiltration in the peribronchial tissue. Other tissues affected were kidney, spleen and colon which demonstrated moderate mononuclear infiltration but no epithelial destruction. It was speculated that i/v injections might induce host anti-donor tolerance in contrast to the non-tolerising i/p route. This difference in outcome could also be explained by differences in draining and circulatory pathways of the donor cells and therefore in the initial alloantigen presentation. The peritoneal cavity drains into the thoracic paraaortic lymph nodes while cells administered via the i/v route home to the spleen (Claesson et al. 1991). The same was observed by other investigators when i/v injections of up to $40x10^6$ completely mismatched donors cells from CBA mice into SCID mice resulted in tolerance while the i/p route caused fatal and progressive GvHD (Renz et al. 1996), (Fraziano et al. 1994). Surh also observed this i/v tolerance and attributed this to the depletion of host APCs with irradiation and tolerance from thymic epithelial cells (Surh et al. 1997). The only contradictory report (Reddy et al. 1994)

was the transfer of splenocytes from NOD or Swiss mice into SCID mouse (80-100x10^6 for i/p and 40x10^6 for i/v route) resulting in more consistent and aggressive GvHD for the i/v compared to i/p route. These mice were however not irradiated and were also given growth hormone. John Dick has extensively studied human haematopoiesis in immunodeficient mouse and has encountered little GvHD except via the i/p route into newborn SCID mice who suffered from infiltrates in skin, liver and gut (Dick et al. 1991). That the i/p route can induce xenogeneic GvHD has also been reported by other groups (Bankert et al. 1989), (Krams et al. 1989), (Purtilo et al. 1991). In summary, it appears that the i/p route is probably more effective in inducing GvHD.

7.1.6 The SCID mouse in allogeneic GvHD prevention strategies:

The SCID mouse has been used as an allogeneic model of GvHD to explore GvHD prevention strategies (Vallera et al. 1996). The model used purified C57BL/6 (H-2^b) lymph node cells injected intravenously into MHC disparate SCID (H-2^d) mouse in the absence of irradiation. Irradiation was avoided so that they could examine GvHD effects in the absence of irradiation toxicity. The GvHD strategy involved the construction of a truncated form of the diphtheria toxin linked to an anti-CD3sFv chain recognising the CD3ε present on all T cells. Survival studies showed that while control mice suffered 83% mortality by day 80, administration of the immunotoxin improved survival to 86% although histologically, GvHD was still evident. The pattern of GvHD observed included infiltrates in the liver, colon, kidney and pancreas as well as crypt dropout in the intestines and interstitial pneumonitis in the lungs. The SCID mouse model was also used by the same investigators to test a combined ex-vivo and in-vivo CD40-40L blockade approach (Blazar et al. 1997a), (Blazar et al. 1998).

There are as yet, no reports in the literature using the NOD/SCID mouse as a model for allogeneic GvHD prevention strategies. The NOD/SCID mouse should prove superior to the SCID mouse because the greater immunosuppression inherent in the former would allow for better engraftment. It is also a useful model to study GvHD because of the absence of detectable host lymphocytes. It is still uncertain whether host lymphocytes play any major role in the pathogenesis of allogeneic GvHD. By eliminating any interaction between donor and host lymphocytes, any component of GvHD that may be critically dependent on this interaction would be missing and this difference may manifest in the severity of GvHD, the subsets involved , the time frame and the target organs. Extensive study of murine models of GvHD other than in immunodeficient mice have already provided us with invaluable information towards the greater understanding of the complexity of the disease process (Korngold and Sprent 1999). This NOD/SCID model would therefore complement other well defined murine models in trying to dissect the various processes and interactions that eventually lead to the development of allogeneic GvHD.

7.2 Materials and methods:

7.2.1 Mice strains used and the MHC systems:

The mice strains used in the set of experiments presented here were: CBA, BALB/c, NOD and NOD/SCID (also known as NOD/LtSz-scid/scid or NOD/LtSz-scid).

The MHC system in the mouse is called the H2 complex, located on chromosome 17. It is organised into a number of regions encoding Class I, II and III gene products with the former 2 being the classical MHC molecules. The Class I complex consists of regions K ,D, L Qa and Tla with the latter two considered as non-classical The Class II region is denoted by IA and IE.

Because many laboratory mice strains are inbred, they are homozygous for all alleles as both parental haplotypes are identical. Hence, they can be designated simply by their haplotypes. The mice relevant to the project and their corresponding haplotypes were:

CBA:	haplotype k ($H2^k$):	$K^k D^k IA^k IE^k$	
BALB/c:	haplotype d ($H2^d$):	$K^d D^d IA^d IE^d$	
SCID:	haplotype d ($H2^d$):	$K^d D^d IA^d IE^d$	congenic to C.B17-($H2^d$)
NOD:	haplotype I-A^{g7}($H2^{g7}$) :	$K^d D^b IA^{g7} IE^{null}$	
NOD/SCID	haplotype I-A^{g7}($H2^{g7}$):	$K^d D^b IA^{g7} IE^{null}$	congenic to NOD-($H2^{g7}$)

The g^7 haplotype consists of a class II I-A^{g7} α-chain identical to the I-A^d α-chain while the β-chain is unique in that it has Ser at position 57 whereas others share Asp at this position. The I-Aβchain of NOD differs from the I-Aβ^d by 5 amino acids. As a result of this substitution, the I-A^{g7} molecule binds peptides with a negatively charged

carboxy terminal not bound by other MHC molecules. The I-Eαis identical to the H-2^b but includes a deletion within the first exon of the Ea locus such that no I-E chains are produced (Serreze & Leiter 1994).

Thus, the donor CBA strain is completely mismatched to the recipient NOD/SCID mice while the BALB/c mouse is partially matched.

7.2.2 Housing and husbandry of animals:

The NOD-SCID mice were bred and maintained in pathogen free conditions in the animal facility (Western Laboratories) at Institute of Child Health. This colony of NOD/SCID mice was established in December 1995 from breeding pairs obtained from the Ontario Cancer Institute, Princess Margaret Hospital, Toronto. They were housed in sterile microisolator cages in a horizontal laminar-flow cabinet system called a Maximiser (Thoren. Hazleton, Pa.) and given pre-packed autoclaved sterile food, water (Harlan, UK) and bedding. A maximum of 5 were housed per cage. All manipulations were carried out in a laminar flow hood. The overall purpose was to minimise exposure to potentially life threatening infections which may also alter and confound experimental results. Mice maintained in this way (single user, SPF and barrier protected room) could survive to 9-12 months of age. Other precautions that were undertaken to prevent introduction of pathogenic viruses and bacteria included:

i) strict limitation of staff authorised to enter the NOD/SCID colony facility

ii) records kept of all procedures

iii) sterile overshoes and foot disinfectant on entry

iv) gown, mask, hat and sterile gloves for all handling procedures

v) autoclaved or chemical sterilisation of all equipment used for procedures

vi) mice showing signs of infection humanely sacrificed along with cage mates.

SCID and NOD/SCID mice were bred without difficulty and breeding pairs produced an average of 6 litters per female, with 6-10 offspring per litter, during their reproductive lives. Young mice were weaned at 21-24 days. Mice selected for the experiment were between the ages of 6-10 weeks. As for the rest of the stimulator (NOD and BALB/c) and donor (CBA) mice, these were maintained under normal laboratory conditions, fed normal food and water ad libitum.

7.2.3 Harvesting of splenocytes:

For the setting up of MLCs, lymphocytes from donor (responder) and recipient (stimulator) mice were obtained by harvesting of their spleens. This was done by humane sacrifice of 6-10 week old mice with concussion followed by cervical dislocation. This, also called Schedule 1 killing ensured that mice were quickly and painlessly sacrificed. Using aseptic techniques, the peritoneal cavity of the mouse was then exposed and the spleen removed by teasing out its capsule and cutting off its connecting stalk of vessels. Any remaining capsular, fibrous or fatty tissue was then carefully teased away using a pair of fine surgical forceps. A few methods were tried in order to achieve optimal mononuclear cell recovery and cell viability.

The first method involved cutting the spleen into smaller pieces and placing them into an autoclaved wire mesh. Sterile cell suspensions were then prepared by gently pressing the spleen fragments against the mesh with the aid of a piston from a 5ml disposable syringe. Although initial cell recovery was good, this method was found to be too traumatic and cell viability was not optimal with considerable cell death after 24 hours in culture. The second method involved puncturing the spleen at one end with a syringe and 21G needle and gently perfusing it with HBSS. This was done repeatedly

and the splenocytes flushed out. This method was better in terms of viability but suffered from poorer yield. For both methods, density gradient separation and RBC lysis wash were also tried to obtain an optimal cell suspension but neither made a significant improvement. The third method proved to be the best in terms of cell recovery and viability. Here, a small part of the spleen was cut at one end with a pair of surgical scissors. With the aid of 2 pairs of watchmaker forceps, one holding the spleen firmly down at one end, the curved end of the second was used to gradually tease the lymphocytes out. This was done repeatedly using a gentle and smooth action leaving the hollow splenic capsule behind. With this method, there was no need for further density gradient separation, extra spinning or RBC lysis. Clumps of debris were allowed to settle out or alternatively, the lymphocytes were passed through a 100*u*m filter to remove any large clumps and a single cell suspension obtained. Viability was checked using trypan Blue exclusion and the cells counted.

7.2.4 Culture media used:

A variety of media were tried to obtain optimal culture conditions:

RPMI1640 with Glutamax (Life Tech), 5% Penicillin/Streptomycin (P/S) (Gibco) and 10% Foetal Calf Serum (FCS)(Gibco)

Dulbecco's modified eagles medium DMEM/F12 (Life Tech) with 5% P/S, 10% FCS, 25uM HEPES and 50uM 2-Mercapto-ethanol (2ME)

Both of these media however did not prevent cell death from occurring in short term culture and the optimal culture medium was found to consist of the following: Iscove's modified Dubelcco's Medium (Sigma IMDM I3390) containing 25uM HEPES and NaHCO3 with the following added: 10% myoclone (FCS), 10mls of 200mM L-glutamine (T7153, Sigma), 1.74ul 2-ME (50uM), 0.25mls of transferrin (Sigma T1428,

10mg/ml), 5% penicillin/streptomycin. All cell culture work was therefore performed and maintained under these conditions.

7.2.5 Setting up of the NOD/SCID allogeneic GvHD model:

The four variables thought to be important in determining the pathogenesis and characteristics of GvHD were:

i) degree of histoincompatibility

ii) cell dose

iii) mode of administration

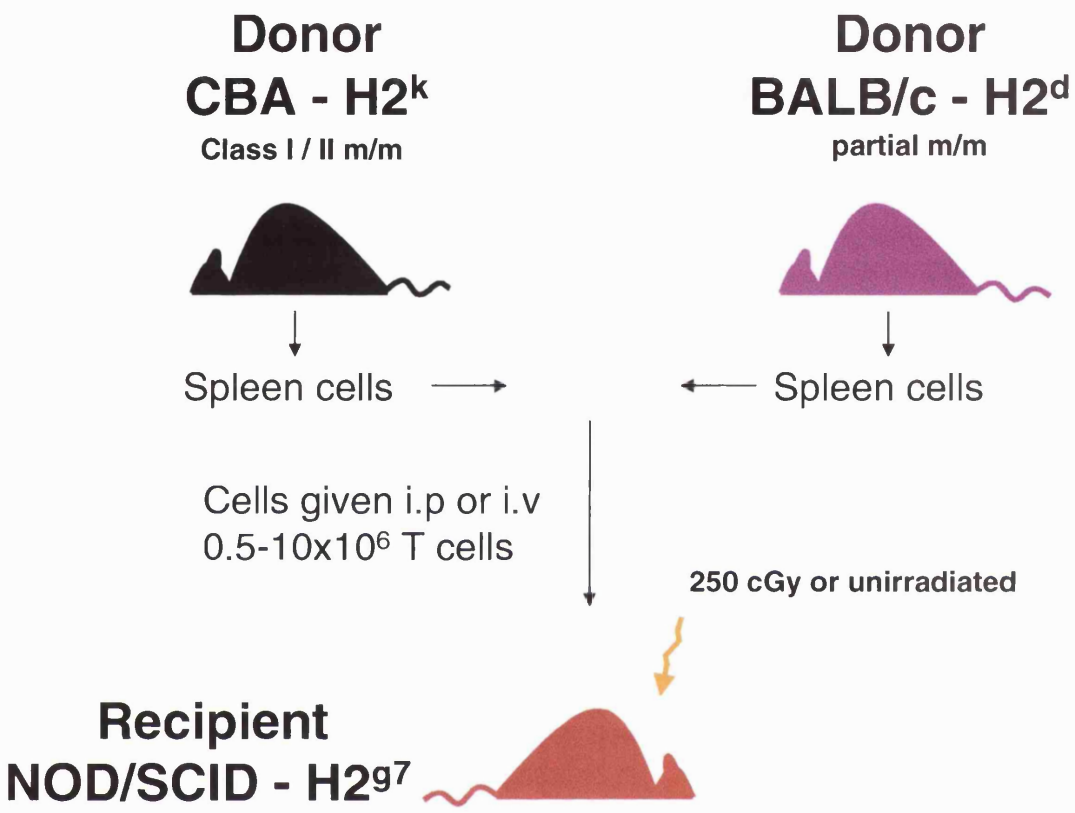
iv) role of irradiation

Preliminary experiments were therefore conducted to investigate the role of these four variables and the protocol illustrated in the Figure 7.1. In all experiments, each mouse was individually marked using an indelible pen by a series of spots on the tail, and re-marked when necessary. All cages were also meticulously labelled indicating the number of mice and procedure undertaken.

7.2.6 Murine CD69 allodepletion strategy:

The allodepletion strategy for the prevention of GvHD is illustrated in Figure 7.9. It is similar to the in-vitro strategy in that alloreactive lymphocytes were identified by the presence of murine CD69 on its surface after in vitro culture in an MLC and subsequently removed by para-magnetic sorting. The protocol is detailed fully in the Section 7.3.4 as it could only be devised after determining the optimal conditions for setting up an allogeneic GvHD model.

Figure 7.1 Protocol for setting up the NOD/SCID allogeneic GvHD model:



The basic protocol illustrated above involved the administration of donor splenocytes into a recipient NOD/SCID mouse. The variables were:

i) completely mismatched (CBA) cells or partially mismatched (BALB/c) cells

ii) range of cell doses 0.5-10x10^6 T cells

iii) i/p or i/v route of administration

iv) non-irradiated recipient or sublethally irradiated with 250cGy

7.2.7 Paramagnetic bead (MACS) sorting:

The CD69 positive alloreactive lymphocytes were removed by paramagnetic bead sorting using a MACS 'AS' depletion column similar to that described in section 2.2.4. Murine cells in the MLC were labelled either with CD69-FITC (ImmunoKontact or Pharmigen) or CD69-PE (Pharmigen) to identify CD69 positive alloreactive cells.

7.2.8 Intraperitoneal injections for the administration of cells:

Donor cells in suspension were aspirated into a 1 ml syringe attached to a 25G needle. Mice were grasped by the scruff of the neck with the left hand, then rotated in the same hand with the little finger used to restrain the tail, and hence movement of the trunk and hind legs. The peritoneal cavity was thus exposed and the mice readily settled into this position. The spine of the animal was then gently extended to both force abdominal organs posteriorly and to tense the abdominal skin. After the area was swabbed, the cells were then inoculated using the right hand into the right or left flank and briskly injected. This method caused little stress, involved a sterile technique and no anaesthesia was required. Due to the large size of the peritoneum, there were few limitations on the number of cells that could be inoculated using this route.

7.2.9 Intravenous injections:

The i/v route was used to compare against the intraperitoneal mode of inoculation. Prior to inoculation, the mouse was exposed to an infra-red lamp suspended over its cage for approximately 5 minutes. This resulted in considerable vasodilatation of its tail veins and facilitated inoculation. The mouse was then suspended above a hollow

Perspex tube mounted on a stand by its tail and by reflex, it crawled into the tube. Once in position, it was restrained at its head end by a flexible bung designed with an air hole, whilst the tail was restrained and exposed by a moveable shutter. There were 4 veins through which intravenous injection could be attempted: 2 lateral, one dorsal and one ventral vein. Once suitably restrained, the end of the tail was grasped with the left hand and slightly tensed. Donor cells in suspension were aspirated into a 1 ml syringe attached to a 25G needle. This was then introduced into the vein and as parallel as possible with the vein, special care being taken as they were extremely superficial and fragile. If cannulation was successful, blood could be aspirated as the syringe plunger was pulled and one could then proceed to inject the donor cells into the vein.

7.2.10 Tail vein sampling:

The same technique used for intravenous injections described in the previous paragraph was equally applied to tail vein sampling. About 50-150ul of blood could be slowly aspirated from the veins into a syringe lined with 10ul of preservative free heparin, appropriately stained and analysed flow cytometrically.

7.2.11 Cardiac puncture:

Tail vein sampling, although relatively simple, yielded at most 150ul of blood and could also cause trauma and deformation to the lymphocytes. A larger sample of peri-mortem blood for flow cytometric analysis could be obtained just prior to schedule 1 termination of the animal. This was done by exposing them to rising concentrations of CO_2 (starting at 2L/min) and when unconscious just prior to death, aspirating blood from the cardiac chamber by percutaneous puncture of the chest wall just under the

xiphisternum. Confirmation of death was ensured by cervical dislocation of the neck. This method could yield up to 500-1000ul of blood.

7.2.12 Assay of engraftment:

It was important to establish that donor allogeneic cells injected engrafted in the NOD/SCID recipient mouse. Otherwise, this could lead to erroneous interpretation of results. This was done by tail vein sampling, described above, at weekly intervals commencing 1 week post injections looking for onset and durability of engraftment. This was confirmed by flow cytometric analysis of the sampled blood. Monoclonal antibodies used were anti-CD3, anti-CD4, anti-CD8 and anti-H-$2k^k$ (CBA). Control mice receiving i/p PBS were used as the negative control. As NOD/SCID mice inherently lack T cells, presence of a population of CD3+, CD8+ or CD4+ cells would indicate their donor origin and successful engraftment. The detection of H-$2k^k$ lymphocytes using anti-H-$2k^k$ antibodies would also indicate the donor origin (CBA) of the cells.

7.2.13 Assessment of animals and the end point of experiment:

All experimental animals were independently observed and assessed by the animal technicians who would recommend termination by humane means if the animal was deemed to be too ill, either from GvHD or other causes. Otherwise, the endpoint of the experiment was decided at 70 days after injection of allogeneic cells and all animals, even if well were humanely sacrificed at this point. Termination of the animals, termed schedule 1 killing involved either exposing them to rising concentrations of CO_2 or concussion followed by cervical dislocation.

7.2.14 Assessment of animals for signs of GvHD:

GvHD was assessed in 2 ways: clinical and histopathological. Clinically, all animals were observed carefully everyday for classical signs of GvHD which included the following: hunched posture, loss of appetite, poor fur texture and inactivity and as far as possible, weighed every week ensuring that they remained within a sterile environment. To avoid any bias, these were independently checked by the animal staff who had no prior knowledge of what treatment each NOD/SCID mouse received. The clinical observation and assessment of GvHD was confirmed by post mortem histopathological examination. The organs and tissue samples collected were spleen, kidney, liver, ileum, colon, skin and lungs. All organs were placed in formalin and examined independently by the histopathologist who again had no prior knowledge of what treatment each animal received.

7.2.15 Monoclonal antibodies and flow cytometry:

T cells were identified by anti-CD3 PE mAbs (clone KT3; Harlan Seralab or clone 17A2; Pharmigen). 4.0ul was added for every 10^6 cells (concentration 0.2mg/ml). For T cell subsets: anti CD4-FITC (clone GK1.5) and anti CD8-PercP (rat anti mouse, clone 53-6.7) were used (both Pharmigen). 2ul of the FITC mAb (concentration 0.5mg/ml) and 4ul of the PerCp (concentration 0.2mg/ml) were added for every 10^6 cells. NK cells were identified with anti CD56-FITC (clone DX5; Pharmigen). Different mouse NK cell antibodies work on different strains and DX5 has been demonstrated to be effective in the CBA mouse strain. 2ul of the mAb (concentration 0.5mg/ml) was added for every 10^6 cells. For the staining of activated cells, anti-CD69-FITC (ImmunoKontact and Pharmigen) and anti-CD69-PE (Pharmigen) were

used. All 3 antibodies were from the same clone H1.2F3 and derived from hamster anti-mouse. 2ul of the FITC mAb (concentration 0.5mg/ml) and 4ul of the PE (concentration 0.2mg/ml) were added for every 10^6 cells. The CBA mouse strain is characterised by the MHC haplotype H-2^k and identification of lymphocytes belonging to the CBA strain was done using anti H-2^k-FITC (clone 36-7-5; Pharmigen). 2ul of the mAb (concentration 0.5mg/ml) was added per 10^6 cells.

Some samples were also pre-incubated with F_c block (0.2mg/ml; Pharmigen and 4ul added for every 10^6 cells) at 4°C for 5 minutes. This F_c block or CD16/CD32 ($F_c\gamma$III/II receptor) reacts specifically with a common non-polymorphic epitope on the extracellular domains of the mouse $F_c\gamma$III(CD16) and $F_c\gamma$II(CD32) receptors expressed on NK cells, granulocytes, monocytes and B cells. The purpose was to eliminate any non-specific binding of the antibodies via their F_c receptors to the $F_c\gamma$ bearing cells resulting in high background level. There was no difference with the addition of the F_c block and hence, in later staining, that step was omitted altogether.

The antibody staining protocol and acquisition / analysis by flow cytometry have been described in Material and Methods. For tail vein and cardiac puncture samples, the additional step of FacsLyse was included. Careful titration of the above antibodies was essential as there was a smaller window between under and over-staining as compared with human cells. All recommended volumes listed were found to be the optimal ones.

7.2.16 Histopathologic examination:

Skin, colon, ileum, liver, spleen, kidney, lung were excised from dead animals and fixed in 10% buffered formalin immediately after autopsy. Sections of skin, ileum and

colon were taken from the same area from all animals. The tissues were then embedded in paraffin, sectioned at 4-6μm and stained with haematoxylin-eosin. The histologic analysis was performed by a pathologist blinded to the treatment regimens.

7.2.17 Statistical analysis:

Data were pooled from at least 5 separate experiments and median survival times calculated. Survival data were plotted by the Kaplan Meir method and analysed by the log-rank test. If two data sets were entered, the log-rank test was equivalent to the Mantel-Haenszel test. This test generates a P value testing the null hypothesis that the survival curves were identical in the study group vs the control. In other words, the null hypothesis was that the treatment, in this instance the allodepletion strategy did not alter survival. A p value<0.05 was considered significant and this value was two-sided.

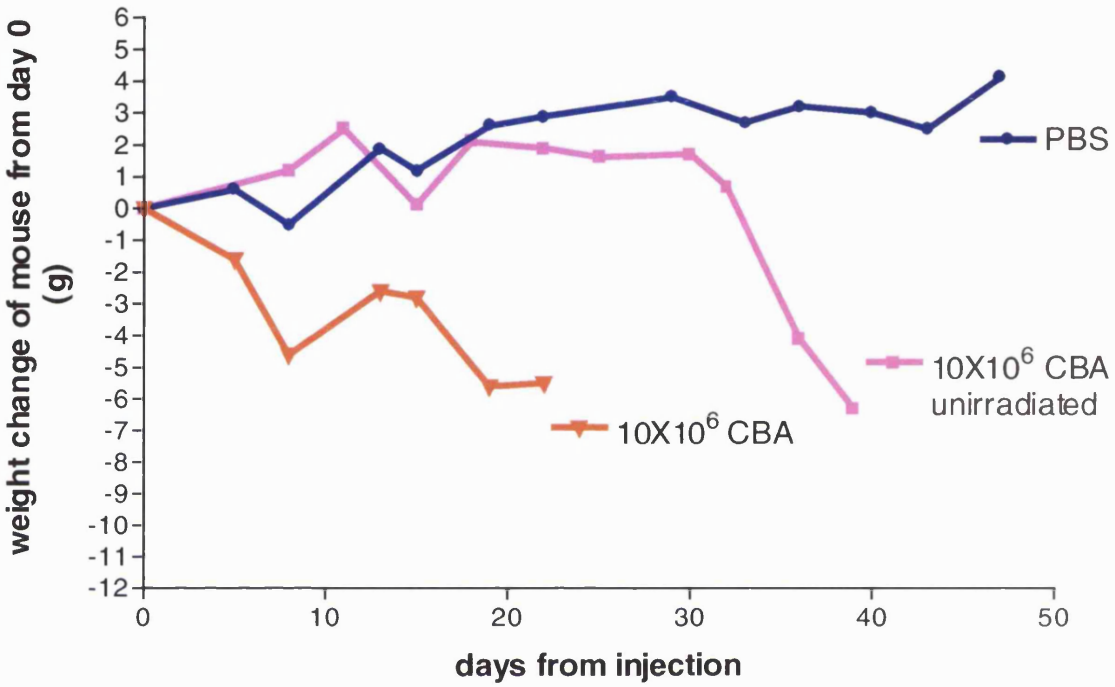
7.3 Results:

7.3.1 Setting up the NOD/SCID GvHD model- role of irradiation:

Preliminary experiments showed that the NOD/SCID mice were unusually sensitive to radiation and any dose above 350cGy was uniformly lethal. A lower, sublethal dose of 250cGy was therefore administered and in control mice given this dose alone, all except one survived to beyond d70 and were well and healthy. The mice were not ill at any time after irradiation and had also gained 4g (20% of its original body weight) during the observation period. The weight chart of a typical mouse sublethally irradiated and injected with PBS is shown in figure 7.2. For comparison, the weight of a similar mouse but receiving completely mismatched cells showed progressive weight loss over 3 weeks of up to 6g (30% of its original body weight).

The effect of sublethal irradiation at 250 cGy was investigated by comparing irradiated vs non-irradiated NOD/SCID mice receiving an i/p injection of $10x10^6$ complete MHC-mismatched allogeneic T cells from donor CBA mice as outlined in Figure 7.1 in Material and Methods. The irradiated mice demonstrated classical signs of GvHD as early as d7 with marked loss of weight, ruffled coat texture, inactivity and hunched posture and were sacrificed soon after. Histology confirmed the presence of GvHD in the target tissues. In contrast, the unirradiated recipient mice demonstrated a slower tempo of disease progression albeit it was still fatal. The median time to lethality was 18 days in the irradiated mice and 39 days in the unirradiated mice. This difference could not be attributed to graft rejection as both demonstrated evidence of engraftment with the donor lymphocytes being CD4/8 +ve and H-2^k+ve.

Figure 7.2 Weight chart comparing unirradiated NOD/SCID mice receiving MHC-mismatched CBA splenocytes, sublethally irradiated (250cGy) mice receiving PBS alone and irradiated mice receiving mismatched splenocytes



3 groups of NOD/SCID mice were compared:

i) unirradiated mice given an i/p injection of $10x10^6$ MHC-mismatched CBA T cells

ii) sublethally irradiated (250 cGy) mice given an i/p injection of PBS alone

iii) sublethally irradiated (250 cGy) mice given an i/p injection of $10x10^6$ MHC-mismatched CBA T cells.

Mice were weighed weekly to twice weekly and the change in weight noted. 0 on the y-axis refers to the weight at the time of injection of cells.

7.3.2 Setting up the NOD/SCID GvHD model- effect of the route of administration

The i/p route of administration was compared to the i/v route. The results indicated that the i/p route resulted in a quicker induction of fatal GvHD compared to the i/v route for sublethally irradiated (250cGy) NOD/SCID recipients receiving $10X10^6$ completely mismatched cells (CBA). The median survival was 18 days for the i/p route and 27 days for the i/v route. Histology of the tissues demonstrated no obvious difference in the pattern of infiltration.

7.3.3 Investigating the effect of varying cell dose and histocompatibility differences:

Preliminary experiments suggested that sublethally irradiated (250cGy) NOD/SCID mice given allogeneic lymphocytes via the i/p route provided the optimum conditions for the development of GvHD. The next step was to determine the cell dose needed and if a threshold for induction of GvHD existed as in human allogeneic transplantation where a T cell dose of $<1X10^5$ /kg in a matched setting eliminated the occurrence of GvHD in all cases (Kernan et al. 1986). Varying cell doses were injected via the i/p route: $10X10^6$, $5X10^6$, $1X10^6$, $0.5X10^6$ T cells (defined by CD3+ or CD4+ and CD8+)

Another question asked was how the degree of histoincompatibility would affect the tempo of GvHD in this animal model. To address this, T cells from a completely mismatched donor ($H\text{-}2^k$) were compared to a partially mismatched BALB/c mouse ($H\text{-}2^d$) which shares some similarity in its MHC to the NOD/SCID recipient (the K^d allele is shared by both while the I-A molecule is similar in its α chain and the β chain differing only by 5 amino acids). Moreover, the *scid* mutation of the NOD/SCID is derived from the CB-17 ($H\text{-}2^d$) strain. The same range of cell doses were used and the

protocol is outlined in Figure 7.1 (Material and Methods). Figure 7.3 illustrates the results obtained from this set of experiments. As a control, one set of recipient NOD/SCID mice was given donor lymphocytes from the NOD strain. As the NOD/SCID mice are derived from the NOD strain, this would almost constitute a syngeneic transfer of lymphocytes with a difference only at the *scid* locus mutation and any possible minor antigens that may be present at that locus.

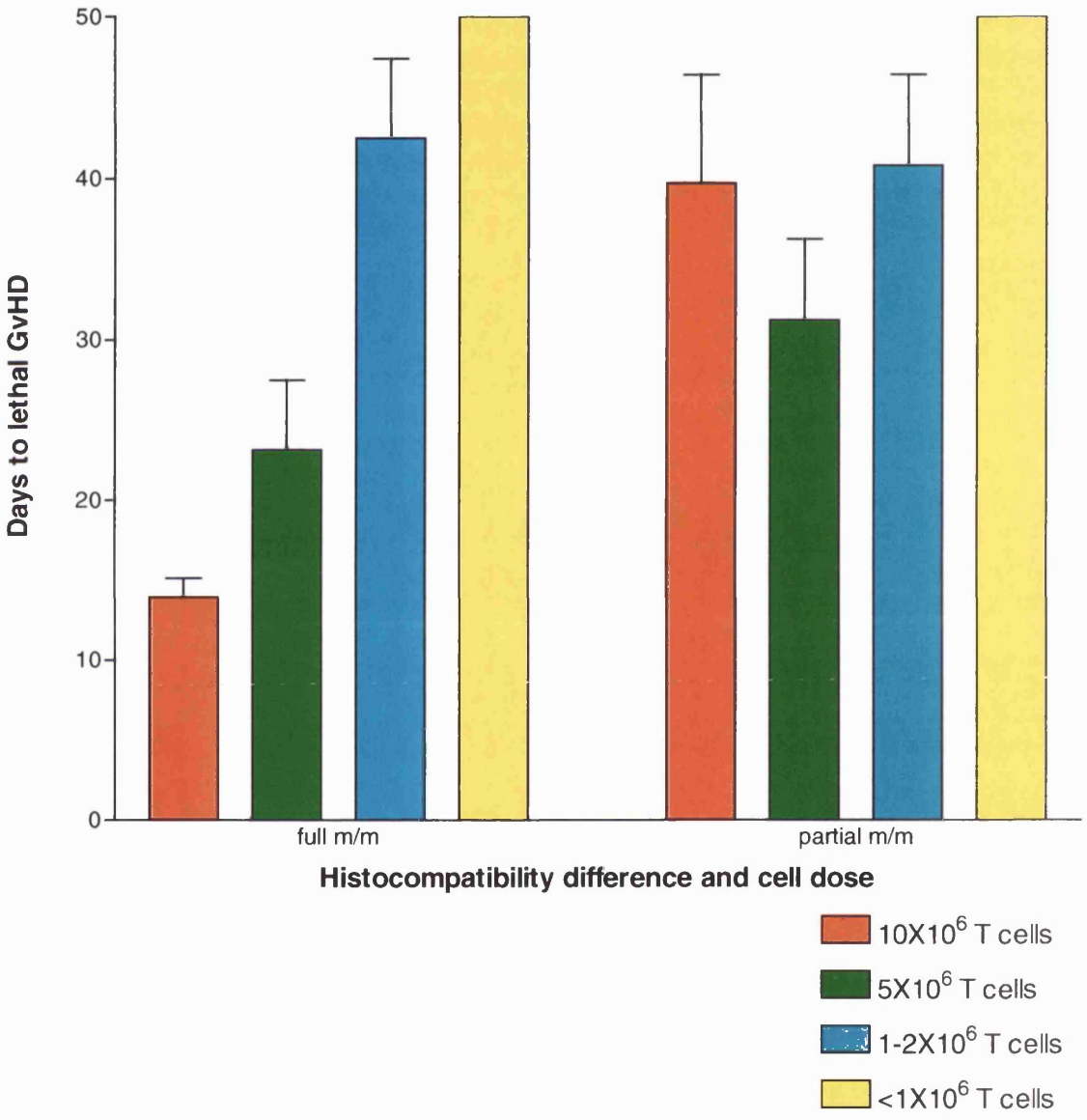
The bar chart in Figure 7.3 illustrates the following important points:

i) there was a threshold for the induction of GvHD. At T cell doses below $1X10^6$, all mice survived to d50 (end of observation period) and were well and healthy.

ii) In the completely mismatched model (CBA as donor), a clear cell dose effect existed in that i/p injection of $10X10^6$ cells induced a more rapid induction of fatal GvHD compared to $2X10^6$ T cells ($p<0.0003$). However the relationship was more complex than a simple linear one. As mentioned, a threshold existed but beyond a certain cell dose, the tempo of fatal GvHD was similar. Hence, $5X10^6$ induced a fatal GvHD with almost equal rapidity compared to $10X10^6$ T cells: 14 days ($10X10^6$) vs 23 days ($5X10^6$) which did not attain statistical significance on t-test ($p>0.05$).

iii) In the partially mismatched model (BALB/c as donor), the same trends were also present although not as clear-cut. All mice receiving a cell dose below $1X10^6$ remained well and healthy. For mice receiving greater than $2X10^6$ T cells, a cell dose effect was also present but significantly, although some of the NOD/SCID mice succumbed to GvHD, others were alive at the end point of observation. Those that survived exhibited poor weight gain, hunched posture and 30% developed fibrotic and scaly skin lesions that resembled chronic GvHD as shown in Figure 7.4.

Figure 7.3 Investigating the effect of varying cell dose and MHC histoincompatibility on the tempo of GvHD in NOD/SCID recipient mice



Sublethally irradiated (250 cGy) NOD/SCID mice received a range of cell doses from either a complete MHC-mismatched donor (CBA) or a partially mismatched donor (BALB/c) via the i/p route. The cell doses administered were $10x10^6$, $5x10^6$, $1\text{-}2X10^6$ and $0.5X10^6$ T cells (as defined by being CD3+ or CD4+ and CD8+). Results presented as mean $\pm$ s.d.

i) The degree of histoincompatibility was important in that for each given cell dose, the median survival was always shorter for mice receiving completely mismatched cells compared to partially mismatched cells. At a cell dose of $10X10^6$ T cells, the mean survival was 14 days (CBA as donor) vs 40 days (BALB/c as donor) which was highly significant (0=0.005) and for $5X10^6$ T cells, the survival was 23 days (CBA as donor) vs 32 days (BALB/c) (p=0.08).

ii) NOD/SCID (H-2^{g7}) mice receiving NOD (H-2^{g7}) donor cells were well and showed good weight gain after 70 days. There were no clinical or histological signs of GvHD.

7.3.4 Clinical features and organ distribution of GvHD:

The NOD/SCID mice exhibited classical signs of acute GvHD which were: weight loss, inactivity, hunched posture, loss of appetite and poor fur texture. The main histological changes were seen in the liver, lungs and gut. Other organs collected such as kidney, spleen and skin were reported as unremarkable by the histopathologists. Some animals suffering from acute GvHD were also noted to have exudative inflammation of the eye and this has been previously reported in the literature (Asai et al. 1998). The main target organs for GvHD were therefore similar to that of classical acute GvHD except for the absence of skin involvement. The pattern was less consistent with the partially mismatched cells as mentioned above. In 30% of mice receiving cells from the partially matched donor (BALB/c), lichenification changes of the skin was seen. It was thick, fibrotic and scaly as clearly shown in Figure 7.4 and resembled chronic GvHD. The areas most affected were the face, neck, tips of ears and around the eyes.

A gross specimen of the caecum (Figure 7.5) and histological sections of the target organs are shown in Figure 7.6, Figure 7.7 and Figure 7.8. Patchy single cell necrosis, crypt dropout and lymphocytic infiltration were noted in the gut. In the liver, there was evidence of endotheliatis and periportal lymphocytic infiltrate. The lung also showed evidence of endotheliatis and a lymphocytic infiltrate in the peribronchial and perivascular areas. The histological changes were however morphologically less severe than the clinical signs which was progressively lethal. It was also less extensive than that described in other murine GvHD models.

Figure 7.4 Fibrotic, scaly skin changes developing in NOD/SCID mice receiving partially mismatched donor splenocytes (BALB/c)

The picture shows two recipient NOD/SCID mice sacrificed at the end of the experiment. The mouse on the left is the negative control which received i/p PBS and remained well and healthy with no skin changes. The mouse on the right received $5x10^6$ partially matched BALB/c donor cells and it can be seen that fibrotic, scaly changes in the skin have developed around the facial area.

Figure 7.5: Caecum from a NOD/SCID mouse with acute GvHD

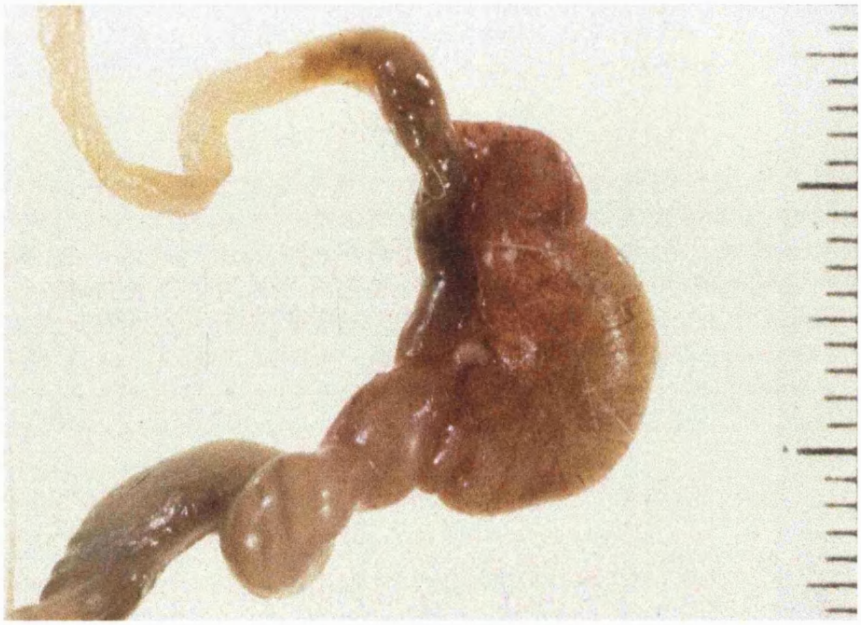


Figure 7.6: Section of the large intestine from a NOD/SCID mouse with acute GvHD

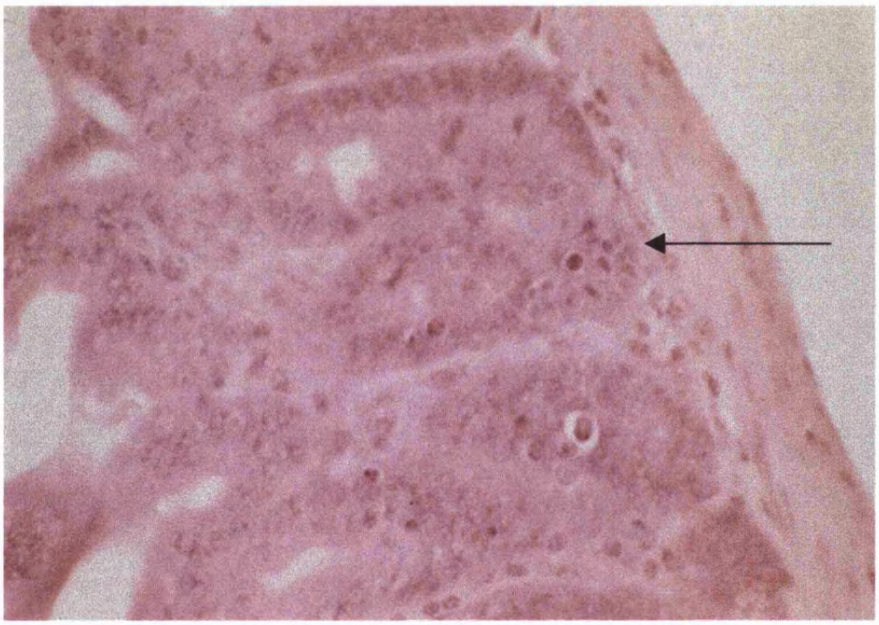


Figure 7.5 shows an acutely inflamed and oedematous caecum from a NOD/SCID mouse that had received $10x10^6$ CBA T cells. It was terminally ill and sacrificed. Figure 7.6 is a section of the intestine from another mouse receiving the same cells. Single cell necrosis and a patchy lymphocytic infiltrate (arrow) could be identified.

Figure 7.7: Section of the liver from a NOD/SCID mouse with acute GvHD

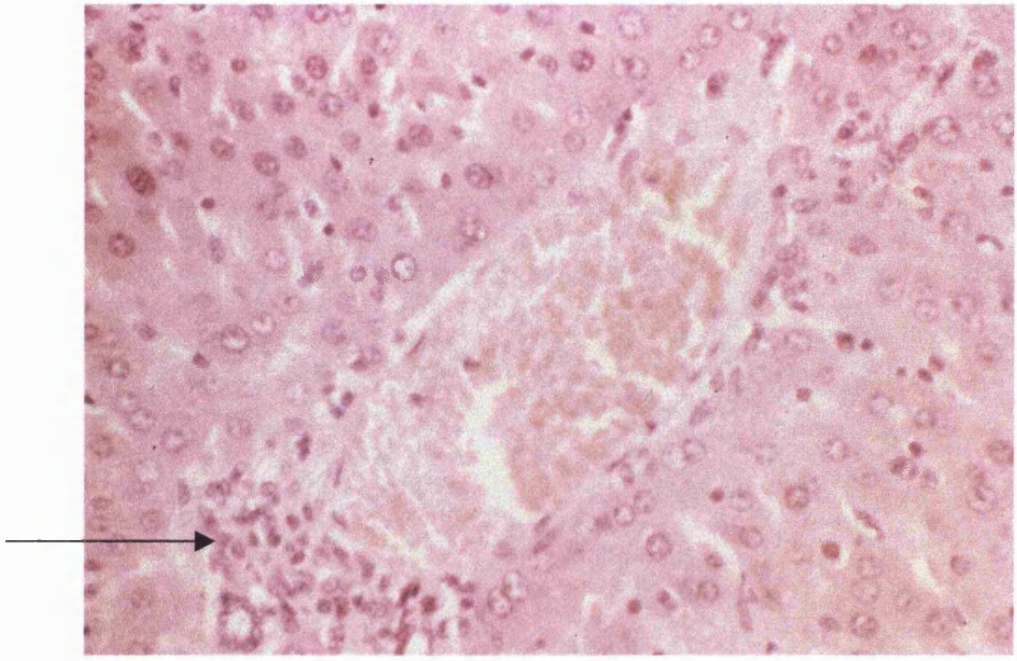


Figure 7.8: Section of the large intestine from a NOD/SCID mouse with acute GvHD

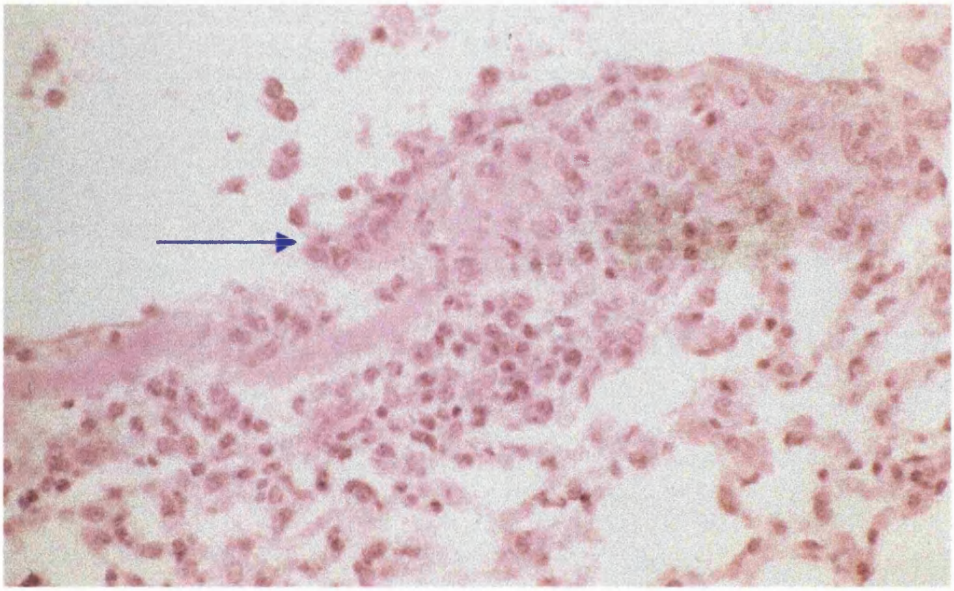


Figure 7.7 shows a section of the liver with evidence of endotheliatis and a moderate periportal lymphocytic infiltrate. The lung in Figure 7.8 shows evidence of endotheliatis and a lymphocytic infiltrate in the peribronchial and perivascular areas. Both sections were from mice given 510^6 mismatched CBA T cells and with clinical signs of GvHD.

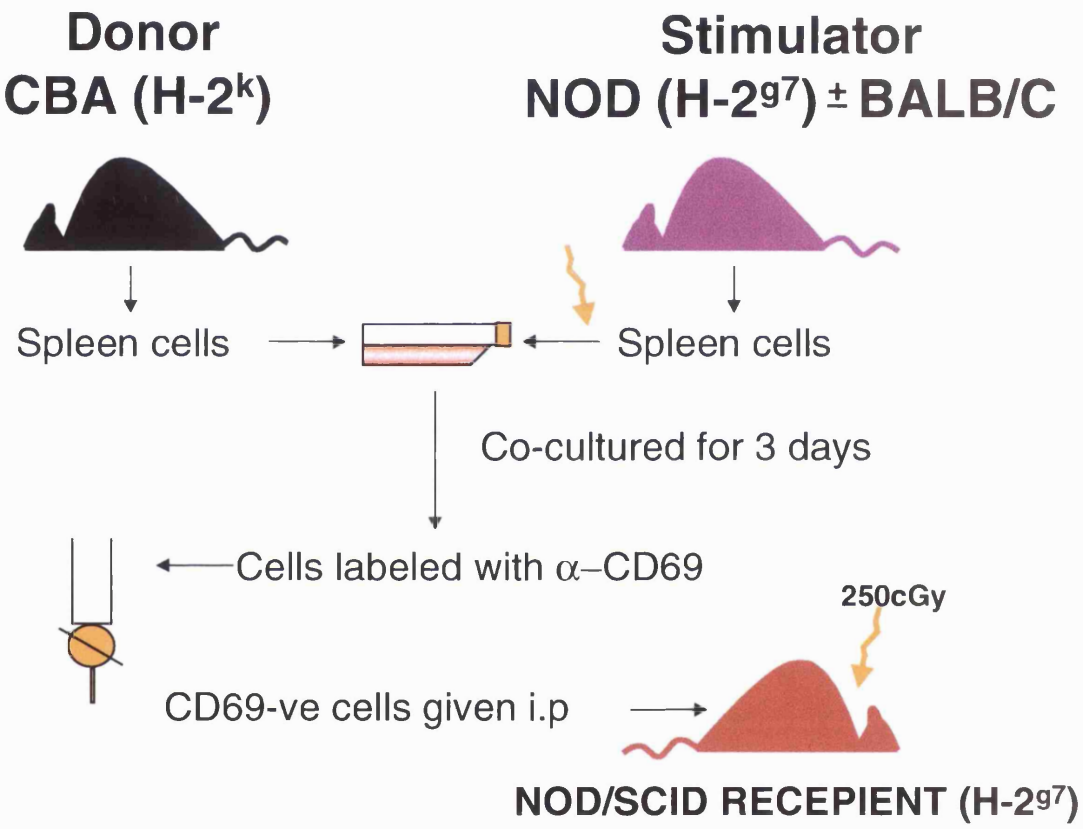
7.3.5 Results of GvHD prevention with the CD69 allodepletion strategy:

The murine system described above using completely mismatched donor cells (CBA) was shown to reliably induce lethal GvHD in recipient NOD/SCID mice and was therefore chosen to be the model of lethal GvHD. The aim was to examine if selective depletion of CD69+ alloreactive cells and subsequent administration of the non-alloreactive fraction into NOD/SCID mice would prevent GvHD and fatality.

The protocol devised is illustrated in Figure 7.9. Briefly, this involved collecting splenocytes from donor CBA (responder) and stimulator NOD mice. All cells were counted either with a haemacytometer or a coulter counter machine and resuspended in "CM" at $3X10^6$ cells/ml. A one way MLC was set up with donor and irradiated (33Gy) stimulators in equivalent numbers in Nunc 80ml culture flasks (each containing 25 mls of cells or $37.5X10^6$ responders and $37.5X10^6$ stimulators). The MLC was incubated at 37^0C with 5% CO_2. This is designated as time t=0 hours. In parallel, an autologous control was set up consisting of donor splenocytes and equal numbers of irradiated donor cells. In addition a tissue flask containing donor splenocytes alone at a similar concentration was also set up in parallel for use later in i/p injections.

Flow cytometric analysis was done at this stage to determine the baseline levels of CD4+, CD8+, CD3+, CD56+ and CD69+ lymphocytes. Based on time course experiments on murine CD69 expression in an allogeneic setting described later, the optimal time for the depletion experiments was found to be at 72 hours. At this time point, the MLC was harvested, activated alloreactive donor cells identified by CD69 expression and depleted using the MACS 'AS' columns as previously described.

Figure 7.9 Protocol for the murine CD69 allodepletion strategy in a complete MHC mismatched model



After depletion, 2 cellular fractions were obtained:

i) the depleted fraction depleted of alloreactive anti-recipient lymphocytes- termed **"the allodepleted fraction"**,

ii) the positive fraction that is enriched for CD69 containing the alloreactive cells- termed **"the enriched fraction"**.

In addition, 3 other cellular components were prepared:

iii) freshly isolated unmanipulated donor cells termed **"the unmanipulated fraction"**,

iv) donor cells that have been left in culture alone without alloantigen stimulation for an equivalent period of time termed **"the old donor fraction"** and

v) unsorted MLC fraction consisting of donor cells that have been in the MLC but not been passed through the 'AS' columns termed **"the old MLC fraction"**

All cells were counted and phenotyped prior to i/p injection into recipient mice.

NOD mice were chosen as stimulators (kindly provided by M. Peakman, Immunology, Kings College Hospital and P. Beale, Endocrinology, St Barts.) as they share the same MHC haplotype as the recipient NOD/SCID mice and the crucial component of the stimulator cells was in its MHC background. This was due to the technical difficulty of obtaining appreciable numbers of lymphocytes from the spleens of immunodeficient NOD/SCID mice. In initial experiments, a mixture of NOD and BALB/c stimulators were used with the rationale that as the scid mutation was derived from the BALB/c ($H\text{-}2^d$) background, adding splenocytes from the BALB/c strain would include any minor antigens that might be associated with the scid mutation and also present in the NOD/SCID mice. Later results showed no difference in terms of survival whether NOD or a mixture of NOD and BALB/c splenocytes were used as stimulators. This demonstrated that any minor antigens that could have been carried over with the scid

mutation were probably not clinically important in establishing an alloresponse which was also confirmed by the fact the NOD into NOD/SCID cell transfer did not produce any symptoms of disease as both were syngeneic.

The recipient mice were divided into 6 groups depending on the cells they received:

Group 1: mice receiving phosphate buffered saline (PBS) as a negative control

Group 2: mice receiving "the depleted fraction" which was the group of interest

Group 3: mice receiving "the enriched fraction" which was another group of interest

Group 4: mice receiving "the unmanipulated fraction" which was the positive control

Group 5: mice receiving "the old donor fraction"

Group 6: mice receiving "the old MLC fraction"

The optimum conditions for the induction of GvHD was employed: all groups received 5-10X10^6 T cells injected intraperitoneally into sublethally irradiated recipient mice.

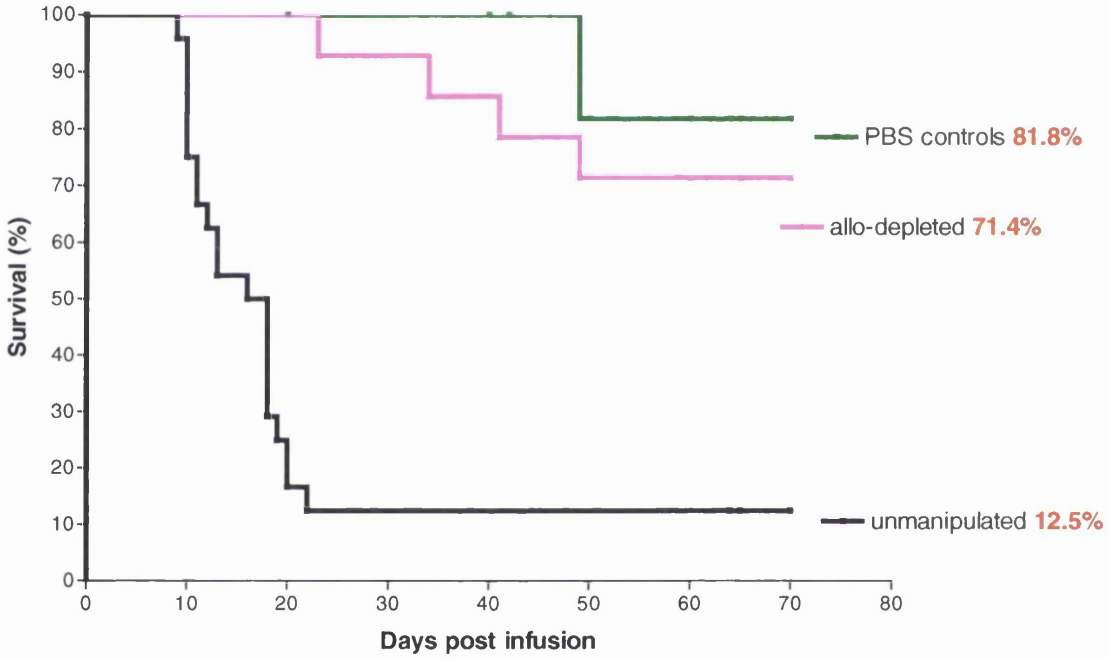
The number of mice in each group are listed below.

Group of mice	Group1: PBS control	Group2: Depleted fraction	Group3: Enriched fraction	Group4: Un-manipulated fraction	Group 5: Old donor fraction	Group6: Old MLC fraction
Nos.	18	14	5	27	5	2

The survival curves shown in Figure 7.10 demonstrated the efficacy of this depletion strategy. In group 1 (negative control), 16 out of 18 mice were well and healthy at d70. The two deaths were attributed to undiagnosed infection following manipulations undertaken in these mice (weights and tail vein injections) despite best efforts at sterility. Post mortem histological analysis of the organs revealed no evidence of GvHD. Hence, even in the negative controls where GvHD would not be expected to

occur, the survival rates were not 100% but 81.8%. This was important when comparing the results against that of the allodepletion strategy. In the positive control (group 4), 24 out of 27 mice (87.5%) succumbed to lethal GvHD with a median survival of 18 days. Of the three surviving mice at d70, histology of tissue sections showed clear signs of GvHD in two of the three giving an incidence of GvHD of 96.3%. In contrast, NOD/SCID recipient mice receiving allodepleted CD69-ve cells (group 2) demonstrated a clear survival advantage over unmanipulated cells. 10 out of 14 mice (71.4%) remained well and healthy with no clinical evidence of GvHD when sacrificed at D60-70, the final time-point of the experiment. Of the 4 that died, 2 had histological evidence of GvHD while 2 did not, and it was likely that the latter 2 mice could have succumbed to lethal infections as in the negative PBS control. The survival figures for the 3 groups were therefore as follows: unmanipulated 12.5% vs allodepleted 71.4% vs PBS controls 81.8%. The median survival of 18 days in the unmanipulated group contrasted against a median survival of beyond 70 days (end point of experiment) for both the allodepleted group and the PBS negative control group. When the survival curves were compared for statistical significance, the survival advantage of the allodepleted strategy over unmanipulated cells was highly significant ($P<0.0001$) while the difference in survival between NOD/SCID mice receiving allodepleted cells and PBS controls was not significant ($P=0.37$).

Figure 7.10 Selective depletion of CD69+ cells and its effect on the survival of NOD/SCID recipient mice



The survival curves compares the effect of CD69 allodepletion on the survival of recipient NOD/SCID mice. Recipient mice received either PBS, 5-10x10^6 unmanipulated CBA derived T cells or 5-10x10^6 allodepleted CBA derived T cells. Survival curves were compared using the log-rank test.

7.3.6 Exclusion of possible confounding factors in the allodepletion strategy:

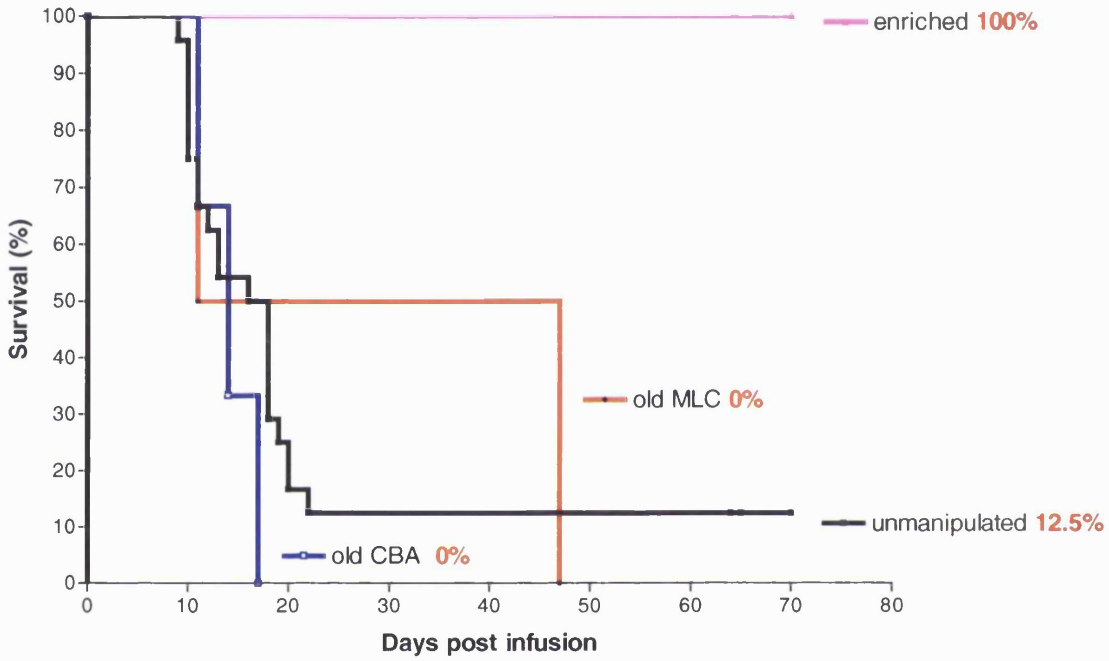
There were several possible important confounding issues concerning the allodepletion strategy that were addressed by the mice in groups 5 and 6. Mice in group 5 addressed the first question: whether the in-vitro culture of cells in media containing FCS would alter the donor cells' capacity to induce GvHD. This was an important confounding factor to exclude because one other difference between group 2 mice and the positive control mice of group 4 was the fact that one consisted of freshly isolated cells while the depleted fraction had been subjected to in-vitro culture. If the in-vitro culture with FCS did not alter the functional capacity of the donor cells, then cells injected into group 5 mice should induce GvHD at the same tempo as the positive control. Mice in group 6 addressed one additional question: whether in-vitro exposure to irradiated recipient cells could induce anti-recipient tolerance in the donor cells. If this postulate was true, then perhaps the depletion strategy might be a redundant step because the donor cells would already have been tolerised either due to a lack of vital cytokines or the absence of appropriate co-stimulatory molecules; and infusion of these anergic cells would therefore not induce GvHD. The CD69 work done in the human in-vitro system would suggest otherwise as 3rd party reactivity was adequately maintained. Nonetheless, it was an important question to address, and if tolerance was not induced, then mice in group 6 should suffer from GvHD at the same tempo as the positive control mice in group 4.

The survival curves shown in Figure 7.11 answered the secondary questions posed earlier. The fact that all cultured cells (group 5) induced lethal GvHD at the same tempo (median survival 14 days vs 18 days for fresh cells in group 4) implied that the effect of culturing the cells in complete medium (RPMI with FCS) did not alter the

potency of the allogeneic lymphocytes in causing GvHD. The survival curves were not significantly different (p=0.13). The other question of possible tolerance induction or anergy was answered by group 6 recipient mice which were injected with CBA derived cells that had been cultured for 70 hours in an MLR with lymphocytes from the NOD strain. That the group 6 mice uniformly succumbed to lethal GvHD excluded the possibility that the CBA derived donor lymphocytes were rendered anergic by exposure to recipient lymphocytes and inappropriate antigen presentation (e.g. lack of costimulatory molecules). The median survival for this group of mice (old MLC) was 29 days vs 14 days in the fresh unmanipulated group and the survival curves were not significantly different (p=0.83). The only difference therefore between the cultured "old MLC" cells and the allodepleted cells was the removal of the alloreactive CD69+ cells by bead sorting. A comparison of their survival characteristics achieved statistical significance (p=0.01).

All 5 mice receiving cells from the "enriched fraction" (group 3) remained alive at d70. In this group, two mice received $1X10^6$ T cells, one had $2.5X10^6$ T cells, one received $5X10^6$ T cells and one $10X10^6$ T cells. The results shown with the administration of the "enriched fraction" was initially surprising. It had been expected that this alloreactive fraction would have the capacity to induce progressive fatal GvHD in the recipient mice, more rapidly than unmanipulated cells and perhaps requiring smaller T cell doses. However, all NOD/SCID recipients were well and healthy at the end of the experiment. Examination of tail vein and cardiac puncture samples from these mice and flow cytometric analysis showed that in contrast to mice receiving unmanipulated or allodepleted fractions, the enriched population did not seem to have engrafted; presumably due to the in-vivo complement-, or reticuloendothelial system mediated clearance of cells labelled with anti-CD69 mAb.

Figure 7.11 Effect of in-vitro culture conditions and administration of CD69+ cells on the survival of recipient NOD/SCID mice:



The survival curves compares the effect of in-vitro culture conditions on the survival of recipient NOD/SCID mice. Recipient mice received either 5-10x10^6 unmanipulated CBA derived T cells, cells obtained from the"enriched fraction" after selective depletion, 5-10x10^6 CBA derived T cells left in culture for 72 hours or 5-10x10^6 CBA derived T cells in an MLC with irradiated NOD cells for 72 hours. Survival curves were compared using the log-rank test.

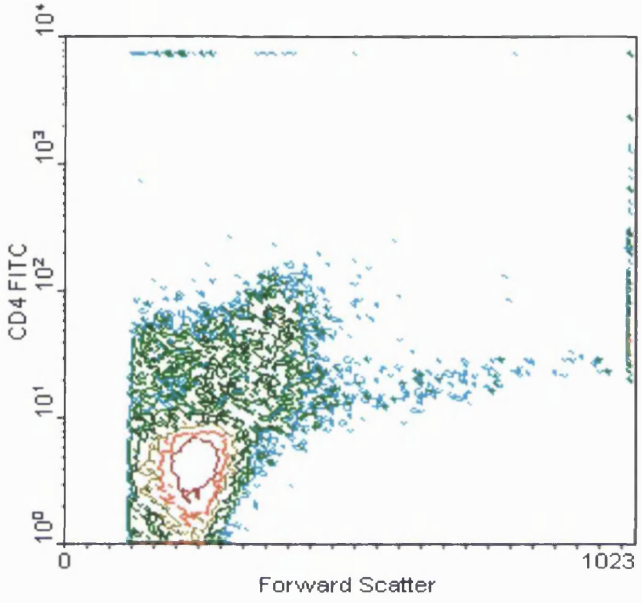
7.3.7 Results of engraftment studies::

Fig 7.12 demonstrates the presence of a distinct population of CD4 cells in a NOD/SCID recipient mouse that received CD69 negative allodepleted cells. This tail vein sampling was done at 35days. In contrast, the control NOD/SCID mouse receiving PBS was characterised by an absence of circulating CD4+ T cells. Engraftment could be seen by 7 days and the sampling done at 35 days indicated that the engraftment was durable.

The next series of dot plots are from a cardiac puncture taken from a terminally ill NOD/SCID mouse with GvHD that had received unmanipulated allogeneic cells 14 days previously. Figure 7.13 demonstrates the presence of a sizeable population of CD3+ lymphocytes which is absent in the normal NOD/SCID mouse. These CD3+ cells were H-2^k positive, indicating conclusively the donor origin (CBA mouse) of the lymphocytes. When further stained for CD4-FITC, CD69-PE and CD8-PERCp, the majority of these donor CD8+ and CD4+ lymphocytes were also CD69+ve, indicating expansion of the donor alloreactive lymphocytes.

For mice receiving "the enriched fraction" consisting of CD69+ alloreactive cells, there was no evidence of engraftment seen.

Figure 7.12 Mice receiving allodepleted cells show engraftment of CD4+ T cells in the peripheral blood



Blood was taken via tail vein sampling at 35 days from a negative control (PBS) NOD/SCID mouse (top dot plot) and from a mouse receiving $10x10^6$ allodepleted T cells (lower dot plot). The blood was stained with anti-CD4 FITC and anti-CD8 PerCp and analysed flow cytometrically. Both dot plots are FSC (*y-axis*) against CD4 FITC (x-*axis*).

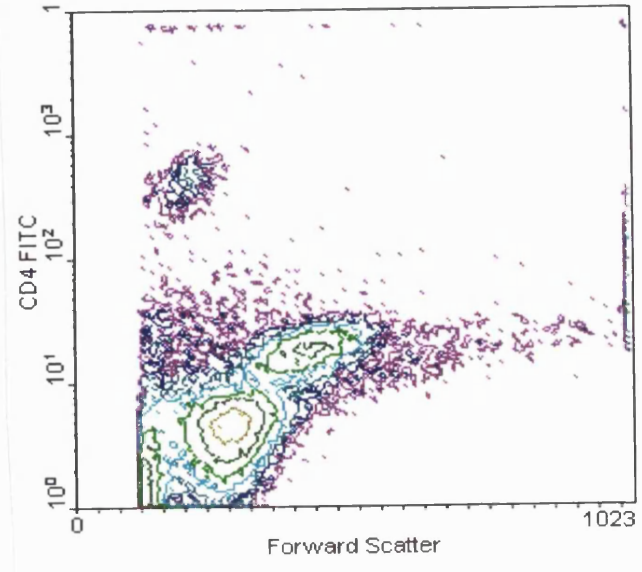
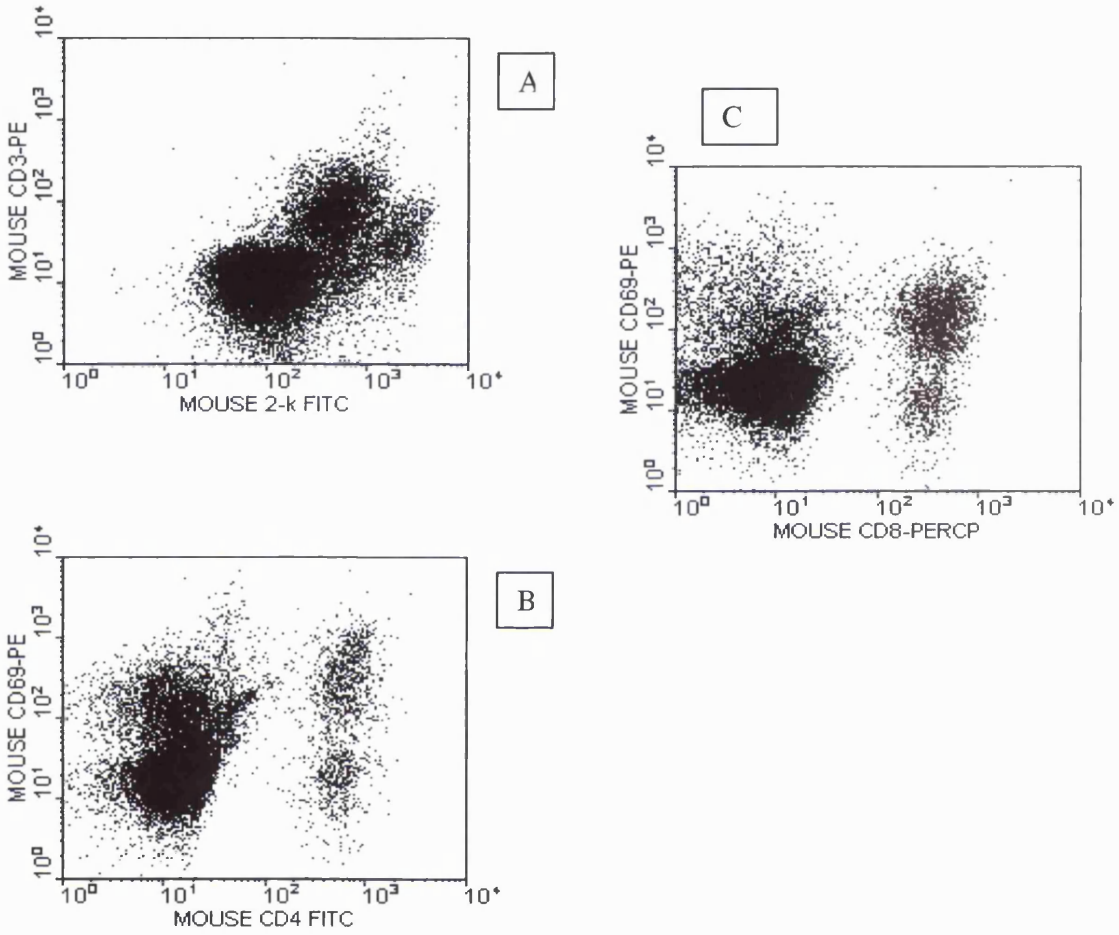


Figure 7.13 Mice receiving unmanipulated cells show engraftment of T cells in the peripheral blood

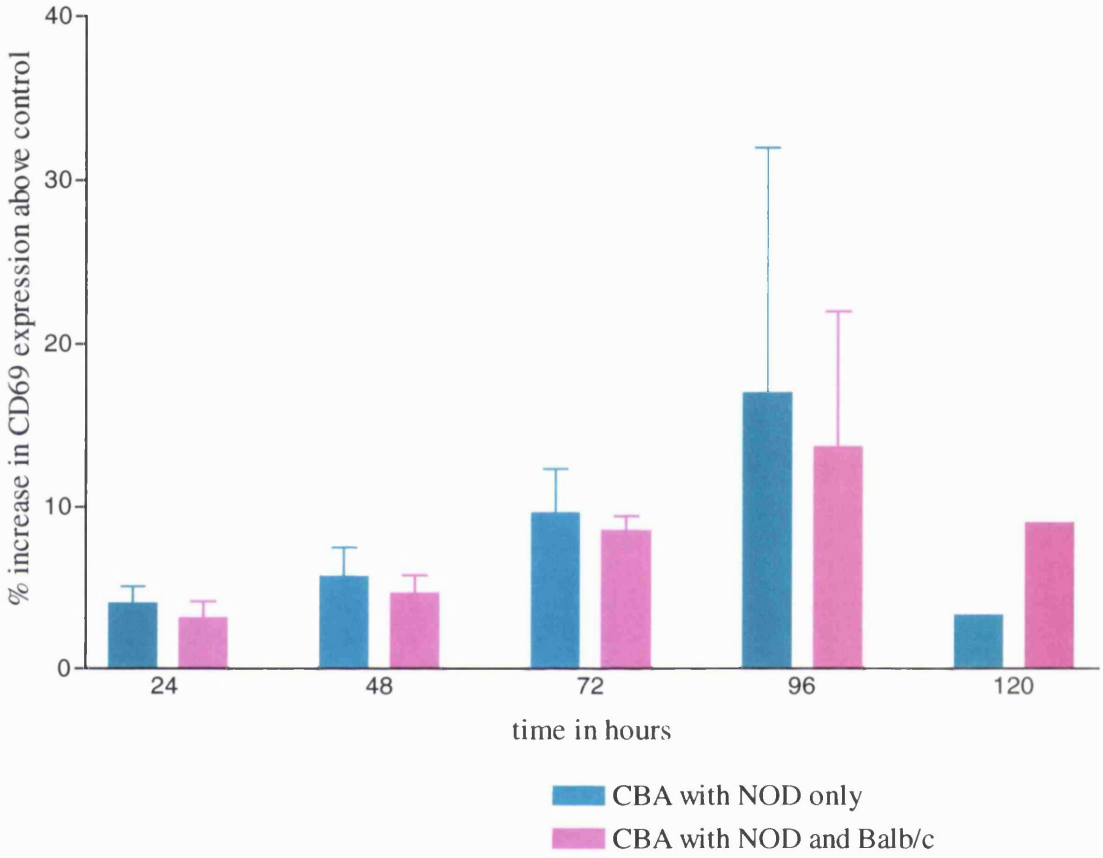


Blood taken via cardiac puncture from a terminally ill NOD/SCID mouse just prior to schedule 1 termination at day 14 post i/p administration of $10x10^6$ T cells. The series of 3 dot plots show a population of CD3+ cells that were H2-k+ (dot plot a). These CD3+ cells comprise both CD4+ and CD8+ cells, the majority of which express CD69 (dot-plots b and c). The blood was stained with anti- H-2k FITC and anti-CD3 PE in tube 1, anti-CD4 FITC, anti-CD69 PE and anti-CD8 PerCp in tube 2.

7.3.7 CD69 activation profile in MLC:

The temporal dynamics of murine CD69 expression in a complete MHC-mismatched MLC paralleled that seen in the human system described in section 3.2.3 with an increase in CD69 expression above autologous control first seen at 24 hours. There was then a progressive increase peaking at 96 hours. Murine responder cells remained viable in culture for the first 72 hours but at 96 hours onwards, increasing numbers became apoptotic. There was no significant difference in CD69 expression whether NOD splenocytes were used as stimulators alone or in combination with BALB/c splenocytes. At t=70h, the time point prior to depletion, there was a mean of 9.6 $\pm$ 5.5% increase in CD69 expression in the MLC using NOD splenocytes as stimulators compared to 8.5 $\pm$ 2.6% using equal numbers of NOD and BALB/c cells as stimulators. The characteristics of this temporal expression are illustrated in Figure 7.14.

Figure 7.14 Temporal dynamics of CD69 expression in a murine MLC between complete MHC-mismatched pairs



A murine MLC was set up using equal numbers of CBA splenocytes and irradiated stimulator cells. These stimulators were either NOD splenocytes alone or NOD and BALB/c splenocytes in equal ratios. NOD mice share the same MHC background as NOD/SCID mice and BALB/c stimulators were used to include any clinically important mHags that could have been carried over with the *scid* mutation. The % increase in CD69 expression was the level of CD69 expression above baseline autologous controls. All figures are expressed as mean $\pm$ s.d. There was no statistically significant difference in the CD69 expression between the two stimulators used ($p=0.6$).

7.4 Discussion:

The first part of the work in this chapter describes an allogeneic model of GvHD using the NOD/SCID mouse as the recipient and appears to be the first description of such a model in the literature. Due to host immunodeficiency, stable and efficient engraftment was seen without the need for lethal irradiation. It was also easy to confirm engraftment as any CD3+ cells must be donor in origin due to the complete lack of functional T cells in the NOD/SCID mouse. Due to the absence of host lymphocytes, this model would also complement and extend the existing knowledge about GvHD from other well described murine models. For example, the distribution of affected organs in the NOD/SCID mouse with GvHD differed from other animal models in the absence of skin involvement. It could be that the host lymphocytes are a major target for initiation of GvHD in the skin and to a much lesser extent in the other target organs. The absence of lymphocytes in the NOD/SCID mouse would account for the lack of pathology in the skin while stromal and interstitial elements may be good targets in the liver, gut and lung.

The histopathological findings in terminally ill mice with acute GvHD were less florid than other comparable murine models. Murine GvHD has been graded into I-IV depending on the severity of the lesions (Xun et al. 1994):

I-II: acantholysis and patchy pyknosis of nuclei of epidermis and skin appendages, single cell necrosis of bowel epithelium and focal cellular infiltration and

III-IV: thinning of epidermis, degeneration and loss of hair follicles and accessory glands, crypt abscesses of bowel and confluent loss of villous architecture and marked cellular infiltration. The majority of affected target organs in the NOD/SCID mouse would be graded as I-II. Clinically however, they showed similar signs and symptoms

and to the same severity namely, weight loss, poor appetite, hunched posture, poor skin texture and decreased activity.

Irradiation is used in experimental models of transplantation to allow engraftment of allogeneic cells. Graft rejection is not a problem with NOD/SCID mice which possess impaired NK cell activity combined with other immune defects allowing for easy engraftment. They were therefore chosen over SCID mice which retain potent NK cell activity that can mediate graft rejection. The purpose of sublethal irradiation in this murine model was therefore not for the prevention of rejection but rather to participate in the inflammatory process contributing to GvHD. In keeping with the cytokine storm induced by TBI in human transplantation, sublethal irradiation would induce the release of cytokines leading to the upregulation of adhesion and MHC molecules, thus priming both immune affector/effector mechanisms and target tissue. Besides irradiation, optimum induction of GvHD in this murine model utilised the i/p route which could be due to differences in the draining and circulatory pathways of the donor cells and in initial alloantigen presentation.

The rest of the work in this chapter addressed the issue of translating the in-vitro studies with the CD69 allodepletion strategy into an in-vivo model and the demonstration of the efficacy of that strategy. NOD/SCID mice receiving allogeneic cells using this allodepletion technique showed a clear survival advantage over mice receiving unmanipulated cells. In fact the survival approached that of control mice receiving PBS suggesting that this method would result in the complete abrogation of GvHD. That this strategy was effective in a completely MHC-mismatched system where the risk for severe GvHD is greatest implied that it would be equally effective in partially matched and fully matched situations as well.

The murine work has also addressed other remaining issues from the in-vitro work. It corroborated the use of the cytokine based modified MLR as an accurate predictive tool for GvHD in the in-vitro studies. By demonstrating the GvHD inducing capacity of donor cells that had been left in an MLC but not subjected to selective depletion, the murine work dismissed the possibility that the CD69 strategy could simply have worked by inducing tolerance in an MLC and hence the attendant risk that tolerance could be broken. The safety and efficacy of the allodepleted cells also dismissed the possible concern that ex-vivo culture in an MLC might 'prime' donor cells and hence cause greater and more accelerated damage when infused into the recipient.

The 100% survival of NOD/SCID mice receiving "the enriched fraction" consisting of alloreactive CD69+ cells was initially perplexing. This however could be explained by two possibilities. First, insufficient numbers might have been infused leading to non-engraftment. Second, the alloreactive CD69+ve fraction would also have retained the anti-CD69 mAb. When infused, the F_c portion of the antibody may have bound to macrophages of the reticuloendothelial system bearing F_c receptors on their surface leading to phagocytosis and clearance or direct complement activation.. Activation induced cell death was less likely as this would have also occurred in mice receiving "old cultured MLC cells" but was not so, as demonstrated above.

Finally, recent years have seen an explosion in research using the NOD/SCID mouse, especially in cell transfer work. The work in this chapter has also highlighted that allogeneic cell transfer can result in the development of GvHD and perhaps be a potential confounding factor.

8. Chapter 8. Final discussion and future studies.

8.1 Clinical application of the CD69 allodepletion strategy:

Until the late 1980s, GvHD preventive approaches were limited in number and the main modalities were restricted to either pan T cell depletion or post transplant immunosuppression. The latter has often proved ineffective; the problems of pan TCD have been emphasised and both approaches are non-specific, resulting in a host who remains severely immunosuppressed.

The work presented in this thesis has attempted to highlight the advantages of a more specific approach. It recognises the crucial function of donor lymphocytes contained in the graft in post transplant immune surveillance against leukaemia and viruses; and has aimed to retain as many of these useful immune effector cells as possible. By exploiting the concept that GvHD is an alloreactive process mediated by activated lymphocytes, a selective depletion graft engineering strategy was developed that effectively abrogated the anti-host response, maintained anti-CMV and anti-EBV activity, preserved 3rd party reactivity suggesting a conservation of the anti-leukaemia response and significantly improved survival in a murine model of lethal GvHD.

Having established this novel CD69 allodepletion strategy, the next step would be to take this experimental system into the clinical arena, as illustrated in a flow diagram, Figure 8.1.

CD34+ve stem cells would be separated from the donor graft using currently available commercial clinical-grade cell sorters and the stem cells either infused directly into the recipient or if necessary, further T cell depleted to attain a T cell content of $<1.5x10^5$/kg and then infused into the recipient. The remaining CD34 negative fraction would contain all the lymphocytes and useful immune effectors needed for hastening immune recovery. This would be incubated ex-vivo with cytokine-treated irradiated recipient normal bone marrow or peripheral blood mononuclear cells for 96 hours and further sorted on the basis of CD69 expression. Both the CD69 depleted and enriched fractions would be recovered and collected with aliquots tested for residual alloreactivity. CD69-depleted fractions with residual patient-specific alloreactivity of <5% (below the predictive threshold for clinical GvHD using the modified MLC assay) would be re-infused in dosed aliquots at 14 day intervals after the initial CD34+ve cell transplant, perhaps in escalating doses starting at $1X10^6$ lymphocytes/kg The remaining CD69-depleted and CD69-enriched samples would remain cryopreserved as sources of mature functional donor lymphocytes (DLI) for the treatment of patients in the event of relapse.

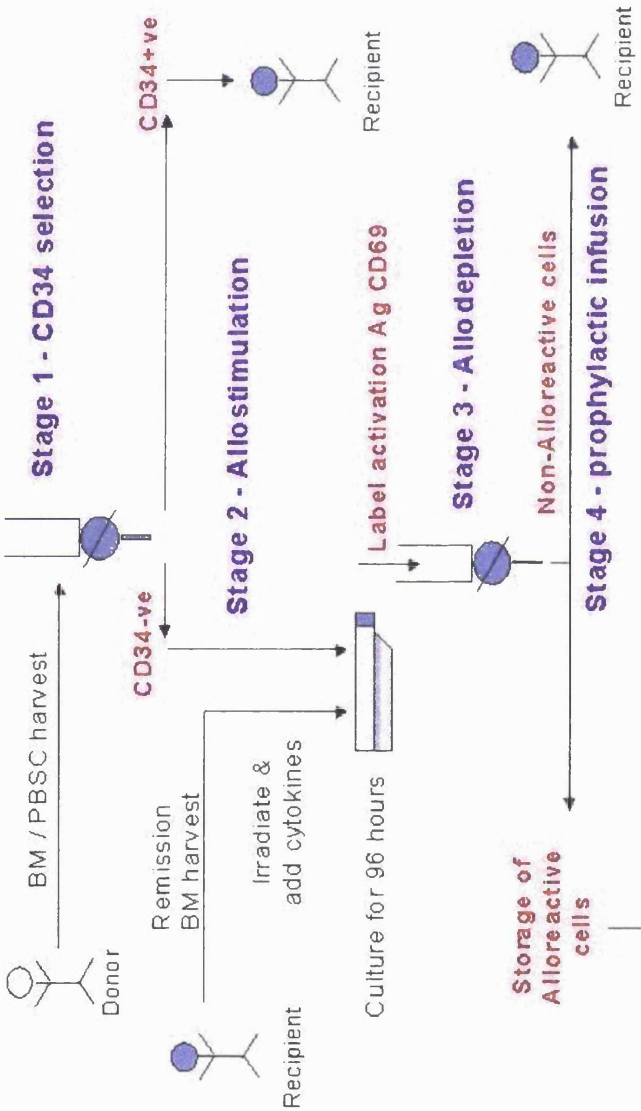
The CD69 depleted fraction possessing 3rd party reactivity would be the treatment of choice in most relapsed patients, especially those having only minimal residual disease as the enriched fraction containing alloreactive cells possess the potential for causing severe GvHD. Use of the latter could be envisaged, however, in frank relapses where urgency and potency of treatment would be a priority. The results of conventional DLI have been disappointing in the treatment of relapsed ALL where the GvL effect is less marked and attainment of remission might well be dependent on a strong alloreactive component. The attendant risk of lethal GvHD however should not be ignored and use

of a thymidine kinase "suicide gene" to switch off the alloreactive process if required would be an attractive proposition.

This method of selective depletion was shown to be effective in both HLA-mismatched and matched pairs in vitro and in a murine mismatched model in vivo. A logical clinical extension would be the use of this method in haploidentical related donors and in 1, 2 or 3 antigen mismatched transplants. This would considerably widen the potential pool of donors since, in most cases, a haploidentical related donor is readily available. It would be particularly relevant to ethnic minority groups where lack of availability of HLA-matched unrelated donors is a major problem. It would also allow further sub-selection on the basis of donor age, parity, CMV status and other important transplant variables. Haploidentical transplants are increasingly being performed, generally with stringent pan T cell depletion or removal of αβ TCR specific T cells to prevent GvHD (Aversa et al. 1998), (Henslee-Downey et al. 1997). With improved conditioning regimens and higher stem cell doses achieved with PBSCTs or "mega-dose transplants", graft rejection has become less of an issue with attainment of over 90% engraftment rates. The main problem remains a severe impairment in immune reconstitution (Aversa et al. 1998). This might prove the ideal scenario for the CD69 allodepletion strategy as over 90% of the mature functional donor lymphocyte pool is retained after depletion.

Since the first allotransplant was performed for acute leukaemias, the range of diseases amenable to treatment by allogeneic bone marrow transplantation has broadened considerably to include lymphomas and non-malignant haematological (sickle cell disease, thalassemias) and non-haematological conditions (metabolic disorders,

Figure 8.1 Flow diagram illustrating the intended clinical application of the CD69 allo-depletion strategy



autoimmune disease). For many of the non-malignant diseases, weighing the potential benefits against the mortality and morbidity of allotransplants is a key issue, perhaps more so than in leukaemias where urgency of treatment is the main priority. As transplants can be a potentially curative procedure for some of these conditions, if the twin problems of GvHD and fatal infections from impaired immune recovery could be resolved by this strategy, the improved safety profile would make allogeneic transplantation an especially attractive procedure to the patient population.

8.2 Future studies regarding the CD69 allodepletion strategy.

The results in this thesis have shown that the lymphocytes mediating anti- CMV and anti-EBV activity are largely distinct from the alloreactive lymphocytes involved in GvHD. The in vitro studies demonstrating substantial preservation of 3rd party reactivity imply that the pool of non-alloreactive cells are capable of other useful immune functions, perhaps including GvL activity. CTLs recognising specific target antigens found only on leukaemic cells should be retained by the allodepletion strategy, as with the anti-viral effectors. These leukaemia-specific antigens have already been discussed in section 1.3.2 and Table 1.1. Future studies however would need to be performed to confirm this. One method would be to test this in a murine model. Recipient mice inoculated with MHC compatible leukaemia would ~~the~~ be treated either with unmanipulated allogeneic cells, allodepleted lymphocytes or PBS as a control and survival compared. Parameters examined would include disease free survival, presence of GvHD in all animals, leukaemia burden and cause of death, whether from leukaemia or lethal GvHD. If the assumption regarding preservation of 3rd party reactivity were to hold true, animals receiving allodepleted cells should have an improved survival in the absence of GvHD and without detectable leukaemia, or at least with a reduction in the

tumour burden. This would provide proof of principle that the GvH and the GvL reaction are separable and distinct processes.

Retention of anti-CMV and anti-EBV activity was demonstrated with the use of tetramers and the ELISPOT assays. Similar studies could be extended to other infectious agents including adenovirus, human herpes virus 6 (HHV6), candida and aspergillus which are other major causes of morbidity and mortality post transplantation. If appropriate tetrameric-peptide complexes from these organisms could be isolated and made, one could readily see if the selective removal of CD69+ alloreactive cells retains reactivity to these infectious agents. However, as already discussed, the occasional argument against the tetramer assay is that it may not always predict functionality of the tetramer binding lymphocytes. To confirm that the anti-CMV and anti-EBV specific lymphocytes are not anergic or effete, immunogenic peptides such as CMV pp65 could be fed to the allodepleted product and subsequent expansion of these specific lymphocytes looked for.

In developing this allodepletion strategy, the reasons why CD69 was chosen over other markers were discussed. An area of future investigation might be to explore if a double depletion using CD69 and CD25 would confer additional benefit over CD69 alone. This "double marker" depletion approach was shown to result in a greater decrease in alloreactivity compared to a "single marker" depletion although this was only tested in HLA-mismatched pairs (Fehse et al. 2000). Analysis of the patterns of upregulation has demonstrated that CD69 peaks before that of CD25 and remains sustained before beginning to tail off. The main potential advantage with double depletion might be to exploit this window when CD25 is being upregulated as CD69 expression is tailing off. CD69 and CD25 may also represent partially differing activation pathways with

mutually exclusive expression on some activated cells (Fehse et al. 2000). Double depletion would remove cells that were singly positive (either CD69 or CD25) and thus, achieve a higher level of specific depletion. Whether this would result in the removal of many non-specifically bound lymphocytes at the expense of anti-viral and anti-leukaemic reactivity would have to be investigated. Another obvious disadvantage would be the cost involved, if translated into a clinical trial, of producing another clinical grade monoclonal antibody.

8.3 Advances and new perspectives in GvHD prevention strategies.

Advances in the understanding of T cell immune responses and the pathophysiology of GvHD have led to exciting new strategies for prevention, with the primary aim of dampening the GvH reaction while harnessing general immune function post transplantation. Recipient conditioning including the cytokine storm, donor T cell activation and effector mechanisms which comprise the 3 phases of acute GvHD are all potential targets for intervention.

8.3.1 Targeting phase 1: recipient conditioning

The crucial role of the cytokine cascade in recipient conditioning has already been discussed. The knowledge that inflammatory cytokines can contribute to the tissue damage seen in GvHD has led to the development of inhibitors or monoclonal antibodies directed against these cytokines, albeit with varying success in clinical application. An anti TNF-α mAb ameliorated GvHD in a murine model (Piguet et al. 1987) while TNF-receptor p55 deficient mice showed reduced mortality post transplant from GvHD (Speiser et al. 1997). A clinical phase I-II study, using an anti

TNF-α mAb during pretransplant conditioning as an additive prophylaxis of acute GvHD after BMT, resulted in some beneficial effects (Herve et al. 1992). Other attempts to ameliorate GvHD by manipulating Th1 cytokines have similarly been mixed. It has to be appreciated that the role of these cytokines are complex, involving multiple interactions at different time points. Hence the timing and duration of administration of either cytokines or their antagonists are crucial because they might even exercebate the occurrence of GvHD. The concept of "cytokine shields" has also recently emerged (Hill & Ferrara 2000). These are mainly Th2 cytokines like IL4, IL10 and TGF-β which are thought to inhibit the pathophysiology or inhibit the deleterious effects of GvHD. Pre-conditioning levels of IL10 were shown to predict for GvHD with high serum levels conferring protection (Holler et al. 2000). But again, the mechanism of interactions is complex and a recent study demonstrated that IL10 administration had the contradictory effect of decreasing survival (Blazar et al. 1995b). Moreover, the issue of polarisation towards a Th2 phenotype and possible loss of GvL reactivity needs to be addressed. Animal studies have shown the GIT system plays a critical role in the amplification of systemic damage with damaged GI tract allowing an increase in the translocation of inflammatory stimuli like LPS (Hill et al 1997b). "Cytokine shields" like IL11 and keratinocyte growth factor (KGF) which fortifies the GI mucosal barrier, thus reducing GIT damage have shown promise in the prevention of GvHD while preserving a graft versus leukaemia effect (Krijanovski et al. 1999), (Hill & Ferrara 2000). This mucosal protection was also associated with a reduction in serum TNF-α levels and an inhibition of LPS translocation. KGF however does not completely ameliorate GvHD but merely reduces its severity.

Reduced conditioning regimens or so called "non-myeloablative transplants" have also been devised to reduce the inflammatory damage released during the conditioning

process. This has been made possible by a greater understanding of the immune mechanisms of graft vs host and host vs graft tolerance, by improvements in graft rejection rates and the introduction of more specific T cell immunosuppresive agents like fludarabine (Slavin et al. 1998), (Khouri et al. 1998). The purpose of pre-transplant conditioning is to create a state of immunological host tolerance to the donor graft allowing the induction of a graft versus leukaemia effect as the primary treatment modality. As a state of tolerance and usually, mixed chimerism is maintained, this allows for further infusions of donor lymphocytes to maximise the GvL effect. This form of therapy is especially attractive because less intensive conditioning (hence, the term mini-transplants) translates into decreased toxicity, improved immune reconstitution, avoidance of TBI and decreased incidence of GvHD (Ferrara 1993).

8.3.2 Targeting phase 2: donor T cell activation

Strategies aimed at targeting phase 2 of the GvH reaction have concentrated either on removing any activated lymphocytes or to prevent the T cell from becoming activated at all. Removal of activated alloreactive lymphocytes has underlined the development and the results of the thesis presented here while the latter strategy concentrates on the signal transduction pathways leading to T cell activation.

A CD40 ligand (CD40L) or CD154 antibody has been demonstrated to be effective in the prevention of GvHD. Blazar demonstrated that ex-vivo culture of donor and recipient T cells with CD40L antibody induced the donor CD4+ cells to become tolerant to host alloantigens while retaining the capacity to respond to nominal antigens (Blazar et al. 1998). This confirmed the importance of the CD40-CD40L interaction between the APC and the T cell. Another reason also appeared to be that in the absence

of CD154, donor T cells were unable to expand or generate high level anti-host CTL activity, hence, leading to the exhaustion or deletion of alloreactive CD8+ clones necessary for the induction of GvHD (Buhlmann et al. 1999).

Co-blockade of vital costimulatory pathways: CD28/CTLA4:B7 and LFA1:ICAM1 pathways prevents productive antigen signalling and leads to a state of anergy for donor T cells that have been exposed to recipient alloantigens. Single blockade using cytotoxic lymphocyte antigen-4 (CTLA4-Ig) was found to be less effective than double blockade with anti-LFA1 antibody and CTLA4-Ig given ex-vivo in an MLR and administered in-vivo as well (Blazar B.R. et al. 1994). This corroborated work showing that in an MLR, blockade of the costimulatory pathway resulted in a 50-85% reduction of the proliferative response, a 1000-fold reduction in the HLTp frequency and induction of alloantigen-specific anergy (Tan et al. 1993), (Gimmi et al. 1993). CTLA4 engagement has also been shown to inhibit both CD69 and CD25 activation indicating that it restricted progression of T cells to an activated state (Krummel & Allison 1996). However, the chief drawback to the use of the CD40L and other similar strategies is that these alloreactive GvHD inducing cells will still present in the infused graft and does not take into account the massive tissue destruction and release of inflammatory cytokines that are present at the time of conditioning. Anergy or tolerance induction can be reversed, as shown in experiments with IL2. It should also be borne in mind that the alloreactive interaction is not a primary response but a cross reactive secondary response. The importance of costimulatory molecules in a primary response is without question, what is less certain is their role in a secondary response. Costimulatory blockade may therefore be less effective in this circumstance. Moreover, if the antibodies were also administered in vivo, this might affect other important antigen specific responses as evidenced by a diminished GvL response

(Blazar et al. 1997b). To bypass the latter problem, an ex-vivo approach using CTLA-4 to induce anergy in a MLC was recently brought into a clinical trial with some success although only small numbers were treated (Guinan et al. 1999). Other APC-T cell costimulatory pathways that are important include CD2:CD48 (Gonzalez-Cabrero et al. 1999) and VLA4:VCAM. Inhibition of the latter prevented murine GvHD across minor histocompatibility barriers (Schlegel et al. 1995).

Peptides have been found to inhibit allorecognition as well as the MLR and CTLp across several major and minor histocompatibility differences in a specific and dose dependent fashion. CD31 derived peptide, when used in a murine model, did not prevent but significantly delayed the onset of GvHD and improved long term survival. The mode of action of CD31 is still not exactly clear although data has suggested that it plays an important role in T cell activation (Chen et al. 1997). Copaxone or GLAT, a synthetic random basic copolymer with promiscuous binding to class II MHC molecules, thus blocking TCR-MHC interactions and antigen presentation has been shown to inhibit T cell responses, delay murine GvHD and is presently in phase I clinical trials (Schlegel et al. 1996), (Vogelsang 2000). These again depend on the induction of tolerance but the fact that GvHD still occurred, although less severe, suggested it was incomplete and if used, would have to be combined with other modalities.

Instead of concentrating on the viewpoint of the T cell in APC-T cell interactions, Shlomchik WD found that in a murine model, only host-derived APCs and not donor derived APCs initiated GvHD (Shlomchik et al. 1999). This was in keeping with the fact that peptides presented to CD8+ T cells are derived primarily from endogenously expressed genes (Germain 1994). Cross presentation of host antigens by donor APCs

is probably insufficient in itself to generate GvHD. Strategies could therefore be developed to specifically target the host APCs, possibly by depleting them prior to transplantation. GvL reactivity however, might also depend critically on the presence of host APCs.

Instead of ex-vivo identifying and targeting CD69, CD25 or OX40 activated cells, monoclonal antibodies against CD25 administered in-vivo have been used for the prevention or treatment of GvHD (Anasetti et al. 1990), (Herve et al. 1988). Initial results for treatment were promising (Belanger et al. 1993), (Anasetti et al. 1990) but a randomised controlled trial for prophylaxis demonstrated no benefit (Anasetti et al. 1991), (Blaise et al. 1995), (Blaise et al. 1991). Furthermore, a higher relapse rate has been reported in allotransplanted patients given anti-IL2R antibody for the prevention of GvHD (Blaise et al. 1995). This was not too surprising considering that the antibody would target all activated cells in vivo, including GvL effectors. A recent report used daclizumab, a humanised monoclonal IgG1 directed against the α chain of IL2-R to treat ongoing GvHD with greater success than in prophylaxis (Przepiorka et al. 2000).

Expression of OX40 has been observed in aGvHD and in a P to F_1 murine model, a blocking monoclonal antibody against OX40L significantly reduced the lethality of acute GvHD and other manifestations of the disease. In vitro assays confirmed marked hyporesponsiveness to host alloantigens and modulation of the Th1 and Th2 cytokine profiles. It is thought that the mAb most likely blocked the interaction between OX40+ alloreactive T cells and OX40L expressing APCs. (Tsukada et al. 2000).

Steroids and cyclosporin have remained the mainstay of treatment and prophylaxis for GvHD. Many newer drugs have since been developed and most, like cyclosporin, are

signal transduction pathway inhibitors of T cell activation. These include FK506, an inhibitor of calcineurin activity, mycophenolate mofetil (MMF), rapamycin and tresperimus (Blazar et al. 1997d), (Vogelsang 2000). Wortmannin which inhibits PI-3 kinase was shown to prevent GvHD in a murine model (Taub et al. 1997).

8.3.3 Targeting phase 3: Inflammatory effector mechanisms

The differential importance of the Fas, perforin and TNF-α effector pathways in the GvH and GvL reactions has already been highlighted and this is being exploited by various investigators (Tsukada et al. 1999), (Speiser et al. 1997). Blocking the Fas pathway was shown to ameliorate GvHD without impairing GvL while perforin was crucial for the GvL effect. TNF-α was also important for GvL and this was also confirmed in a recent study (Hill et al. 1999). A metalloproteinase inhibitor (KB-R7785) which inhibits TNF-α and FasL release prevented GvHD in a parent to F1 spleen cell transfer model (Hattori et al. 1997) and in a murine BMT model (Hattori et al. 1999) without compromising engraftment. Nonetheless, these strategies should be approached with caution as phase I and II of the GvH cascade would already have proceeded uninhibited.

8.3.4 T cell addback and DLIs.

DLIs were initially used to treat relapsed leukaemias but increasingly, T cell addback has been incorporated into part of an integral transplant procedure as in the proposed clinical application of the strategy described in this thesis or in the addback of EBV or leukaemia specific lymphocytes. The main complication of any T cell addback is the

risk of GvHD. Ways to surmount this potential complication have involved adjusting the dose, timing and content of the DLIs (Johnson & Truitt 1995), (Barrett et al. 1998).

In CML patients relapsing after T depleted transplant, $1X10^7$ CD3+ T cells/kg was found to result in excellent efficacy without causing significant GvHD (Mackinnon et al. 1995). What was less clear however was if this same dose level applied to non-T cell depleted transplant recipients as well. Using a murine model, (Truitt & Atasoylu 1991a) found that moderate numbers of T cells caused only mild GvHD while still providing a significant GvL effect. With regards to the timing of the DLI, a study showed that 1/7 receiving $0.5X10^6$ CD3+ T cells developed GvHD in contrast to 3/4 at <90days developed GvHD requiring steroids (Small et al. 1999b), (Barrett et al. 1998). Moreover, a threshold of 10^5 T cells/kg at time of transplant was associated with a significant risk of GvHD in sibling grafts but if the T cell addback was delayed by 9-12 months, up to 10^7 cells/kg could be given without developing GvHD (Mackinnon et al. 1995). This has given rise to the concept of escalating doses of T cell addback whereby increasing number of cells are given the further away from the transplant procedure. The mechanisms responsible for this reduction in GvHD risk are not clear but most certainly is due in part to the relatively lower sensitivity of host tissue to GvH induced injury late after the conditioning regimen. Other possible reasons include the development of donor T cells derived from the marrow graft capable of modulating the function of alloreactive lymphocytes in the DLI or the replacement of host APCs capable of stimulating an alloreactive response with tolerant donor derived cells.

Other groups have attempted to either deplete CD8+ (Giralt et al. 1995) or enrich for CD4+ cells to reduce GvHD with the aim of retaining a GvL effect. In a recent series of 40 patients, defined doses of CD4+ donor T cells were infused with good disease

control. Nonetheless, 52% of the responding patients still developed GvHD (Alyea et al. 1998). It would therefore appear that rather than crudely depleting either CD4 or CD8 cells, the concept of T cell addback would nicely complement the CD69 allodepletion strategy. If the alloreactive cells were selectively depleted, then more donor lymphocytes could be given and early in the post transplant period without incurring the risk of GvHD and these cells would contribute to an improved immune recovery

An approach that has generated considerable interest has been the transduction of donor lymphocytes with the herpes simplex thymidine kinase (HS-TK) "suicide" gene which renders the cells sensitive to ganciclovir. The study demonstrated the efficacy of the transduced lymphocytes, their persistence in vivo for up to 1 year and the ability to control GvHD in some cases although significantly, not in all with the administration of ganciclovir (Bonini et al. 1997). The obvious disadvantage however, is the loss of ganciclovir as a therapeutic option for CMV disease post transplant. Moreover, this approach only intervenes after GvHD has been initiated; by then, secondary effector mechanisms might already be in place and elimination of donor cells at this point achieving only minimal effect. Other crucial issues include immunogenicity of the gene product, sustainability of expression and the extent of transduction needed in the T cells. Nonetheless, this approach could be applied to the CD69 allodepletion strategy as discussed above.

8.4 Harnessing the immunotherapeutic potential of the GvL effect

The CD69 allodepletion strategy has demonstrated the ability to retain over 70% over the original 3rd party response. The removal of alloreactive cells would also allow for greater numbers of donor lymphocytes to be infused without risk of severe GvHD and at regular intervals, thus producing a more potent and sustained specific anti-leukaemic effect.

Although this implies clear separation of GvHD from GvL, an overlapping alloreactive component is likely to exist (van Lochem et al. 1992) and this would inevitably be lost as part of the allodepletion strategy. Hence the approach as presented in this thesis could be further optimised with continuing efforts to harness the immunotherapeutic weaponry needed to achieve long lasting cure rates in haematological malignancies.

The most obvious strategy would be to develop techniques to further characterise and expand specific GvL effectors. Some of these have already been identified, as discussed, and the challenge would be to expand these cells for clinical infusions to test their anti-leukaemia potential. Whether these are 'universal' anti-leukaemia effectors or applicable only to certain disease subtypes remains to be elucidated. Moreover, the timing of infusion may also be a critical issue as some of these cell types (NK, CD4, CD8) have also been implicated in GvHD. Another way of identifying specific anti-leukaemia effectors would be to generate them using tumour antigens. CD4 and 8 T cell lines (CTLs) have been detected or generated against tissue restricted mHags (Goulmy 1997), lineage and differentiation specific (proteinase 3) (Molldrem et al. 1996) or leukaemia specific (PML-RARA in M3) antigens (Gambacorti-Passerini et al. 1993). CD8 clones specific for a variety of mHags can be detected in the majority of

patients post transplantation, an example being a novel H-Y protein which is HLA-B8 restricted, selectively expressed on haematopoietic cells and suitable as a GvL target. The same applies to HA-1 and HA-2, minor antigens expressed exclusively on haematopoietic cells.(Goulmy 1997) In a situation where the donor was HA-1 or HA-2 negative and the recipient positive, HLA-A2 restricted CTLs generated against these mHags were found to selectively lyse only the recipient leukaemia and haematopoietic cells, while sparing donor haematopoietic cells, recipient fibroblasts, keratinocytes and hepatocytes.(Mutis et al. 1999b) The characteristic leukaemia specific fusion oncogene PML/RAR from AML M3 (Gambacorti-Passerini et al. 1993) is another attractive option and corresponding trials using bcr-abl peptides in CML are underway. (Osman et al. 1999) Various methods of generating these CTLs have been tried including bulk cultures,(Falkenburg et al. 1993) limiting dilution assays(Smit et al. 1997) and dendritic cell stimulation(Mutis et al. 1999b). The emergence of tetramer technology has allowed visualisation, selection and expansion of specific responding CTLs bound to these MHC-tumour peptide complexes and has been successfully used to identify mHag specific CTLs (Mutis et al. 1999a). The exciting possibility of using the catalytic subunit of telomerase as a universal tumour antigen (Vonderheide et al. 1999) has also recently been described. CTLs raised were able to recognise and lyse a variety of leukaemias, lymphomas and myelomas in a HLA-restricted manner. With reference to the allodepletion strategy, it could be envisaged that these tumour-specific CTLs would be present in the CD69 depleted product, although probably in low frequencies. While aliquots would be infused into the patient and others stored for possible future use, remaining samples could be used to expand these CTLs ex-vivo.

Conversely, immunotherapeutic approaches can explore the GvL effect from the perspective of the afferent arm: enhancing tumour antigen presentation, improving

vaccination techniques and examining how tumours evade the host immune system. Dendritic cells (DCs) are the most efficient APCs and one method of optimising the immune response is to pulse DCs with peptides, proteins or tumour lysates to obtain specific T cell effectors (Mayordomo et al. 1995), (Ockert et al. 1999), (Mutis et al. 1999b). Significant strides have been made in isolating, identifying and expanding DC populations from monocytes and from CD34 progenitors. Others have tried using these peptides and dendritic cells for in vivo vaccination strategies (Zeis et al. 1998) and this may improve responses compared to vaccination with peptides alone, proteins or irradiated leukaemic blasts (Zeis et al. 1998). Activating anti-CD40 antibodies have been used to modulate the function of DCs. This augmented responsiveness to tumour vaccination strategies as well as enhanced and primed specific anti-tumour CTL response (Sotomayor et al. 1999), (Diehl et al 1999), (French et al. 1999)

Leukaemic blasts subvert the host immune surveillance by myriad mechanisms which include decreased expression of MHC molecules, secretion of immunosuppresive cytokines, lack of costimulatory molecules, (Hirano et al. 1996), (Delain et al. 1994) downregulation of CD3ζ and tyrosine kinase expression (Buggins et al. 1998). This results in the failure of 'correct' antigen presentation' to the T cell and hence a failure of the immune response. This has led to the development of gene transfer techniques using viral vectors for expression of MHC molecules, cytokines like IL12 (Anderson et al. 1997), costimulatory molecules such as B7.1(Hirst et al. 1997) and other accessory molecules like CD40 onto the cell surface of the malignant blasts and used as effective leukaemia vaccines (Di Nicola et al. 1998).

8.5 Concluding remarks

The era of pan T cell depletion and global immunosuppression is gradually being eclipsed as the immunological basis of allogeneic transplantation becomes elucidated. The strategy described in this thesis will allow us to undertake "intelligent" T cell depletion where the issue is no longer how many T cells can be removed from the graft but rather, how many can be retained without inducing GvHD. This and the other recent advances in GvHD prevention add to the growing conviction that donor lymphocytes are vital to the success of allotransplantation. As ex-vivo techniques improve, component therapy in the form of prophylactic or therapeutic non-alloreactive tumour specific and pathogen specific donor lymphocytes will become commonplace, forming an integral part of the graft engineering procedure.

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Temporal dynamics of CD69 expression on lymphoid cells

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Temporal dynamics of CD69 expression on lymphoid cells

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Abstract

Lymphocyte activation remains an area of intense interest to immunologists and cell biologists and the dynamics of expression of surface molecules during the process are widely studied. The CD69 C-type lectin is reportedly the earliest activation antigen on lymphocytes and can be detected within hours of mitogenic stimulation. Recently reports have described differential activation dynamics with respect to different antigenic or mitogenic stimuli. This study has investigated the dynamics of CD69 expression over time after mitogenic, allogeneic, cytokine and target cell mediated activation of T-cell and NK cell subsets. It is apparent that the dynamics of CD69 expression differ with respect to the cell type and the method of stimulation. Mitogenic stimulation resulted in the most rapid expression of CD69 on both T- and NK cells while alloantigen stimulation induced a far slower response. Target cell stimulation of NK cells gave paradoxical results in that the CD69 + ve subset increased as a proportion of the total NK cells but did not increase in number. This was due to the selective binding of CD69 − ve NK cells to the target cells and their subsequent loss from the lymphoid gate. We confirmed this by showing that CD69 + ve NK cells do not lyse K562 target cells. This observation demonstrates the caution needed in the analysis of flow cytometric data based solely upon relative proportions of cells within discrete subsets. © 1997 Elsevier Science B.V.

Keywords: CD69; Activation antigens; Flow cytometry; Techniques

1. Introduction

The human CD69 antigen (AIM, Leu-23) is a type II integral membrane protein belonging to the family of C-type lectin receptors (Testi et al., 1994). It is one of the earliest cell surface markers induced in resting T-lymphocytes and natural killer (NK) cells following their activation by mitogens, cytokines or contact with target cells. CD69 has been shown to function as a co-stimulatory molecule in conjunction with either the phorbol-12-myristate-13-acetate (PMA), or with anti-CD3 antibody (Zeigler et al., 1994) although its precise biological role is unknown. Cytolytic function of natural killer cells can be triggered through CD69 signalling (Lanier et al., 1988) but this is not true of T-cells (Testi et al., 1989b).

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Stimulation of lymphoid cells results in the de novo synthesis of CD69 mRNA (Testi et al., 1989a). Using Northern blot analysis, CD69 transcripts can be detected in T-cells within 30 min of PMA stimulation: detectable surface expression of CD69 antigen occurs within 3 h of stimulation (Lopez-Cabrera et al., 1993) and peaks in activated T- and NK cells after 12 h (Testi et al., 1989a). Upregulated expression of CD69 antigen on T-lymphocytes may be followed by cell proliferation (Cebrian et al., 1988) and an increase in secretion of IL-2 and IFN-gamma.

The extracellular changes associated with CD69 expression in activated T-cell populations have been followed using single and dual-colour fluorescence flow cytometry (Testi et al., 1989a). More recently, Maino et al. (1995) have used a rapid multiparametric (three-colour) flow cytometric method to assay expression of CD69 in the activated T-lymphocyte fraction of both whole blood and peripheral blood mononuclear cell (PBMC) populations. Induction of CD69 antigen in the CD3 + T-cells following stimulation by pokeweed mitogen occurs after 2 h as assessed by three-colour immunofluorescent staining, peaking at 6 h.

In this study we have used three-colour flow cytometry to analyse the time-dependent expression of CD69 in T- and NK cell subsets simultaneously following a variety of stimuli. We have assessed the responses to the mitogenic lectin phaseolus haemaglutinnin (PHA-P), phorbol ester (PMA), cytokine stimulation, contact with the erythroleukaemic cell line K562 and alloantigen stimulation. This provides a comprehensive analysis of the temporal expression of CD69.

2. Materials and methods

2.1. Peripheral blood mononuclear cell samples

Peripheral blood was obtained from healthy donors and the mononuclear fraction (PBMC) was isolated by density centrifugation (Lymphoprep, Nycomed Pharma, Norway). The PBMC were washed in RPMI 1640 supplemented with 10% FCS, 2.5 mM L-glutamine and 100 units/ml penicillin and streptomycin (complete medium, CM) and cell density adjusted to 10^6 cells/ml.

2.2. Mitogen stimulation

2.2.1. Phytohaemaglutinnin (PHA)

2×10^6 cells were placed in polystyrene tubes (Falcon-Becton Dickinson, Oxford, UK) and incubated with and without PHA (2 $\mu g/10^6$ cells) (Sigma, Poole, Dorset). Cells were analysed at 1, 2, 4 and 24 h and phenotyped for CD69, CD56, CD28, CD4 and CD8 expression using fluorochrome conjugated monoclonal antibodies (Becton Dickinson, Oxford, UK). Three different combinations were used, CD69/CD56/CD8, CD69/CD28/CD4 and CD69/CD28/CD8 where the first antibody was FITC-labelled, the second labelled with PE and the third conjugated with PerCp. Cells were incubated at 21°C for 15 min and washed twice with PBS before analysis by flow cytometry (FACScan with Lysis II software-Becton Dickinson, Oxford, UK). 10,000 events were collected as list mode data in Lysis II after live gating of lymphocytes and lymphoblasts by FSC and SSC signals.

Cells were electronically gated on CD56 PE expression and then CD8 and CD69 expression of this population was analysed on a FL1/FL3 dot plot. Quadrant statistics were used to determine the relative proportions of CD8wk + /CD69 − , CD8wk + /CD69 + , CD8 − /CD69 − and CD8 − /CD69 + NK cells. CD8 positive T-cells were analysed on the same basis after electronically gating on FL3 signals from the CD69/CD28/CD8 labelled samples after exclusion of CD8wk + ve cells which we had found to be exclusively NK cells in the CD69/CD56/CD8 monoclonal antibody combination. CD69 + cells were expressed as a proportion of the total cells in each subset.

2.2.2. Phorbol myristate acetate (PMA)

2×10^6 cells were placed in polystyrene tubes (Falcon-Becton Dickinson, Oxford, UK) and incubated with and without PMA (10 ng/ml) (Sigma, Poole, Dorset). Cells were analysed at 1, 2, 4 and 24 h and phenotyped as above with CD69FITC/CD56PE/CD3PerCp and with CD4FITC/CD69PE/CD8PerCp. NK cells were determined as CD56 + /CD3 − and T-cell subsets were analysed on the basis of CD4 and CD8 expression. CD69 + cells were expressed as a proportion of the total cells in each subset.

2.3. Cytokine stimulation

2.3.1. Interleukin-12

1×10^6 cells, suspended in X-Vivo 10 serum-free medium (Biowhittaker, Walkersville, Maryland) supplemented with 100 units/ml penicillin and streptomycin, were placed in 24 well plates (Nunclon, Nunc, Denmark) and incubated with and without IL-12 (Roche) at a final concentration of 25 ng/ml. The cells were incubated in serum free medium to ensure that any activation that occurred was due to the exogenous cytokine and not serum-derived cytokines. The cells were analysed at 0, 24, 48 and 72 h to assess IL-12-induced activation, using a panel of monoclonal antibodies CD69FITC, CD56PE and CD3PerCP (Becton Dickinson, Oxford, UK). At each time point an aliquot of cells was incubated with the monoclonal antibodies for 15 min at 21°C in the dark and washed twice in PBS before analysis by flow cytometry (FACScan with Lysis II software-Becton Dickinson, Oxford, UK). 10,000 events were collected as list mode data in Lysis II after live gating of lymphocytes and lymphoblasts by FSC and SSC signals.

Cells were electronically gated on CD56 expression (excluding any CD3 + /CD56 + cells) and on CD3 expression and then these populations analysed on a dot plot showing CD69 expression against FSC. Quadrant statistics were used to determine the proportions of NK or T-cells which expressed CD69.

2.4. K562 stimulation of NK cells

Exponentially growing K562 cells (ECACC-Porton Down) were recovered from continuous culture and washed once with complete medium by centrifugation at $200 \times g$. The cell density was adjusted to 10^6/ml in complete medium and equal volumes of the PMBC suspension were added. Parallel cultures of PBMC alone were also set up. Aliquots were analysed by flow cytometry at 10, 60, 90, 120, 180 and 240 min after labelling with CD69/CD56/CD8 as described above. 20,000 cells with lymphoid morphology as determined by FSC and SSC signals were acquired and all events above the FSC threshold were stored in list mode. K562 stimulator cells were excluded from the NK cell analysis by electronic gating on FSC and SSC signals. NK cells were selected from within the lymphoid gate on the basis of CD56 expression. CD69 and CD8 expression on these NK cells were arrayed in 2-dimensional dot-plots and the relative proportions and numbers of cells in each quadrant were recorded.

2.5. Measurement of NK cell-mediated cytotoxicity on sorted NK cell fractions

NK cells were selected from PBL fractions from three normal donors. Briefly, PBLs were incubated with CD56 FITC (Becton Dickinson, Oxford, UK) followed by anti-FITC micro-magnetic particles (MACS, Miltenyi Biotec, Teddington, Middx) and positively selected using a magnetic cell sorter (MACS). The NK-enriched cell suspensions were then labelled with CD69 PE (Becton Dickinson, Oxford, UK) and sorted by flow cytometry (FACS Vantage, Becton Dickinson, Oxford, UK) into CD56 + /CD69 − and CD56 + /CD69 + fractions. The selected cell populations were used as effector cells in a flow cytometric NK cell cytotoxicity assay (Hatam et al., 1994) at an effector:target cell ratio of 1:1. K562 cells in continuous exponential growth phase were recovered from culture and washed in Hank's balanced salt solution (HBSS) and resuspended in 1.0 ml PKH-26 labelling diluent (Sigma, Poole, Dorset) at a concentration of 4×10^6/ml. A 4 μl aliquot of PKH-26 (Sigma, Poole, Dorset) was added to 1.0 ml labelling diluent and then added to the cell suspension for 2 min at room temperature. The labelling reaction was stopped by the addition of 1.0 ml neat foetal calf serum for 1 min. Finally the labelled cells were washed twice in CM and resuspended in CM at 10^6/ml.

At least 2×10^4 effector cells were incubated in triplicate with equal numbers of labelled targets for 4 h at 37°C. After the incubation period the cells were resuspended in a solution of propidium iodide (Sigma, Poole, Dorset) in PBS (1 μg/ml) and analysed by flow cytometry (FACScan or FACS Vantage, Becton Dickinson, Oxford, UK). At least 10,000 target cells were acquired with 1024 channel resolution after electronic gating on red fluorescence and the mean proportion of propidium iodide positive cells from the triplicate samples determined. Background target cell death was determined from cells incubated in the absence of effector cells. Cell-medi-

ated cytotoxicity was reported as percentage killing over background cell death averaged from the three samples:

Mean(% necrotic in test − % background necrosis)

2.6. Alloantigen stimulation of lymphoid cells

PBMC from two unrelated normal donors were used for each experiment, one labelled with PKH-26 as described above and termed the stimulator population and the other being the responder population. Stimulator and responder cells were mixed in an equal ratio. Non-stimulated responder cells were used as controls in each experiment. In a single experiment responder cells were incubated with PKH-26-labelled autologous PBMC to exclude the possibility that the membrane dye could be responsible for the stimulation. Samples were incubated at 37°C/5% CO_2 and analysed at 4, 24, 48, 72, 96, 120, 144 and 168 h. At the completion of each incubation the cells were labelled with monoclonal antibodies in the following three colour combinations, CD69, CD56, CD3 and CD4, CD69, CD8 where the antibodies were conjugated with FITC, PE and PerCp, respectively. 10,000 responder cells were acquired as list mode data with 1024 channel resolution. Stimulator cells were excluded from the analysis by electronic gating of high FL2 and FL3 signals. The CD56PE and CD69PE signals always fell within the first 500 channels of a 1024 channel distribution whilst the PKH-26 signals consistently appeared above channel 800 (Fig. 1). Similar differences in fluorescence signals were observed in FL3 between CD3PerCp or CD8PerCp and PKH-26. This technique permitted analysis of a one-way mixed lymphocyte reaction.

Data were analysed at each time interval by quadrant analysis of 2-dimensional dot plots after electronic gating on PKH-26 −ve events. The percentage of CD69 + cells within both the CD3 + and CD56 + subpopulations were determined both for the non-stimulated control and for the mixed lymphocyte culture. Percent T-cell activation in both cell suspensions was defined as the proportion of activated CD3 cells (CD3 + /CD69 +) divided by the total proportion of CD3 + cells (CD3 + /CD69 + and CD3 + /CD69 −). NK cell activation was assessed in the same fashion. The difference in activation between the MLR (responder population in the presence of stimulator cells) and the control responder population alone was calculated and the percent difference in activation was equal to % activation in MLR − % activation in control.

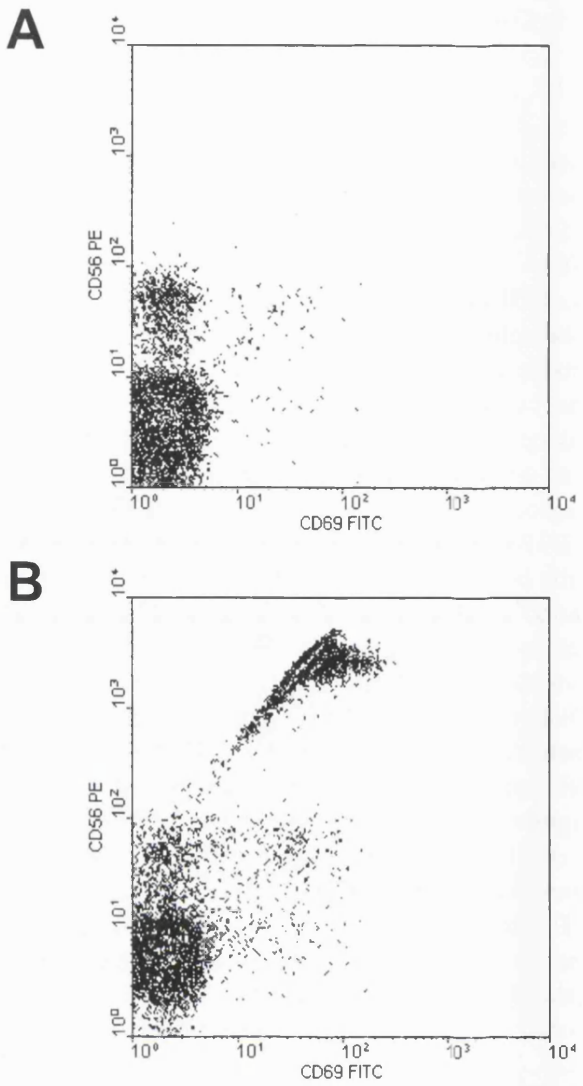

Fig. 1. Resolution of PKH26 labelled mononuclear stimulator cells from monoclonal antibody labelled responder cells. (A) Responder cells alone labelled with CD69 FITC and CD56 PE. (B) Responder and stimulator cells mixed at a 1:1 ratio showing clear resolution of PKH26 labelled stimulator cells in region 1.

3. Results

3.1. Effect of PHA stimulation

PHA induced rapid expression of CD69 on CD4 + T-cells with an increase over the non-stimulated

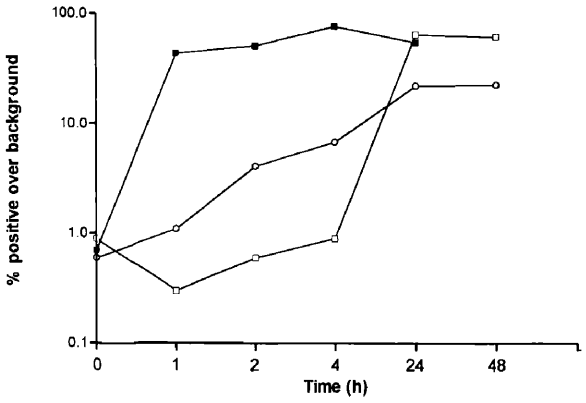

Fig. 2. Temporal expression of CD69 on CD4+ve and CD8+ve lymphocytes after PHA stimulation. CD4+, ■; CD8+/28+, □; CD8+/28−, ○.

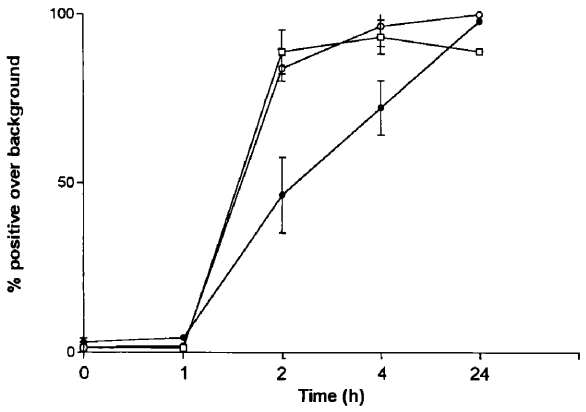

Fig. 4. Temporal expression of CD69 on T-cells and NK cells in response to PMA. CD4+, □; CD8+, ○; CD56+, ●. Data are presented as means and standard deviations.

control cells detectable after 1 h (Fig. 2). All CD4+ ve cells expressed CD28 and it was thus not possible to determine the kinetics of CD69 expression on CD4+/28− cells. CD8+ T-cells were considerably slower to respond to PHA. Both CD28− and CD28+ CD8+ cells required 24 h of stimulation to achieve the level of CD69 expression shown in the CD4 subset after only 1 h. The mitogen induced CD69 expression on both the CD28− and CD28+ subpopulations of CD8+ T-cells. The CD28+ fraction represented a smaller starting population than the CD28− cells but the rate of increase in CD69 expression over the first 3 h was comparable.

CD56+ natural killer (NK) cells also showed a rapid upregulation of CD69 in response to PHA. This was identical in both the CD8− and the CD8wk+ subpopulations (Fig. 3). At 24 h post stimulation and beyond, the proportion of CD8wk+ NK cells expressing CD69 fell whilst the CD8− NK cell population expressing CD69 increased. This was not associated with any change in the total percentage of CD69+ NK cells and thus presumably represents shedding of the CD8 alpha chain as a consequence of activation.

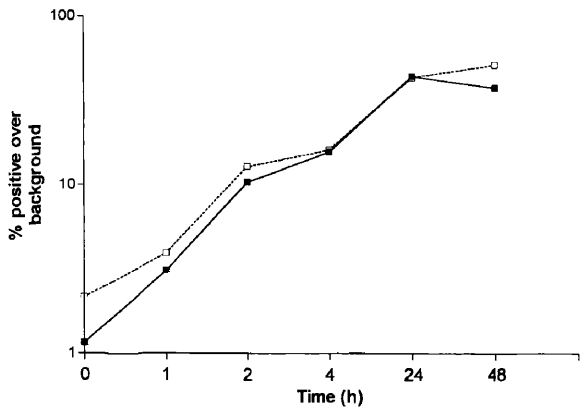

Fig. 3. Temporal expression of CD69 on CD56+ve lymphocytes after PHA stimulation. CD8+ve, ■; CD8−ve, □.

3.2. Effect of PMA stimulation

PMA stimulation led to activation of both T- and NK cells between 1 and 2 h post incubation (Fig. 4). Activation of T-cells was more marked than of NK cells but, in contrast with PHA, there was no difference between the rate of CD4 or CD8 responsiveness.

3.3. Effect of IL-12 stimulation

IL-12 induced a marked increase in CD69 expression on NK cells at 24 h post incubation; peaking at 48 h. Neither CD4 nor CD8 cells showed a response during 48 h of incubation (Fig. 5).

3.4. Effect of K562 stimulation

Initial analysis of CD69 expression on NK cells in the lymphoid gate after co-incubation with K562 cells for periods of greater than 2 h suggested an increase in the positive fraction. However, in all cases, the total NK cell population within the lym-

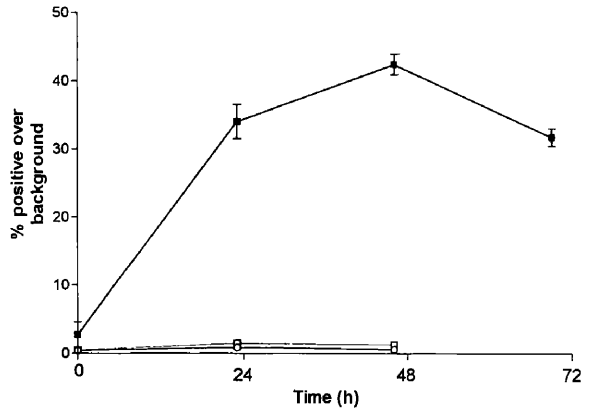

Fig. 5. Temporal expression of CD69 on T-cells and NK cells in response to IL-12. CD4+, □; CD8+, ○; CD56+, ■. Data are presented as means and standard deviations.

phoid gate was reduced. This reduction in NK cells in the lymphoid gate was consistently within the CD56 + /CD69 − population (Fig. 6), leading to an indirect increase in the proportion of CD69 + NK cells. At 2 h post incubation the absolute number of CD56 + /CD69 − cells within the lymphoid gate fell by an average of 48.99% (standard deviation, 15.78) and by 47.71% (standard deviation, 8.86) at 4 h. We were unable to demonstrate any increase in CD69 + NK cells during a 4 h incubation. Division of NK cells into subsets on the basis of CD8 expression showed no consistent pattern of reactivity, the cell populations dividing on the basis of presence or absence of pre-existing CD69 expression (data not shown).

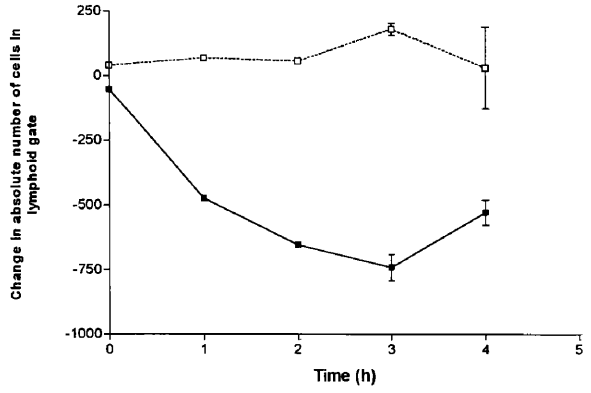

Fig. 6. NK cell subset response to K562. CD69+ve, □; CD69− ve, ■. Data are presented as means and standard deviations.

3.5. Measurement of NK cell cytotoxicity

CD56 + /CD69 − NK cells from all three normal donors exhibited normal levels of K562 lysis (mean cytotoxicity = 28%). In contrast, the CD56 + /CD69 + cell fractions tested synchronously showed no significant increase in cytotoxicity above background (mean cytotoxicity = 2.25%).

3.6. Effect of alloantigen stimulation

3.6.1. CD3 + T-cells

The proportion of CD3 cells in the control cell cultures expressing CD69 was low and remained relatively constant over the time course. The mean percent positive ranged from 1–1.3%. There was a consistent fall in the CD69 + fraction in the control cultures as the time course experiment progressed to 120 h and beyond. This may have been due to downregulation of the CD69 molecule or the apoptosis of the innately activated T-cells through lack of essential cytokines.

A small proportion of CD3 T-cells responded to the alloantigen stimulus. This was detectable from 24 h post stimulation and rose to a peak at 96 h, maintaining a plateau to 168 h before falling. Even at the peak of the response the proportion of activated T-cells never reached more than 3% of the total CD3 + subset (Fig. 7).

In one pair of experiments it was noted that there was an unusually high percentage of activated CD3 + /CD69 + cells at the start of the experiment (17%). In this pair, rather than showing a progressive rise in CD69 expression over time in the MLR, both control and MLR demonstrated a progressive drop in CD69 expression over time (data not shown).

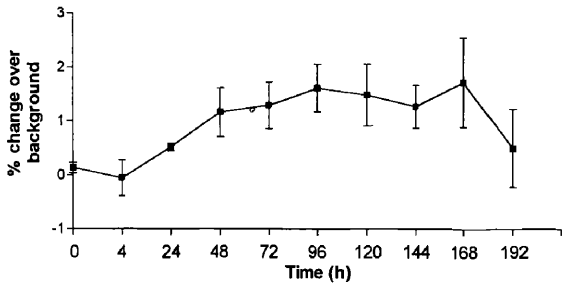

Fig. 7. Temporal expression of CD69 on CD3+ T-cells after alloantigen stimulation. Data are presented as means and standard deviations.

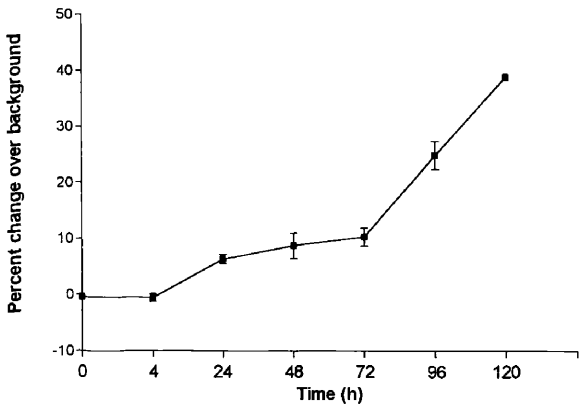

Fig. 8. Temporal expression of CD69 on NK cells after alloantigen stimulation. Data are presented as means and standard deviations.

3.6.2. CD56 + NK cells

The background incidence of CD69 expression was higher in the CD56 + cell population than in the T-cell population. This ranged between 5 and 20%. Following incubation with the irradiated stimulator cells the CD56 + population showed no increase in CD69 expression after the first 4 h but did show a response after 24 h. This was consistently more marked than that shown by the CD3 + cell population at the same time points and, in contrast to the T-cell population the proportion of activated NK cells peaked at 120 h (Fig. 8).

To exclude the possibility that PKH 26 labelling may directly affect CD69 expression, a time course experiment was repeated using PKH 26 labelled autologous stimulator cells. This showed no increase in CD69 expression as compared to the control unlabelled responder population (data not shown).

4. Discussion

The results presented above confirm previous findings that CD69 is an early activation antigen expressed on lymphoid cells following a variety of stimuli. The studies with PHA and PMA stimulation showed a surprisingly fast upregulation of CD69 on both T- and NK cells with a detectable increase above background levels within 2 h of stimulation. This implies that the molecule can reside within the cytoplasm and expression can occur without de novo protein synthesis.

We studied the relative responses of CD28 − and CD28 + T-cells to PHA. A previous comparison of CD28 + and CD28 − T-cells found no difference in activation when measured between 24 and 48 h post stimulation (Lucas et al., 1995) and our results show that this is also true over the first 3 h post stimulation. This implies that functional maturity has no effect on the speed of response to stimulation as determined by CD69 expression.

In a comparison of the relative PHA responses of T-cells versus NK cells it was consistently apparent that NK cells showed more rapid expression of CD69 than did T-cells from the same donor. However, the NK cell activation reached its plateau earlier than the T-cell response leading to equivalent total activation in the two subsets. The earlier response by the NK cells may have been due to higher levels of expression of CD2 on the NK cells since this is one of the cellular receptors for PHA (Leca et al., 1986). The CD8wk + NK cells appeared to shed the CD8 molecule upon activation as evidenced by the relative increase in CD56 + /CD8 − and the concomitant decrease in CD56 + /CD8wk + subsets in the absence of any demonstrable increase in total NK cell number. The shedding of CD8 alpha chains from CD8 + T-cells following activation is well known (Tomkinson et al., 1989).

We have studied the effect of IL-12 on lymphocyte subsets. IL-12 is a multifunctional cytokine with effects on T- and NK cells with many similarities to IL-2. The effect of IL-2 on CD69 expression on lymphocytes has been studied in detail (Lanier et al., 1988) but this is not the case for IL-12. In our studies IL-12 induced rapid CD69 expression on NK cells with no direct effect on CD4 or CD8 cell subsets during the period of study. This is in keeping with previous reports in which it has been shown that T-cells require prior stimulation with antigen or mitogen for three to five days before IL-12 signalling through STAT4 can be detected (Bacon et al., 1995a,b).

The K562 stimulation studies demonstrated that CD69 expression by NK cells identifies a population which neither lyses nor conjugates with K562 target cells. In our initial analyses of these experiments we found a relative increase in CD69 + cells within the CD56 + population and assumed that these were the result of co-incubation with K562 cells. However, it

was apparent that the total number of CD56 + cells within the electronic gate was reduced after co-incubation and the relative increase in CD69 + cells was due to the selective depletion of CD69 − cells from the lymphoid gate. Subsequent analyses showed that the CD69 − NK cells had conjugated with the K562 cells, thus removing them from the lymphoid gate. Positive selection of CD69 + and CD69 − subsets of NK cells from normal donors demonstrated that the lytic capacity resided within the CD69 − fraction. We were unable to determine whether the CD69 − fraction attained CD69 after conjugation and lysis since the K562 target cells were persistently autofluorescent which masked any specific fluorescence from monoclonal antibody labelled NK cells within the conjugate. However, it has been reported previously that NK cells express CD69 upon conjugation with target cells and are subsequently incapable of further K562 lysis (Jewett and Bonavida, 1995). In our studies we have used NK cells which inately expressed CD69 and cells which have been mitogen stimulated to express CD69 and in both cases they are incapable of K562 lysis. This suggests that CD69 expression identifies cells in a state of anergy post function and not cells which are 'pre-activated' and ready to function.

Alloantigen stimulation of normal lymphocytes resulted in low incidence, but nonetheless detectable, expression of CD69 above background. This was true of both T- and NK cell subsets. This finding is in contrast to a previous report in which no CD69 expression was detected on T-cells during 7 days of allogeneic stimulation despite detectable responses to a variety of mitogenic stimuli (Simms and Ellis, 1996). This study utilised irradiated stimulator cells and excluded them from the electronic analysis gate by virtue of their light scatter characteristics. We have undertaken similar experiments and found that the change to an apoptotic morphology by the irradiated stimulators requires three days of culture. In some culture systems this is associated with a degree of background cell activation, presumably in response to cytokines within the foetal calf serum. It is possible that such background activation may screen the low level alloantigen-specific activation and this could explain the previous findings. Another study of CD69 expression in response to alloantigen stimulation by irradiated cells did detect expression (Hara et al., 1986). The earliest time point studied was 60 h post initiation of the mixed lymphocyte culture and the response peaked at 108–156 h. This is comparable to the time point of maximal response in this study It was our experience that the dynamics of the alloantigen response were markedly different from the mitogen responses of both T- and NK cells. In all cases the CD69 expression was delayed in comparison with the mitogen response and, unremarkably, was at considerably lower incidence. It was a common finding with the alloantigen responses that a higher proportion of NK cells responded than did T-cells in each culture pair. We interpret this as a reflection of the high incidence of mismatches at the appropriate HLA-C and HLA-B loci involved in NK cell regulation (Colonna and Samaridis, 1995). The observation supports the concept that NK cells are involved in graft rejection and graft-versus-host disease after allogeneic bone marrow transplantation (Scott et al., 1995).

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Selective removal of alloreactive cells from haematopoietic stem cell grafts: graft engineering for GVHD prophylaxis

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Summary:

One of the main goals in allogeneic bone marrow transplantation is the abrogation of graft-versus-host disease with the preservation of antileukaemia and antiviral activity. We have established a novel system for the selective removal of alloreactive lymphocytes from donor grafts while retaining an effective allogeneic response to third-party stimulator cells. Initial feasibility studies were done with unrelated HLA-mismatched pairs and then extended into the matched setting. Mononuclear cells from HLA-matched donors were cocultured with irradiated recipient cells prestimulated with cytokines (γ-IFN and TNF-α) in a modified mixed lymphocyte culture (MLC). Alloreactive donor lymphocytes were identified by expression of CD69, an early activation marker and selectively removed by paramagnetic bead sorting. The remaining 'non-alloreactive' lymphocytes were tested in proliferative assays against the original matched recipient and to a third-party donor. A mean depletion of proliferative capacity to 11.5 ± 9.9% of the original matched recipient response was achieved while the residual third-party response was largely preserved at 77.8 ± 20.9% which should translate into improved immune reconstitution and preservation of antiviral activity. The non-alloreactive lymphocytes could also possess functional antileukaemia activity. Moreover, the alloreactive cells are easily recoverable in this selective T cell depletion strategy for cryopreservation and ready for immediate access as therapeutic donor lymphocyte infusions in cases of frank relapse post transplant.

Keywords: T cell depletion; alloreactivity; CD69; allogeneic BMT; GVHD; graft-versus-leukaemia

It is established that mature immunocompetent donor lymphocytes present in the graft are responsible for the pathophysiological effects of graft-versus-host disease GVHD[1,2] either due to major or minor histocompatibility antigen disparity or radiation-induced cytokines or both. Activated T cells proliferate, secrete further cytokines and recruit other inflammatory effector cells leading to the development of GVHD. Pan T cell depletion strategies have generally been successful in reducing the incidence and severity of acute and chronic GVHD;[3] unfortunately, they have been associated with a concomitant rise in the incidence of graft rejection, leukaemia relapse and delayed immunological reconstitution.[4–7]

So far, successful use of T cell depletion has required the establishment of an appropriate balance between the residual immune response of the recipient, which can be achieved by enhanced conditioning, and the adjustment of the T cell content of the donor bone marrow.[8,9]

That T cells play an integral role in the mechanisms of graft rejection, GVHD and in GVL activity is no longer in doubt. What has proved to be more difficult has been the identification of specific subsets of cells involved in each of these activities. Murine models have variously implicated either $CD4^+$ or $CD8^+$ cells as effectors in GVHD.[1,10] These observations have been strain-specific and dependent on the degree of donor–recipient histocompatibility. They have also not translated as clearly into the human situation. Similarly, $CD4^+$, $CD8^+$ and NK cells have all been implicated in GVL activity without causing GVHD,[11–13] these however may only be applicable to certain leukaemias and not others.[14] The advent of donor leucocyte infusion (DLI) has provided the strongest evidence so far of the importance of a GVL effect,[15,16] and again attempts have been made to identify distinct populations of cells involved in GVL without inducing GVHD.[17,18]

An alternative approach to pan T cell depletion is to selectively deplete only those T cell subsets responsible for graft-versus-host reactions while retaining lymphocytes capable of GVL and antiviral activity in the graft.[19–23] Immune recovery may also be improved with the increased numbers of residual T cells in the graft leading to a more diverse repertoire.

Here we present a selective T cell depletion strategy based upon the physical separation of the alloreactive T cells from the donor graft whilst retaining the non-alloreactive T cells. In this system donor T cells were stimulated *in vitro* with recipient mononuclear cells (representing alloreactive GVHD targets). After defined periods, alloreactive responder cells were identified by virtue of their expression of an activation marker and then physically depleted from the remaining cells using magnetic cell sorting (MACS, Miltenyi Biotec, Cologne, Germany).

We have chosen to use CD69 as the activation marker in this study. This antigen is a type II integral membrane protein belonging to the family of C-type lectin receptors.[24] It is one of the earliest cell surface activation markers that is expressed on lymphocytes and NK cells after mitogenic

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stimulation. Upregulated CD69 expression on T lymphocytes may be followed by cell proliferation and an increase in secretion of IL-2 and IFN-gamma.[25] The kinetics for the expression of CD69 have been investigated by us and previously published. We have shown that CD69 is upregulated in an allogeneic MLC in a time-dependent fashion,[26] peaking at 96 h and could be used as a marker of alloreactivity and indirectly of GVHD.

Materials and methods

Patients and subjects

In the initial feasibility study involving mismatched pairs, blood was obtained from HLA mismatched normal volunteer donors. For the matched study, the first-party stimulators were patients (recipients) who were undergoing subsequent HLA-matched allogeneic bone marrow transplants. The responders (donors) were either their HLA-identical siblings or matched unrelated donors from a donor registry (Anthony Nolan Research Centre or Bristol Bone Marrow Registry). Peripheral blood was collected from the donors and remission peripheral blood or bone marrow from the patients after informed consent. For the third-party stimulators, blood was collected from HLA-mismatched normal volunteer donors.

The mononuclear cell fraction (MNC) was isolated from heparinised blood or bone marrow by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway), washed in RPMI 1640 and supplemented with 10% fetal calf serum (FCS), 2.5 mM L-glutamine and 100 units/ml penicillin and streptomycin (complete medium (CM), all from Gibco, Paisley, UK). All cell densities were adjusted to 10^6 cells/ml. Cells were either used fresh or cryopreserved in N_2 vapour for subsequent use.

HLA typing

In each case, donor and recipient pairs were serologically typed for HLA-A and HLA-B locus antigens and high resolution sequence specific oligonucleotide typing for HLA-DRB1, DRB3, DRB4, DRB5 and DQB1 alleles (performed at the Anthony Nolan Research Centre, London, UK).

Identification and selection of alloreactive T cells

HLA-mismatched study: In each case, PBMC from three unrelated normal donors were used; one termed the responder population, one the first-party stimulator population and one as the third-party stimulator to assess specificity of alloreactive cell removal. Responder PBMC were mixed in an equal ratio with irradiated first party stimulator cells in a one-way 'primary mixed lymphocyte culture' (MLC). In a parallel culture, responder cells were also mixed in an equal ratio with an irradiated third-party population. All cells were resuspended to a density of 10^6 cells/ml and cultured in tissue grade plastics (Nunclon, Nunc, Denmark).

The controls for each experiment were non-stimulated responder cells alone. All stimulator cells were irradiated for a total of 33 Gy just prior to mixing The 'primary MLCs' were incubated at 37°C/5% CO_2 and samples analysed at 72–96 h for CD69 expression in the responder population.

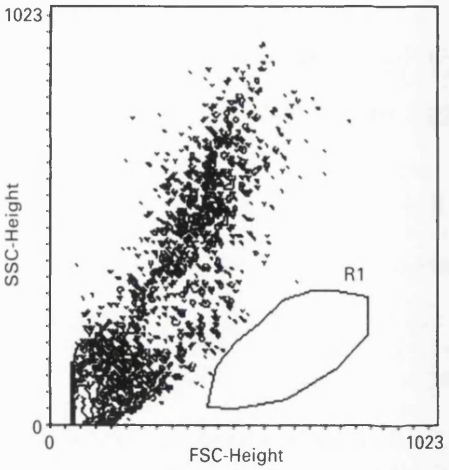

Figure 1 Flow cytometric dot plot showing forward angle light scatter *vs* 90° light scatter of irradiated stimulator peripheral blood mononuclear cells 3 days after irradiation. Region 1 delineates the area in which live, non-apoptotic PBMC should fall.

Using analysis by flow cytometry (FACScan with Lysis II software, Becton Dickinson, Oxford, UK), responder lymphocytes and lymphoblasts were easily identified by live gating on forward angle light scatter (FSC) and side angle light scatter (SSC) signals. By 72 h the irradiated stimulator cells had moved out of this lymphoid gate due to apoptosis and could be easily separated from the responder population (Figures 1 and 2).

CD69 expression was assessed for the responder population in both the first-party primary MLC and the third-party primary MLC. Alloreactive cells in the first-party primary MLC could be distinguished from non-alloreactive

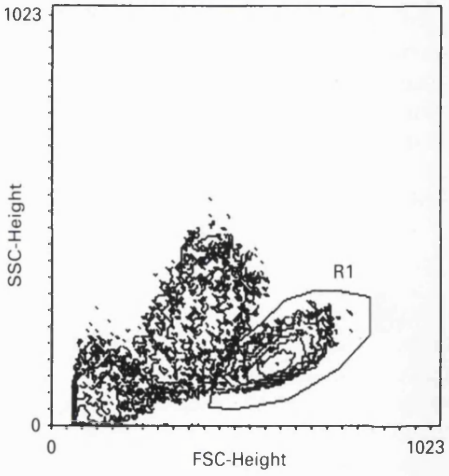

Figure 2 Flow cytometric dot plot showing forward angle light scatter *vs* 90° light scatter of normal PBMC plus irradiated simulator PBMC 3 days after irradiation. Region 1 delineates the area in which the live, non-apoptotic responder PBMC fall and shows the resolution of responder cells from irradiated stimulator cells. Such clear resolution of the cell populations allows electronic gating of the responder population for the assessment of CD69 expression.

cells by the expression of CD69. These CD69+ve cells in the first-party primary MLC were then depleted using a magnetic cell sorting (MACS) AS depletion column (Miltenyi Biotec). This is a sterile technique of cell separation involving the passage of cells labelled with iron dextran microparticles (50 nm diameter) through a ferrous matrix column within an intense magnetic field. Cells were labelled with saturating amounts of anti-CD69 FITC for 10 min as described by the manufacturers. Excess antibody was then washed off before incubation with anti-FITC microbeads for 30 min at 4–8°C after which the labelled cells were passed down an AS column with a 25 G capillary. The buffer used throughout the separation procedure was Dulbecco's phosphate-buffered saline (PBS) supplemented with 2 mM EDTA and 0.5% BSA (MACS buffer).

Non-bound cells were collected and termed the 'depleted fraction'. After briefly washing the column with MACS buffer, the bound cells were eluted by forced flushing of the column outside the magnetic field. The eluted cells were termed the 'enriched fraction'.

The CD69-depleted responder PBMC were then re-introduced with equal numbers of first-party stimulator PBMC in a first-party secondary MLC. The depleted fraction was also mixed in a parallel culture with equal numbers of the same third-party PBMC in a third-party secondary MLC. CD69 expression of the responder population in both secondary MLCs were then assessed at the same time-point prior to sorting as in the primary MLC.

The CD69 expression of the unsorted cells against either first-party stimulator or third-party was normalized to 100%, and the degree of depletion achieved determined by the following formula:

$$\frac{(\text{CD69 expression in primary MLC}) - (\text{CD69 expression in responder alone}) \times 100\%}{(\text{CD69 expression in secondary MLC}) - (\text{CD69 expression in depleted fraction alone})}$$

The degree of depletion to both the first-party stimulator and the third-party was then compared as a percentage of the unsorted response.

HLA-matched pair study: Figure 3 outlines the method in a flow diagram. The same procedure was applied as above with the patient being the first-party stimulator and the matched donor (either sibling or unrelated) being the responder. However, to circumvent the problem that no appreciable CD69 expression or tritiated thymidine (^{3}HTdR) uptake is normally detected above baseline in HLA matched pairs (data not shown), the following modifications to the above procedure were made: *(1) Modified MLC:* All stimulator cells (PBMC from patient, third-party donor and the autologous control) were pretreated with 1000 U/ml of γ-IFN (Genzyme, West Malling, Kent, UK) and 1000 U/ml of TNF-α (Genzyme) for 24 h. The cytokines were washed off twice with PBS and resuspended in CM at 10^6 cells/ml. The stimulator cells were then irradiated for a total of 33 Gy before being mixed with the donor (responder) cells. To exclude any non-specific effect by the cytokines, an autologous control was set up in parallel consisting of responder cells mixed with cytokine pretreated autologous cells. *(2) Proliferation assays:* The ^{3}H-thymidine (Amersham, Bucks, UK) incorporation was used

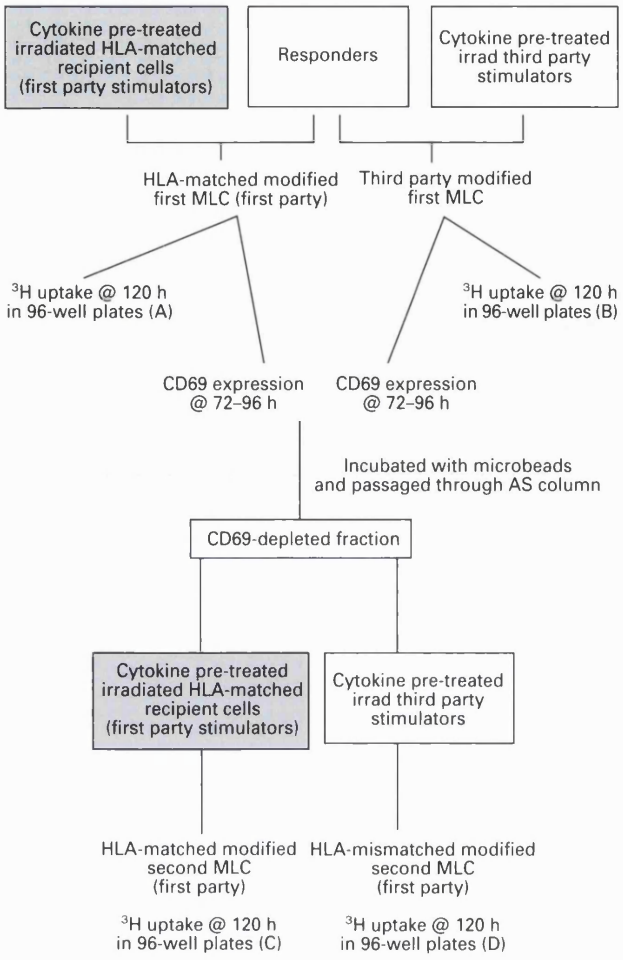

Figure 3 Flow diagram of the experimental protocol for HLA-matched pairs.

as an *in vitro* marker of alloreactivity to compare the results of depletion in matched pairs. In addition to assessing the CD69 expression at 72–96 h, proliferation assays were set up in triplicate in 96-well round-bottom plates using 10^5 donor (responder) and 10^5 cytokine pretreated irradiated patient (first-party stimulator) or third-party cells per well in a total volume of 200 μl/well. 18 U/ml of IL-2 or 1000 U/ml of IL-4 (Genzyme, West Malling, Kent, UK) were added to this modified MLC. The baseline autologous controls were donor cells plus cytokine pretreated autologous stimulators. The other controls present were irradiated stimulators alone. Cultures were incubated at 37°C/5% CO_2 for 5 days, and pulsed for the last 18 h with 1 μCi/well of ^{3}HTdR. Cells were harvested and tritiated thymidine incorporation measured in a Wallac 1205 Betaplate (Wallac, Turku, Finland). Proliferation was measured for both the primary first and third party MLCs. After removal of alloreactive cells via the AS column, proliferation assays were again set up for the depleted fraction in the secondary HLA-matched and third party MLC. The specificity of depletion was assessed by comparing the proliferation assays of the depleted fraction against the pre-sorted cells.

The ^{3}HTdR incorporation of the unsorted cells against either patient (first-party stimulator) or third party was nor-

malized to 100%, and the degree of depletion achieved established from the following formula:

$$\frac{(^3\text{H-incorporationin primary MLC}) - (^3\text{H incorporation in auto control})}{(^3\text{H incorporation in secondary MLC}) - (^3\text{H incorporation in post sort-auto control})} \times 100\%.$$

From Figure 3, the relevant calculation is (C)/(A) and (D)/(B). The ^{3}H-thymidine incorporation in each instance was worked out as the mean of the triplicates.

Cell recovery, purity and depletion efficiency: The responder cell population was counted before and after sorting via the MACS columns. The responder cells could be easily distinguished from irradiated stimulator cells by cell morphology. Responder cells retained their cell shape and nuclear configuration whereas irradiated stimulator cells had lost their membrane and nuclear integrity.

Cell recovery (yield) was calculated as follows:

$$\frac{\text{Number of CD69 neg responder cells post-sorting}}{\text{Number of CD69 neg cells before sorting}} \times 100.$$

Cell purity of the engineered graft is defined as (100 – %CD69 pos cells in the depleted fraction).

Depletion efficiency was calculated by comparing the number of CD69-positive cells in the depleted fraction after sorting with the number of CD69-positive cells prior to sorting. This is expressed as follows:

$$\frac{(\text{No. of CD69 pos cells before sorting}) - (\text{No. of CD69 pos cells after sorting})}{(\text{No. of CD69 pos cells before sorting})} \times 100.$$

Immunophenotyping: All cells were immunophenotyped using fluorochrome conjugated monoclonal antibodies (Becton Dickinson, Oxford, UK). The following three-colour combination was used: CD69-FITC, CD56-PE and CD3-PerCp to differentiate between activated T cells and NK cells. PBMC were incubated with the antibody at 21°C for 15 min and washed twice with PBS before analysis by flow cytometry. (FACScan with Lysis II software, Becton Dickinson). 10 000 events were collected as list mode data in Lysis II after live gating of lymphocytes and lymphoblasts by FSC and SSC signals.

Results

HLA-mismatched pairs

Table 1 shows the increase in CD69 expression above baseline in both first and second MLCs at 72–96 h. It can be seen that following the depletion strategy, the increase in CD69 expression for the first party stimulator MLC falls from 6 ± 2.4% to 1.5 ± 1.2% while that that of the third party MLC is well maintained at 3.9 ± 2.7% in the first MLC and 4 ± 2.4% in the second MLC.

Selective depletion: To calculate the degree of selective depletion, the primary response for both first party stimulator and third party was normalized to 100% and the residual response expressed as a percentage. The mean residual response post-CD69 depletion was 25.6% ± 17.9. The response to third party stimulator cells was retained (mean of 115.7% ± 35.9) (Figure 4). The median cell recovery was 72.5% (range 65–75%) with an associated median purity of 97% (range 96–98%).

The depletion efficiency was a median of 75% (range 65–84%).

Matched pairs

Modified MLC: Following cytokine pre-treatment of stimulator PBMC, proliferative responses were detected in five out of nine HLA-matched pairs. Table 2 shows the ^{3}H-thymidine incorporation by each of the nine donor-recipient pairs in the primary MLC.

All stimulator cells were cytokine pre-treated as described above. We also assessed the background autologous MLC, ie donor PBMC plus cytokine pre-treated auto-

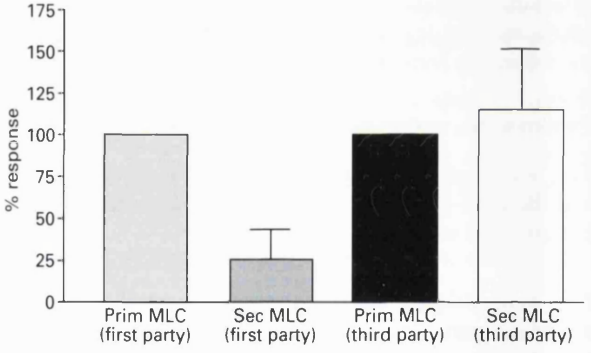

Figure 4 Residual allo-antigen activation response to HLA mismatched stimulator cells after removal of CD69$^+$ responder cells. Results are expressed as mean (±s.d.) percentage of the original response prior to depletion.

Table 1 CD69 expression (% of total responder MNC fraction) in primary and secondary cultures

Donor–recipient pair	*CD69 expression above baseline for first party stimulator primary MLC*	*CD69 expression above baseline for third party stimulator primary MLC*	*Residual CD69 expression above baseline post depletion for first party stimulator secondary MOC*	*Residual CD69 expression above baseline post depletion for third party stimulator secondary MLC*
1	6.1	7.2	2.5	7.7
2	9	1.5	0	1.5
3	8.2	1.4	2.7	2.3
4	4.9	3	1.9	4.1
5	2.9	6.1	0.4	4.3
Mean ± s.d.	6 ± 2.4	3.9 ± 2.7	1.5 ± 1.2	4 ± 2.4

logous PBMC to control for possible non-specific effect from the cytokines.

As detailed in Table 2, the addition of cytokines to the modified MLC did not cause a significant increase in the autologous MLC compared to the responder cells incubated alone ($P > 0.1$). In contrast, the majority of MLC of the matched donor–recipient pairs did generate a proliferative response above that of the autologous control. The trend in all nine experiments for the MLC response was: third-party MLC > matched donor–recipient MLC > autologous MLC > responder alone. This is in keeping with the greater degree of HLA disparity between donor and third party as compared to the matched recipient.

When Bishara *et al*[27] developed the modified MLC as a predictive tool for clinical GVHD, the relative response index (RRI) was used to calculate if the modified MLC response in the matched pair was positive compared to the autologous control. Normalizing the response against third party as 100%, the relative response index (RRI) was calculated as follows:

$$\text{RRI (\%)}: \frac{\text{(matched MLC c.p.m.)} - \text{(autologous MLC c.p.m.)} \times 100}{\text{(third party MLC c.p.m.)} - \text{(autologous MLC c.p.m.)}}$$

A relative response index >5% was considered positive. Table 2 shows the RRI calculated for each experiment: Using the modified MLR, five positive responses were generated out of nine matched pairs (56%).

Effect of cytokines on CD69 expression after HLA-matched MLC: We have previously established the expression of CD69 in an allogeneic setting and confirmed this in our mismatched study. Initial work done with matched pairs without the use of cytokines showed no upregulation of CD69 expression (data not shown). Like the proliferation seen in the modified MLC, adding cytokines to the system did cause upregulation of CD69 expression in the matched pairs. The difference was that there was an upregulation of CD69 in all pairs tested compared to five out of nine for the proliferative assay (Table 3).

The mean baseline CD69 activation of the autologous control at 72 to 96 h was 5.0 ± 2.5%, that of matched pairs 8.9 ± 3% and third party was 10.1 ± 4.1%. The mean increase in CD69 expression above autologous control for matched pairs was 3.8% and that of third party controls was 5.1% above baseline. This increase in CD69 expression for mismatched third party using cytokines compared well with the data for the mismatched study. Pre-treatment of stimulator cells with γ-IFN and TNF-α and addition of low dose IL-2 did not increase the baseline CD69 activation of the autologous controls. In contrast, an appreciable CD69 response with matched siblings was generated above the baseline controls.

Depletion efficiency and cell purity: Table 4 shows the results of magnetic cell sorting using the AS depletion columns. This gave a median depletion efficiency of 80.3% (range 62–98.6%). The cell purity was consistently more than 94% (range 94.3–99.8%). As the columns used were designed for depletion strategies, not surprisingly, the enrichment process was less stringent ranging from 30 to 68% CD69-positive cells in the alloreactive enriched fraction.

Recovery of cells: The median cell recovery was calculated as described in Materials and methods and was 75% with a range of 46–85%.

Selective depletion of alloreactive cells: In five out of nine experimental pairs (1, 2, 3, 7 and 8), a positive proliferative result was obtained in the modified MLC. We could therefore compare the ^{3}H-thymidine incorporation of the PBMC before and after depletion. In the other four experiments however, we could not assess the degree of selective depletion of alloreactive cells since no proliferative response was generated in the primary MLC. Nevertheless, in all cases, we were able to determine whether the third party response had been preserved despite the removal of CD69-positive cells after allostimulation.

Table 5 shows the ^{3}H-thymidine incorporation of the five evaluable pairs post-depletion. The residual response was calculated as described in Materials and methods. A mean depletion of proliferative capacity to 11.5 ± 9.9% of the original first party response was achieved (Figure 5). The RRI (Table 5) of the depleted fraction is <5% in each instance which is below the threshold reported to be predictive of clinical GVHD.[27]

Preservation of third party response: We looked at the residual third party response to determine if the depletion of alloreactive CD69-positive cells was alloantigen-specific. This showed a mean preservation of proliferative response of 77.8% ± 20.9% as illustrated in Figure 5.

Association with clinical GVHD: Table 2 shows the relationship between the results of the modified MLC and the incidence of clinical GVHD in the recipients who have undergone an allogeneic transplant from their HLA-matched donor. All sibling transplants (six patients) were non-T cell-depleted (TCD) and recipients were given either cyclosporin or cyclosporin and methotrexate as GVHD prophylaxis. Two patients who had matched unrelated transplants received Campath 1-M *ex vivo* TCD marrows for GVHD prophylaxis with no additional immunosuppressive therapy. Both also received Campath 1-G prior to transplant as part of the conditioning treatment. The ninth donor–recipient pair did not proceed on to a transplant. As shown in Table 5, a RRI >5%, indicating a positive modified MLC was associated with subsequent clinical GVHD in three of four evaluable patients. Only one of the four patients receiving a bone marrow from a donor showing a RRI <5% (ie negative) showed evidence of clinical GVHD post transplant. The median follow-up period of these patients is 12 months (range 8–13 months).

Discussion

GVHD still represents a major barrier to successful allogeneic transplantation. Pan T cell depletion strategies have reduced the incidence and severity of GVHD. Unfortunately, this has not generally translated into better survival rates largely due to reported increased rates of leukemia relapse, graft rejection and infections from delayed immune reconstitution. The increase in patients requiring allogeneic

Table 2 ^{3}H-thymidine incorporation in primary modified MLCs

Donor–recipient pair	*Donor alone*	*Donor cells + autologous stimulators*	*Donor cells + HLA-matched recipient first party cells*	*Donor cells + HLA-mismatched third party cells*	*Relative response index*	*Positive if RRI >5%*	*Clinical GVHD*
1	37199	28744	60444	74490	71	positive	grade I
2	8019	13027	25823	60175	26	positive	grade III
3	8943	12590	19695	76925	11	positive	grade II
4	16370	17152	18152	63594	2.1	negative	No GVHD
5	6356	4690	3159	30139	0	negative	No GVHD
6	891	NA	857	NA	0	negative	No GVHD
7	25189	29626	36171	71324	12	positive	No GVHD
8	NA	1980	17071	31410	51	positive	NE
9	9849	6375	7084	46403	2.6	negative	grade II

(Mean counts per minute; $n = 3$).
NA = not assessed; NE = not evaluable.

Table 3 CD69 expression in primary MLCs expressed as (% total of responder MNC fraction)

Donor–recipient pairs	*Donor cells alone*	*Donor cells + autologous stimulator cells*	*Donor cells + HLA-matched recipient cells*	*Donor cells + HLA-mismatched third party cells*
1	5.4	5.9	11	10
2	4.6	2.1	7.2	10.3
3	3.6	2.8	6.9	8.9
4	2.2	4.4	10	8.1
5	3.4	4.8	6.2	6.2
6	2.5	2.5	6.1	6.2
7	NA	10	11	19.4
8	6.7	6.7	15	13
9	NA	6.1	6.7	8.8
Mean ± s.d.	4.1 ± 1.6	5.0 ± 2.5	8.9 ± 3	10.1 ± 4.1

NA = not assessed.

Table 4 CD69 expression in fractions pre- and post-sorting (% of total responder MNC)

Donor–recipient pairs	*Pre-sort*	*Depleted fraction post-sort*	*Enriched fraction post-sort*	*Depletion efficiency (%)*
1	11	0.2	68	98
2	7.2	1.2	41	83
3	6.9	1.8	45	74
4	10	2.2	50	78
5	6.2	1.1	63	82.3
6	6.1	1.2	NA	80.3
7	11	1.5	79	86.4
8	15	5.7	NA	62
9	6.7	1.5	30	77.6

NA = not assessed.

BMT has also meant that, in many cases, no fully matched donor is available. Use of mismatched or haploidentical donors is associated with an even higher incidence of clinically significant GVHD and more impaired immune reconstitution.

The T cell depletion strategy we have described here preferentially removes cells recognising GVHD targets by *in vitro* stimulation of donor cells with cytokine-treated irradiated recipient PBMC and the subsequent selection of alloreactive cells on the basis of CD69 expression. By removing the CD69-positive cells via a MACS column, we have shown that it is possible to deplete approximately 90% of the alloreactive component while maintaining >70% of the residual immunity as measured by a third party alloantigen response. The selective depletion of cells may increase haematopoietic engraftment rates post transplant. It has been shown that T cells of donor origin, devoid of anti-recipient activity, may contribute to engraftment by the

Table 5 ^{3}H-thymidine incorporation in secondary MLCs after removal of CD69^{+} cells

Donor–recipient pair	*Donor CD69 depleted cells alone*	*Donor CD69 depleted cells + autologous stimulators*	*Donor CD69 depleted cells + HLA-matched recipient stimulators*	*Donor CD69 depleted cells + HLA-mismatched third party stimulators*	*Residual response (%)*	*RRI*
1	49524	37726	39774	89544	6.5	3.9
2	1827	3464	5825	55921	18.5	4.5
3	16194	21058	21170	82987	1.6	0.2
4	3300	8806	10455	42304	25.2	4.9
5	NA	707	1569	22485	5.7	4.0

(Mean counts per minute; $n = 3$).
NA = not assessed.

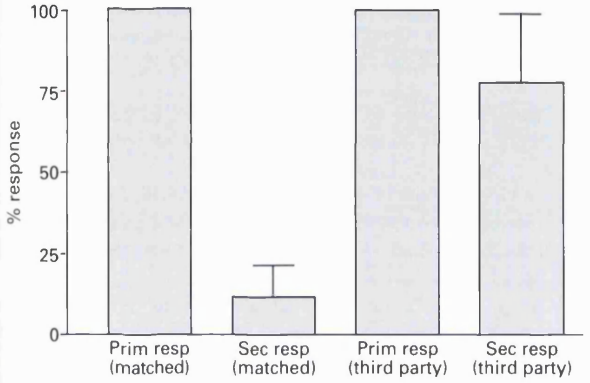

Figure 5 Residual allo-antigen proliferative response to HLA-identical stimulator cells in the modified MLC after removal of CD69^{+} responder cells. Results are expressed as mean ± (s.d.) percentage of the original response prior to depletion.

production of cytokines or by the exertion of a veto effect on host CTLs.[20] We also expect enhanced immune reconstitution since this has been shown to be directly proportional to the number of T cells in the infused graft.[8] This method may also exploit antigen differences on normal tissues and leukaemia cells in that donor responses to recipient MHC and ubiquitous mHA are eliminated while conserving responses to lineage-restricted or even leukaemia-specific antigens. This would translate into a graft-mediated anti-leukaemia activity.

CD69 was chosen as the activation marker because it is expressed on the cells of interest in GVHD-activated T and NK cells. It is upregulated as early as 24 h after allogeneic stimulation and peaks at 96–120 h.[26] CD69 was expressed at sufficiently high density on activated cells for efficient removal by the MACS device. Lamb *et al*[28] have found a good correlation between CD69 expression and ^{3}H-thymidine incorporation in the evaluation of T cell function. Other studies have also shown good correlation in the CD69 and proliferative response to various mitogenic stimuli.[29]

Various groups have used immunotoxins or fluorescent activated cell sorting (FACS) to eliminate activated cells, identified by expression of CD25.[19–23] The use of immunotoxins however results in the loss of the activated alloreactive cells. It also requires the manufacture and use of immunotoxins for clinical use. FACS sorting on the other hand is slow and designed for positive selection rather than depletion strategies.

We believe this method of selective depletion has several advantages over other protocols in that: (1) The alloreactive cells are easily recoverable and can be cryopreserved for ready immediate access as DLI if the patient relapses. This could be of particular value in the treatment of relapsed acute leukaemias, especially if donor availability and recall is a problem. (2) The MACS columns enable a good median depletion efficiency to be achieved (80.3%). (3) The use of immunomagnetic bead columns is already in clinical use for CD34 selection.

We are aware that these alloreactive cells have the potential for causing GVHD, and its use may therefore not extend to relapsed patients having only minimal residual disease on board. However, in frank relapses where the urgency of treatment is a priority, this strategy can be particularly useful. Moreover, the results of conventional DLI have been disappointing in the treatment of relapse of ALL where the GVL effect is less marked[10] and attainment of remission may be dependent on an alloreactive cell strategy like ours.

The method of selective depletion has also proved to be efficient in cell recovery (75%). The depleted fraction demonstrated good cell purity (range 94–99.8%) and is an easy and sterile system to use which can be readily translated into clinical use.

It is well established that in an HLA-matched setting, a conventional MLC is non-reactive and unable to detect minor antigenic differences that exist. Minor histocompatibility antigens (mHA) are the main source of disparity between HLA-matched siblings and contribute a significant part to the antigenic disparity between HLA-matched but unrelated pairs. mHA are thought to be derived from normal but polymorphic cellular proteins which are presented by MHC molecules and recognised in a MHC restricted fashion.[30] Bishara *et al*[27] have demonstrated that pre-treatment of stimulator cells with cytokines and the subsequent addition of cytokines to the MLC can detect fine antigenic disparities that exist between HLA-identical siblings. Our data supported this. In five out of nine pairs (three sibling, two unrelated), a positive MLC was generated while four (three sibling, one unrelated) remained negative despite the addition of cytokines. Furthermore, the results of the modi-

fied MLC were, in general, predictive of the incidence of clinical GVHD following transplantation. Although numbers are small, this does imply some clinical association and a negative modified MLC may predict absence of GVHD. It is equally encouraging that our method of alloreactive cell removal has depleted the response to below the predictive threshold for clinical GVHD (RRI <5%).

It has been shown that TNF-α and γ-interferon can increase the expression of class I and II molecules on GVHD target tissue as well as adhesion molecules important in cell–cell signaling.[31,32] Pre-treatment of stimulator PBMCs with cytokines did increase the expression of these molecules (data not shown) and this could have increased the number of rare MHC/peptide combinations above the threshold necessary for T cell stimulation. Upregulation of adhesion and costimulatory molecules could also reduce the threshold for T cell activation and thus account for the positive reactions seen in the modified MLC.[33] This is also relevant in the clinical situation where cytokines have been implicated in the pathogenesis of GVHD, the so-called 'cytokine storm'.[34,35]

In terms of CD69 expression, the addition of cytokines did not induce any non-specific increase above the baseline controls. The matched pairs and third-party MLCs showed a significant increase in CD69 expression above background. Interestingly, the CD69 expression in the modified (with cytokines) third-party MLCs and autologous controls were no higher than in the simple third party and control MLCs (without cytokines) in our mismatched study. What the cytokines did was to enhance the response mainly in the matched pairs, some almost to the same degree as in the third-party pairs. These data did contrast with the ^{3}HTdR incorporation data in the MLCs where the third party MLCs was consistently and considerably higher than the matched pairs. One must however remember that CD69 is a marker for activation and is not equivalent to the *in vitro* assessment of proliferation. The addition of cytokines, by amplifying minor antigenic differences might be sufficient to trigger a CD69 response, almost equivalent to a third-party response in some instances. What is clear though is that both CD69 expression and proliferation detected by ^{3}HTdR uptake do serve as markers for a specific alloreactive response.

This method of selective depletion has been shown to be effective in both mismatched pairs and in HLA-identical donor:recipient pairs. A logical extension would be the use of this method in haploidentical-related donors and in 1/2/3 antigen mismatched transplants. This would considerably widen the potential pool of donors available since in most cases, a haploidentical related donor is readily available. It would also allow for further sub-selection on the basis of age, CMV status and other important transplant variables. This will allow us to undertake 'intelligent' T cell depletion where the issue is no longer how many T cells can be removed from the graft but rather, how many can be retained without inducing GVHD.

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Immunotherapy of AML: future directions

Mark W Lowdell, Mickey B C Koh

The role of the immune response in the control and eradication of leukaemia after allogeneic bone marrow transplantation is established but there is increasing evidence that the principal mechanism of cure after intensive chemotherapy alone is also immune mediated. The idea of a graft versus leukaemia (GvL) effect in eradicating leukaemia was suggested as early as 1957, when Barnes and colleagues[1] showed in a murine model that leukaemic animals which received myeloablative radiotherapy followed by syngeneic bone marrow transplant all relapsed with the original acute leukaemia and died; in contrast, animals receiving the same radiotherapy but allogeneic bone marrow transplant all developed fatal graft-versus-host disease (GvHD). However, these latter animals survived longer than the recipients of syngeneic marrow and had no evidence of leukaemia relapse at necropsy. This provided evidence both that myeloablative radiotherapy does not eradicate leukaemia, and that an anti-leukaemia immune response was present. Immune mediated cure of residual leukaemia in the clinical setting has been suggested by observations of a reduced risk of leukaemia relapse after allogeneic bone marrow transplant when compared with autologous or syngeneic bone marrow transplant, and an association between graft-versus-host disease (GvHD) and disease-free survival.[2 3] Furthermore, an increased incidence of leukaemia relapse has been reported after aggressive GvHD prophylaxis with cyclosporin or lymphocyte depletion. The most convincing direct evidence of allogeneic antileukaemia activity has been provided by the long lasting remissions following donor leucocyte infusions to treat relapse of chronic myeloid leukaemia (CML) after allogeneic stem cell transplantation.

Specific immunotherapy of acute myeloid leukaemia (AML) is the subject of intense research at present, with major research bodies such as the NIH, the Leukemia Society of America, and the Leukaemia Research Fund supporting large programmes in many centres. The approaches being taken differ widely between groups but can be most readily subdivided into those that rely upon an allogeneic transplant and those that aim to induce or maximise autologous immunity to residual disease.

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Allogeneic responses

In allogeneic transplantation the aim is to maximise the GvL effect while minimising the risk and severity of GvHD. Post-transplant relapse of AML remains a problem, and subsequent survival rates have generally been very poor with either reinduction chemotherapy or second transplants. The advent of donor leucocyte infusions has provided an additional therapeutic option and the fact that complete remissions have been achieved is further "proof of principle" of the GvL effect of mature donor lymphocytes. Donor leucocyte infusions have been most effective in CML (76% complete remission), least in acute lymphoblastic leukaemia (ALL), with AML (29% complete remission) occupying the middle ground.[4] In acute leukaemias, donor leucocyte infusions may need to be given in addition to prior chemotherapy.[5] The full potential of donor leucocyte infusions thus far has been hindered by the concurrent risk of marrow aplasia (34%) and severe GvHD (41%).[4] To minimise these risks, the optimal dose strategy for T cell add-back has been investigated[6] and, in relapsed CML, infusions are given cautiously, starting with doses of 10^5 lymphocytes/kg patient body weight and increasing over several weeks to doses of 1–5 10^7/kg in most cases. Eradication of the Philadelphia chromosome positive cells from the bone marrow of these patients takes many months and multiple lymphocyte infusions. Relapsed AML progresses too quickly for such a delayed response to be effective in most cases. Infusion of higher doses of donor leucocytes may resolve this, but the increased concomitant risk of severe or fatal GvHD is unacceptable.

The use of prophylactic donor leucocyte infusions is an approach being studied by several groups, with the intention both of treating undetectable residual disease and improving the immune reconstitution of recipients of T cell depleted allogeneic grafts. The timing of the infusions will be critical if GvL is to be retained without significant GvHD.[7] Most groups are giving the first reinfusion around day 40 post-transplant but results are too preliminary to determine the effectiveness of this strategy in AML.

Given the aim of eradicating donor derived GvHD while retaining donor GvL and donor immunity to viral infections, some groups are trying to remove only the GvHD effectors from the graft ex vivo or in vivo. Ex vivo CD8 depletion[8] has been tried with some success in CML but no results have been published from AML patients. Most recently, advances in clinical grade cell sorting have led to novel techniques for graft engineering, a field which is becoming known as "intelligent T cell depletion."

Intelligent T cell depletion involves the removal of alloreactive cells which induce GvHD while preserving non-alloreactive cells. The ability to use HLA mismatched donors, ideally haplo-identical parents or children, will revolutionise allogeneic stem cell transplanta-

tion, as the greatest barrier to allogeneic stem cell transplantation remains the lack of suitable HLA identical donors. However, the severity of GvHD is unacceptable unless extreme (> 4 log) T cell depletion is employed. This severely delays immune reconstitution, leading to intense post-transplant immune suppression and deaths from relapses and disease.

Our group has looked at the activation marker CD69 in an ex vivo mixed lymphocyte culture of donor mononuclear cells and irradiated recipient cells as a tool to identify alloreactive GvHD inducing donor lymphocytes. Alloreactive cells can be efficiently depleted using magnetic beads, the allodepleted fraction retaining third party alloreactivity despite the removal of reactivity to the initial recipient cells.[9] This mode of selective depletion only removes a small minority of donor cells and preserves a large pool of lymphocytes capable of anti-leukaemia activity, improving engraftment and hastening immune recovery. This system has been tested effectively in a mouse model of major histocompatibility complex (MHC) mismatched stem cell transplantation, with complete abrogation of clinical GvHD in the recipients of up to 2×10^7 donor lymphocytes. In contrast, 10^7 non-depleted donor cells were fatal in over 90% of animals (fig 1). Plans for a phase 1 clinical trial are under way.

Several other strategies have been used which either eliminate the alloreactive cells by immunotoxin[10] or ricin[11] conjugated to an anti-CD25 antibody, or alternatively anergise alloreactive cells by blocking the CD40:40 ligand[12] or B7:CD28[13] costimulatory pathways. The success of such strategies will rely upon the ability to dissect GvHD effector cells from those mediating GvL—that is, there must be leukaemia specific or leukaemia restricted responses. There is evidence supporting this assumption which is presented below. However, an alternate approach in which the GvHD response may be harnessed has been described by two European groups, and trials are under way: briefly, the donor lymphocytes in the allogeneic graft are transduced with a thymidine kinase (HSV-TK) suicide gene in a retroviral vector before infusion.[14] In the event of clinical GvHD the transduced cells can be eliminated in vivo by administration of therapeutic doses of ganciclovir. The problems with the thymidine kinase transduced gene are that for the strategy to be successful, all alloreactive T cells must contain the gene, expression must be sustained in vivo, and the gene product must not be immunogenic. A further disadvantage is the loss of ganciclovir as a therapeutic option for CMV disease after transplantation.

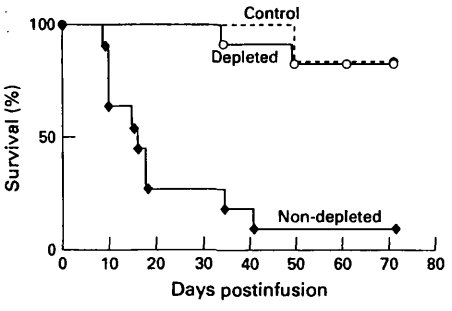

Figure 1 Survival curve for NOD/SCID mice recipients of MHC mismatched donor splenocytes. Mice in the "non-depleted" group (n=11) received 10^7 non-manipulated donor splenocytes intraperitoneally; mice in the "depleted" group (n=12) received 10^7 donor splenocytes which had been depleted of alloreactive lymphocytes; "control" mice (n=8) received phosphate buffered saline alone.

With increased life expectancy, the numbers of elderly patients who may require allogeneic transplantation for AML are growing. The greatest advance for these patients will be the "mini-transplant" or "transplant lite." This has sparked an explosion of interest in the last two years and aims to exploit immune mediated cure in transplantation, as patients do not receive myeloablative pretransplant conditioning.[15,16] The purpose of pretransplant conditioning is to create a state of immunological host tolerance to the donor graft, allowing the induction of a graft versus leukaemia effect as the primary mode of treatment. As a state of tolerance is maintained, further infusions of donor lymphocytes can be given to maximise the GvL effect. Immune mediated treatment is especially attractive because less intensive conditioning is needed (hence, the term mini-transplant), which translates into decreased toxicity, improved immune reconstitution, avoidance of radiotherapy, and decreased incidence of GvHD, all of which are related to the intensity of conditioning.[17]

The safest allogeneic immunotherapy will rely upon the generation of non-alloreactive, donor derived, leukaemia specific or leukaemia restricted immunity. This has been hampered by the lack of known leukaemia specific tumour antigens. Tumour antigens promoting T cell responses have been studied mostly in the context of melanoma, but in AML they can either be alloreactive (MHC), tissue restricted (minor histocompatibility antigens, mHag),[18] lineage and differentiation specific (proteinase 3),[19] or leukaemia specific (PML-RARA in M3).[20] In all the above, cytotoxic CD4 and CD8 T cells have been detected or generated which possess specific antileukaemia activity. CD8 clones specific for a variety of mHag can be isolated from most patients after transplantation, and some are selectively expressed on haematopoietic cells and so are suitable as a GvL target.

Professor Els Goulmy, at the University of Leiden, has been at the forefront of research into mHags for many years and was the first to isolate and sequence a human minor antigen. She has described two such antigens, HA-1 and HA-2, which are expressed exclusively on haematopoietic cells which are HLA-A2 positive.[18] However, HLA-A2, HA-1, and HA-2 are not linked, so individuals who are HLA-A2 positive may not express HA-1 or HA-2 or may express one or both. Imagine a situation where both the donor and recipient are HLA-A2 matched (that is, around 70% of transplants in white patients) and the recipient is HA-1 positive. If the donor is HA-1 negative, the transplanted T cells will recognise the

HLA-A2 molecules carrying the HA-1 mHag on the hosts myeloid cells as "foreign"; they will then generate a cytotoxic T cell response which selectively lyses only the recipient leukaemia and haematopoietic cells, while sparing donor haematopoietic cells and recipient fibroblasts, keratinocytes, and hepatocytes.[21] The difficulty of finding donors who are HLA-A2 matched but HA-1 or HA-2 mismatched with the recipient may be overcome by the generation of specific T cell lines that are HLA-A2 restricted and are specific for each of the mHags to be used for treatment of relapse post-transplant, or for in vivo purging before transplantation. Minor antigens restricted to other HLA alleles will be identified if such an approach is successful in the HLA-A2 matched setting.

Specific fusion oncogenes such as PML/RAR in AML M3[20] or bcr/abl[22] are potential tumour antigens, and various methods of generating cytotoxic T cells to these have been tried, including bulk cultures, limiting dilution assays, and dendritic cells.[22-24] The emergence of tetramer technology, in which complexes of HLA molecules and peptides are manufactured artificially, allows us to visualise and select specific peptide restricted cytotoxic T cells for expansion and therapeutic application. This method has been successfully used to identify mHag specific cytotoxic T cells[25] and a project supported by the LRF Immunotherapy Initiative is using these reagents to derive cytomegalovirus (CMV) specific cytotoxic T cells to treat CMV infection post-transplant (Moss P, personal communication).

The exciting possibility of using the catalytic subunit of telomerase as a universal tumour antigen has also recently been described.[26] T cells generated against peptides of this tumour antigen can recognise and lyse a variety of tumours, including lymphoma and myeloma, in an HLA restricted manner.

Currently, allogeneic antileukaemia activity is recognised and some target antigens have been identified. Problems of donor availability remain acute and research into optimising the safety of multiply HLA mismatched transplants is under way. Rendering the mismatched transplants safe with respect to GvHD may abrogate GvL and thus the optimal treatment may involve combination treatment by passive immunotherapy with leukaemia restricted cytotoxic T cell lines.

Autologous responses

All patients who achieve complete remission after chemotherapy are potential recipients of autologous immunotherapy, with or without concomitant stem cell transplantation. This concept is not new: advent of recombinant interleukin-2 in the 1980s led to a number of clinical trials.[27-30] The overall results—including those from large, randomised, but unreported studies—have been disappointing, and most trials in acute leukaemia have ceased. However, our understanding of the human immune system has advanced enormously since these early trials, and the field is both active and optimistic once more. Particularly encouraging is the evidence that patients can spontaneously develop immunity to residual leukaemia after chemotherapy[31 32] and that this immunity can be measured in vitro and induced in some patients by administration of α interferon.[33] In a recent study from our group, a cut-off level of leukaemia-specific cytotoxicity in peripheral blood samples of patients in remission was determined which predicted disease-free survival beyond two years with high specificity and sensitivity (fig 2). Furthermore, the cells which mediated this activity have been identified as a subset of natural killer (NK) cells and work on their expansion for passive immunotherapy is underway.

Passive immunotherapy with autologous natural killer cells is logistically attractive for many reasons, but for continued immunological memory a specific T cell response is required. Evidence for spontaneous T cell responses to AML is lacking but our current level of understanding of the nature of naive immune responses suggests that we will be able to generate such cells in vitro and or in vivo. Several strategies are being employed.

Dendritic cells are the most efficient antigen presenting cells and these can now be cultured from monocytes and from CD34 progenitors. Dendritic cells can be pulsed with peptides, proteins, or tumour lysates, a strategy that has already been used to feed leukaemia derived peptides (synthetic and eluted) into dendritic cells to obtain specific T cell effectors.[22 34 35] Data were presented recently on the successful generation in vitro of specific cytotoxic T cell to autologous CLL cells using a dendritic cell strategy.[36] Others have tried using these peptides and dendritic cells for in vivo vaccination strategies which may improve responses compared to vaccination with proteins, irradiated leukaemic blasts, or even immunogenic peptides alone.[37] Tumour vaccines will probably work best in a minimal residual disease setting after chemotherapy. However, on many occasions, the tumour antigen may be unknown or it may not be possible to isolate and obtain exact tumour peptides. Moreover, predictions of MHC peptide specificity in vitro may not hold true in clinical practice. To bypass these problems of

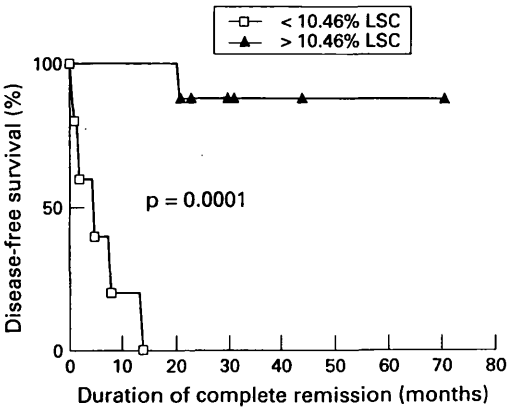

Figure 2 Disease-free survival of patients with acute leukaemia by virtue of the level of autologous leukaemia specific cytotoxicity measured in peripheral blood samples taken in remission after completion of treatment.

peptide generation, cytokines have been used to derive dendritic cells from autologous leukaemic blasts with the intention that these cells will present leukaemia restricted peptides innately.[38] While this is a promising strategy, it is not applicable to all subtypes of AML. An alternative strategy is to fuse the tumour cell with the dendritic cell.[39] This has been successful with carcinoma cells and could be extended into the context of AML. Also, the discovery that dendritic cells shed membrane vesicles called exosomes, which in themselves can function as efficient antigen presenting cells, has led to the pulsing of tumour peptides into exosomes.[40]

The other vital component in the generation of cytotoxic T cells is provided by CD40 on dendritic cells and CD40 ligand on CD4 cells.[41] There has been a flurry of excitement recently in this field using activating anti-CD40 antibodies to modulate the function of dendritic cells. This augments responsiveness to tumour vaccination strategies as well as enhancing and priming the specific antitumour cytotoxic T cell response.[42-44]

AML blasts subvert the host immune surveillance by a myriad of mechanisms which include decreased expression of MHC molecules, secretion of immunosuppressive cytokines, a lack of costimulatory molecules,[45 46] downregulation of CD3ζ, and tyrosine kinase expression.[47] This results in the failure of "correct" antigen presentation to the T cell and hence a failure of the immune response. However, AML blasts can be genetically modified to improve their ability to present endogenous antigens.[48] Our group has produced an adeno-associated virus (AAV) construct capable of simultaneously transducing primary human AML cells with genes encoding IL-12 and CD80, leading to subsequent protein synthesis and expression.[49] There is marked synergism between IL-12 and CD80[50] in the initiation of primary cytotoxic responses. We have termed these transduced cells "tAML" and have shown them to be immunostimulatory in vitro. We are currently using them to stimulate autologous peripheral blood mononuclear cells in vitro and expand reactive cells for analysis and in vitro testing. A passive immunotherapy programme with these reactive cytotoxic T cells is planned for patients in remission after chemotherapy. Ultimately, the tAML cells could be used as a vaccine in vivo to stimulate autologous immunity directly. This approach is being followed by the group at King's College Hospital in London where they are using an adenoviral vector to transduce AML blasts with CD80 and interleukin (IL)-2.

The immune system is regulated by a cytokine network and its importance in the pathophysiology of GvHD is well established.[17] IL-2 increases antigen specific effector function and induces MHC unrestricted natural killer cell and cytotoxic T cell activity[51] but, as discussed above, trials of IL-2 have been disappointing. Recently there has been a resurgence of interest[52 53] and its use in combination with histamine has been proposed as the two are synergistic in cell mediated killing of leukaemic cells and in upregulation of CD3ζ chain expression.[54]

IL-12 is likely to prove a valuable immunotherapeutic cytokine. It drives reactive CD4 cells to a Th1 (cytotoxic) response[55] and has been shown to promote GvL activity independent of GvHD. Despite this, systemic injection results in marked adverse effects and variable long term immunity; thus its successful clinical application will require more physiological delivery systems.

There has been extensive use of α interferon in CML but less so in AML. As described above, we have used it as maintenance therapy in AML, where it has been associated with induction of leukaemia specific cytotoxicity and long term disease-free survival.

Conventional chemotherapy, bone marrow transplantation, or systemic immunotherapy with cytokines are all associated with varying degrees of toxicity to other tissues. In contrast, targeted immunotherapy involves the specific delivery of either toxins, chemotherapy, or radioactive elements specifically to the leukaemia blasts, while avoiding toxicity to other organ systems. This can be done by identifying unique cell surface markers on leukaemia blasts like CD33 (present in 90% of AML blasts), and conjugating specific antibodies to a toxin/radionuclide for specific killing. Selective killing of AML blasts was achieved in a recently published phase I study using anti-CD33-calicheamicin immunoconjugate.[56] Side effects were tolerable: postinfusion symptoms of fever, chills, and hypotension, as well as neutropenia. There was 20% success in this group of relapsed or refractory patients, although nearly all subsequently relapsed. Immunocytokines are another potential way forward, for example fusing IL-2 with a cell surface molecule or IL-4 with an immunotoxin for AML.[34]

AML targeted antibody treatments are not only restricted to cytotoxic effects. Another novel emerging mode of treatment is the use of anti-CD44 to induce differentiation in AML blasts, much as ATRA (ALL trans retinoic acid) is used with such success in the treatment of AML M3. A recent trial has produced promising initial results.[57]

Summary

Immunotherapy in the form of allogeneic GvL has been curing AML patients for nearly 30 years but our understanding of the mechanisms has been poor. Our rapidly evolving understanding of the human immune system and the concomitant technical developments in ex vivo cell manipulation, the vision of funding bodies, the dedication of clinical and research staff, and above all the commitment of our patients, promise substantial progress in the treatment of this disease in the year 2000 and beyond.

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