Cellular mediators of anti-leukaemic activity

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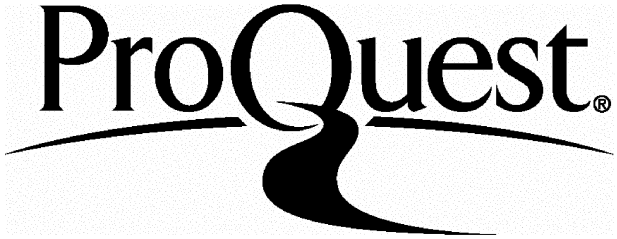

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Abstract

The aim of this project was to study the cells which mediate allogeneic and autologous anti-leukaemic activity. By studying blood samples from patients pre and post bone marrow transplant (BMT) we hoped to establish which cells played an important role in the post-transplant period. Phenotyping analysis was performed on patient samples from one month post-transplant for up to twelve months. From studying the results of the phenotyping data we have acquired a bank of data on immune reconstitution and constructed a hypothesis about possible mediators of graft-versus-leukaemia (GvL).

Patient material was gathered at presentation and each month post-transplant. Cytotoxicity assays were performed by a flow cytometric method using patient material. Some of these experiments were performed on patients who had received chemotherapy and gone into remission, others had received autologous transplants. From these experiments it became apparent that a level of killing of around 17% was required to allow likelihood of survival and a subset of natural killer (NK) cells ($CD56^+/CD8^+$) was identified and appeared to have specific anti-leukaemic potential after autologous BMT.

This subset has been studied extensively in terms of its biology. Both $CD3^-/CD56^+/CD8^+$ and $CD3^-/CD56^+/CD8^-$ subsets were monitored to see if they reacted in different ways to cytokine stimulus and to discover whether they had similar cytotoxic potential. Three different cytokines were employed to generate *ex vivo* expansion of these cells from their precursors. Interleukin 2 (IL-2), interleukin-7 (IL-7) and interleukin 15 (IL-15) were used separately and in combination in an attempt to establish what their role was in the development of NK cells and the survival of NK cells *in vitro*.

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Abbreviations.

ABMT	autologous bone marrow transplant
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
Ara C	adriamycin C
ATG	anti-thymocyte globulin
BMMC	bone marrow mononuclear cells
BMT	bone marrow transplant
Bu/Cy	busulfan/cyclophosphamide
CFU-GM	colony forming units-granulocyte macrophage
CGH	Colchester General Hospital
CGL	chronic granulocytic leukaemia
CLL	chronic lymphoblastic leukaemia
CM	complete medium
CML	chronic myeloid leukaemia
CMV	cytomegalovirus
CR	complete remission
CsA	cyclosporin
CTL	cytotoxic T lymphocyte
DLI	donor leucocyte infusion
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
FLAG/Ida	Fludarabine, adriamycin, GCSF, idarubicin
FSC	forward scatter
FITC	fluorescein isothiocyanate
GCSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
GvHD	graft-versus-host disease
GvL	graft-versus-leukaemia
HBSS	Hank's balanced salts solution
HCl	hydrochloric acid
HLA	human leucocyte antigen
IFNα	interferon-alpha
IFNγ	interferon-gamma
IL-1	interleukin-1
IL-2	interleukin-2
IL-7	interleukin-7
IL-10	interleukin-10
IL-15	interleukin-15
Ig	Immunoglobulin
IT	ricin A-chain immunotoxin conjugation
ITAM	immunoreceptor tyrosine-based activatory motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
kDA	kilodalton
LAK	lymphokine activated killer
LFA	leucocyte function-associated antigen

LFS	leukaemia free survival
LSC	leukaemia specific cytotoxicity
Mabs	monoclonal antibodies
MACS	magnetic cell sorting
MHC	major histocompatibility complex
MHA	minor histocompatibility complex
NCR	natural cytotoxicity receptors
NK	natural killer cell
NKIR	natural killer cell immunoglobulin-like receptor
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSCT	peripheral blood stem cell transplant
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA	phytohaemagglutinin
PI	propidium iodide
PMA	phorbol-12-myristate-13-acetate
PMT	photomultiplier tube
R/D	relapsed/deceased
RFA	Royal Free antibodies
RFH	Royal Free Hospital
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SSC	side scatter
TBI	total body irradiation
TCD	T-cell depletion
TCR	T-cell receptor
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor- α
TRM	transplant related mortality
TRM/D	transplant related mortality/deceased
VUD	volunteer unrelated donor

1. Introduction

Bone marrow transplants (BMT) are now regularly performed in the course of the treatment of chronic myeloid leukaemia (CML), acute myeloid leukaemia (AML) and acute lymphoid leukaemia (ALL) where chemotherapy alone has failed to induce a lasting remission or cure. In the early years of bone marrow transplantation it was thought that chemotherapy and radiotherapy cured the disease and that the transplant repopulated the haemopoietic system. The first human leucocyte antigen (HLA) matched transplant was performed in the 1960s by Fritz Bach, at which time the rationale was to repopulate the haemopoietic system with a non-tumour bearing graft after intensive chemo- and radiotherapy (Fritz Bach *et al.*, 1968). However, previous work in a murine model of leukaemia had suggested that radiotherapy was not curative (Barnes *et al* 1956). This group produced evidence that the allograft itself may have the predominant anti-leukaemic effect in transplant treatment.

In a series of murine studies Barnes and Loutit (1957) showed that mice injected with leukaemia cells followed by myeloablative radiotherapy and then a syngeneic BMT, died from the original leukaemia. In contrast mice that received myeloablative radiotherapy and then an allograft lived longer and died without evidence of leukaemia. These mice appeared to die from wasting following chronic diarrhoea. This response in mice had already been noted by Jacobson *et al* (1950). It later became known as graft versus host disease (GvHD). This was the first indication that allogeneic lymphocytes might be able to produce an immune reaction that was capable of recognising surviving

leukaemic cells as foreign and destroying them, which came to be known many years later as the graft versus leukaemia (GvL) effect. It was also proof that radiotherapy did not necessarily eradicate leukaemia.

Over 40 years later we have enough understanding of the immune response to manipulate the allogeneic graft given to the recipient to reduce the potentially fatal effects of GvHD. Yet the separation of the deleterious effects of GvHD from the benefits of GvL has not yet been perfected. The target antigens involved in allogeneic GvL are as yet unknown. What has been established is that the immunogenetic relationship between the host and the donor is crucial in triggering a response in the patient which may result in a combination of GvHD and GvL and although potentially fatal this response gives the patient a much better prognosis for leukaemia-free survival.

As bone marrow transplantation has developed over the years it has become apparent that the techniques involved need to be balanced against one another. In an allogeneic transplant the host T cells that survive the conditioning regimen can reject the graft, conversely donor T cells can react against histocompatibility antigens on recipient cells to produce GvHD (Butturini *et al.*, 1987). From a treatment point of view the clinician has a number of tools which can be used to influence the outcome for the patient. Immunosuppressive drugs, T cell depletion (TCD), and donor leucocyte infusions (DLI), which will all be discussed later, can affect graft rejection and leukaemia relapse and will influence GvHD and GvL. A balance of treatment must be achieved to give the best outcome but it is often highly patient dependent.

Transplant immunology involves the study of host or recipient immune responses to allogeneic donor tissues, particularly donor organs or skin grafts. In the case of conventional BMT, donor grafts include large numbers of immunocompetent lymphocytes, mostly T cells, which are transplanted with the graft and can be stimulated by the highly polymorphic major histocompatibility (MHC) antigens of the host. In contrast, depletion of the GvHD inducing T cells from the donor graft may lead to graft rejection since the recipient is able to mount an immune response against the donor MHC. An increased incidence of graft rejection in some centres using T cell depleted allogeneic grafts has been overcome by increased immune suppression of the recipient pre- and peri-transplant by manipulation of the conditioning regimen. The reasons for this will be discussed below.

To prevent rejection in allogeneic BMT the patient receives appropriate conditioning to prepare for the transplant. The conditioning of the patients included in this study consisted of high dose cyclophosphamide and total body irradiation (TBI) to 750cGy, given at a fast dose rate (15 μ 2cGy/min) in a single fraction. Some patients who received transplants from HLA-matched unrelated donors underwent additional conditioning. These patients received *in vivo* anti-leukocyte therapy with Campath-1G, a monoclonal rat antibody against the human CD52 antigen which is expressed on all leukocytes.

The difference between BMT and solid organ transplants is that the allogeneic BMT is not only immunogenic but is itself immunocompetent. This means that having conditioned the host to accept the graft there is a chance that the graft will reject the host (GvHD). The likelihood of this is increased by the

conditioning of the host which results in the spontaneous release of high concentrations of inflammatory cytokines such as tumour necrosis factor α (TNFα) and interferon γ (IFNγ) which is known as the 'cytokine storm' and will be discussed later. To try and avoid graft rejection and GvHD the donor recipient pair are matched by human leucocyte antigens (HLA) which will be described in the following section.

1.1 The major histocompatibility complex

The immunological character of an individual is expressed in cell surface proteins encoded by the major histocompatibility complex (MHC). This is a complex of polymorphic genes found on chromosome 6 in the human. The role of MHC proteins known as human leucocyte antigens in humans (HLA) is to allow the body to distinguish between what is native, and foreign invaders (Ferrera and Deeg, 1991).

There are two types of MHC molecules. The MHC class I molecules or proteins present exogenous peptides generated in the cytosol to $CD8^+$ T cells, and the MHC class II molecules present endogenous peptides degraded in cellular vesicles to $CD4^+$ T cells. These proteins may be derived from innate cellular constituents or from infectious micro-organisms. The MHC is the most polymorphic gene cluster in the human genome, having large numbers of alleles at several different loci (Janeway and Travers, 1997).

In humans there are three main class I genes, HLA-A, -B and -C. For class II genes there are three pairs of MHC α- and β-chain genes which are called HLA-DP, -DQ and -DR (HLA-DR has an extra β-chain gene). Each of the class I and

class II molecules are capable of presenting antigen to T cells, and each protein can bind a different range of peptides, therefore with several loci it means a diverse number of peptides can be presented.

For all the human MHC genes apart from DRα there are a number of different alleles at each loci. This means that the chance that the MHC genes on both chromosomes of an individual will encode the same allele is very small. As the products of both alleles are expressed, and function in the presentation of antigen to T cells this can lead to particular problems in transplantation. There is only a one in four chance that an individual will share the same allelic pairing with a sibling.

For BMT it is essential to try to match for class I and class II alleles between donor and recipient. An inability to do this will increase the chances of graft rejection or GvHD. The diversity of these genes described above highlights that the matching of individuals can be extremely difficult. High resolution deoxyribonucleic acid (DNA) typing is used to match donor and recipient for class I and class II antigens.

As well as the MHC there are also minor histocompatibility antigens (mHC). These antigens are presented by HLA class I and class II antigens, and will be discussed later (section 1.6.1.). Even with the best matches possible in MHC molecules, with both HLA-matched unrelated transplants and sibling transplants, mismatches in the mHC cannot be eradicated, and these disparities are likely to be responsible for the development of GvHD in these transplants. Indeed it has become apparent from clinical studies that there is an enhanced

anti-leukaemic effect after HLA-identical sibling donor allogeneic BMT when compared to autologous BMT, or syngeneic BMT (Horowitz *et al.*, 1990), and this is presumably due to mismatches in the mHC.

1.2 Graft-versus-host disease

This is a complication of BMT caused by donor immunocompetent cells, due to antigenic disparities between donor and recipient. Acute GvHD results in skin, gut and liver complications and usually occurs within 100 days of the transplant. Chronic GvHD can evolve from acute and has more systemic complications. Once GvHD has developed it is treated with immunosuppressive agents such as corticosteroids, anti-thymocyte globulin (ATG) (Hoffbrand and Pettit, 1993), or MMF (mycophenolate mofetil).

Cytotoxic T cells were considered solely responsible for GvHD, but now it is thought that cytokines and inflammatory cells assist T cells (Antin *et al.*, 1992). Antin and colleagues put forward a hypothesis that GvHD results from three consecutive events. First the expression of human leukocyte antigen (HLA) and leukocyte adhesion molecules on the target tissues such as skin, intestinal mucosa and liver is upregulated by cytokines. These are inflammatory cytokines produced in response to the conditioning regimen, infection and possibly the disease itself.

Second the cytokines from this cytokine response provide an appropriate environment for the mature donor T cells which react to recipient peptide-HLA complexes (allo-antigens), where either the HLA molecules or the bound peptides are foreign. The host antigen-presenting cells release cytokines and in particular the second activation signal for T cells – interleukin-1 (IL-1). CD4,

CD8, CD44 and lymphocyte function antigens (LFA-1 and LFA-2) encourage interactions between effector and target cells. The presentation of antigen activates individual T cells by multiple intracellular biochemical changes, namely an increase in cytoplasmic free calcium levels and activation of protein kinase C and tyrosine kinases. The result is the activation of the transcription genes for cytokines such as interleukin-2 (IL-2) and the interleukin-2 receptor (IL2-R) which stimulates the proliferation of the cells secreting IL-2, and upregulates the expression of the receptor on other cells (Ferrera and Deeg, 1991).

Third the profusion of IL-2 activates mononuclear cells from the donated marrow to secrete more inflammatory cytokines such as interleukin-1 (IL-1), TNFα and IFNγ. The resulting increase in soluble cytokines can lead to local organ damage (Antin *et al.*, 1992).

If this hypothesis proves to be correct, and there is substantial evidence to support it, it highlights the importance of cytokines. It also answers why it is possible to add back T cells after BMT when the pro-inflammatory cytokine response has dissipated without incurring the same problems (as discussed in DLI section). Finally it also opens up the possibility of manipulation of GvHD by control of the relevant cytokines and may be an area in which to separate the GvL effect from GvHD. Indeed, there is evidence that some individuals are genetically predisposed to produce higher levels of anti-inflammatory cytokines (such as IL-10) and these patients appear to be protected from acute GvHD after allogeneic BMT. In a recent study from Germany high spontaneous production of IL-10 by PBMC at the time of admission (pre-preparative treatment for BMT) correlated with a subsequent low incidence of GvHD and

transplant-related mortality (8%), as compared with patients with low or intermediate IL-10 production (50%, $p<0.01$)(Holler *et al.*, 2000).

1.3 Graft-versus-leukaemia

Since Barnes and Loutit (1957) first saw the significance of GvHD in the outcome of BMT many have tried to separate GvL activity from clinical GvHD. This has been successful in animal models but has proved to be more difficult in man (O'Kunewick *et al.*, 1995, Bortin *et al.*, 1979). The clinical importance of the GvL effect became apparent from a number of observations: leukaemia remission during GvHD, the inverse relationship between GvHD and leukaemia relapse (Weiden *et al.*, 1979), the increased relapse rates in syngeneic grafts when compared to allogeneic grafts (Weiden *et al.*, 1979, Horowitz *et al.*, 1990), the higher probability of relapse following autologous graft in AML (Talbot *et al.*, 1990), and the increased frequency of relapse following severe T cell-depletion (TCD) as GvHD prophylaxis (Apperley *et al.*, 1986, Bortin *et al.*, 1991).

GvHD has been attributed to T cells (Ferrera and Deeg, 1991) but the effectors of GvL have been more difficult to define and may include NK cells as well (Glass *et al.*, 1996). From as early as 1968 it was apparent that one way of influencing the deleterious effects of GvHD was to manipulate the graft by depleting the T cells (Dicke *et al.*, 1968). T cell depletion was the first attempt to control the lineages of cells returned to the patient, and the development of this technique did eventually allow separate effects of the GvHD and GvL to be observed (Horowitz *et al.*, 1990).

1.4 T cell depletion

The challenge therefore in allogeneic BMT is to salvage as much of the GvL effect as possible whilst reducing GvHD. An approach pioneered in the late 1960s and regularly used today is T cell depletion (TCD) which involves removing the potentially alloreactive cells from the graft before it is given to the recipient. It was first described by Dicke in the 1960's in a murine model of allogeneic BMT (Dicke *et al.*, 1968). This group used selective elimination of immunologically competent cells from the bone marrow. A gradient separation technique was applied to fractionate mouse spleen cells. The different fractions were transplanted into mice and it was discovered that the fraction inducing least GvHD was devoid of lymphocytes. This was the first evidence that an element within the lymphocyte compartment was responsible for GvHD. This paved the way for new and more efficient techniques for the removal of lymphocytes from the graft.

Reisner and colleagues (1981) were one of the first groups to document TCD in the clinical setting. They used soybean agglutination and sheep red blood cells which bind to CD2 molecules on T cells and form rosettes which can then be removed by density gradient separation. They were closely followed by Prentice *et al* using a monoclonal antibody OKT3 (subsequently known as CD3) in BMT for acute leukaemia (Prentice *et al.*, 1982). Donor marrow was pre-incubated with murine OKT3 in a study of 17 patients who received matched or slightly mismatched sibling transplants. By doing this the percentage of those suffering acute GvHD of grade II or worse was reduced from 79% in an earlier

group of patients receiving transplants to only 18% in those receiving OKT3-treated marrow.

A retrospective analysis of patients treated with TCD BMT at the Royal Free Hospital comparing depletion by a cocktail of monoclonal antibodies (comprising of anti-CD6, anti-CD7 and anti-CD8) termed the Royal Free Hospital antibodies (RFA), CAMPATH IgM (anti-CD52 'CAMPATH') or ricin A-chain immunotoxin conjugate (IT) for their impact on GvHD reported the following. Thirty-one matched sibling transplant recipients in first complete remission treated with RFA had more GvHD but a relapse risk of only 4% at 10 years; they did not receive any post-transplant prophylactic immunosuppression. In contrast the relapse risk was 34% for CAMPATH (28 patients) and 30% for IT recipients (10 patients). The disease-free survival for the RFA group was 66% at 10 years (Prentice *et al.*, 1994). It appears therefore that the intensity of the conditioning, and the degree of TCD (partial TCD may be used) can influence the balance between the immune responses of the host and donor. The extent of TCD must be taken into account when administering prophylactic immunosuppressive drugs to allow the graft to function.

One key difference between the results from this centre and those of others may be that in the majority of other centres immunosuppressive prophylaxis for GvHD was given post-transplant and this could be the critical factor (Apperley *et al.*, 1986). Over aggressive immunosuppression of the recipient post-transplant will inhibit the ability of the engrafted donor immune system to eradicate any residual leukaemia; increasing the risk of relapse. The veracity of this is

disputed by some studies which allege that post-transplant immune suppression does not increase the rate of relapse, and may, because of decreased GvHD and rejection, increase leukaemia free survival (Marmont *et al.*, 1991).

In this centre the use of single fraction TBI provided a degree of immunosuppression pre-transplant, which allowed the in-coming graft to be accepted. Encouraged by the results, *ex vivo* TCD has continued to be a mainstay of the BMT regimen in this centre, but due to the unavailability of RFA, Campath IgM (anti-CD52) has been used, with and without the IgG isotype (Campath 1G) for *in vivo* conditioning.

These very positive results were not entirely consistent with the IBMTR registry data (Horowitz *et al.*, 1990). This comparison of 2,254 BMT patients with leukaemia monitored graft-related anti-leukaemia effects with and without GvHD. They were able to show that the type of graft and the development of GvHD were important in the long term outcome with respect to risk of relapse. The absence of GvHD post transplant was associated with a significantly high relapse risk, thus recipients of grafts from syngeneic donors or of TCD grafts from HLA-matched sibling donors together with patients who had not suffered from GvHD, lay at one end of the spectrum. However, at the other end lay those patients who had suffered acute and chronic GvHD, who were far less likely to suffer leukaemic relapse, but had an increased likelihood of dying from transplant related mortality.

Horowitz and colleagues (1990) broke down the anti-leukaemic effect they had isolated from the collated results into three separate categories: (1) anti-

leukaemia activity associated with clinically evident GvHD, (2) anti-leukaemic effect independent of clinically evident GvHD and (3) anti-leukaemic effect as a separate entity from GvHD, modified by TCD.

In the first category they found that although patients with acute and chronic GvHD had the lowest risk of relapse, moderate to severe GvHD meant increased transplant related mortality (TRM). Therefore their overall survival was not increased. They also found that chronic GvHD was an important factor in AML and CML, whereas acute GvHD was more important in ALL.

In the second category GvL, without GvHD was seen in AML patients. This was deduced from the fact that allograft recipients who did not experience GvHD had a lower risk of relapse than patients who had received syngeneic transplants (autologous recipients were not considered). There was a limited GvL effect seen in CML and none in ALL for patients in this category.

In the third category, Horowitz and colleagues looked at the effect of TCD and found these patients had a further increased risk of relapse even allowing for the lack of GvHD, which was particularly significant for CML patients. This suggested an anti-leukaemic effect of the cells removed from the graft. Without these cells the patients were lacking some beneficial element from the transplant, and this increased their risk of relapse and depending on the type of TCD used may have involved cells other than just T cells, such as NK cells.

Campath-1 has been used widely for *ex vivo* TCD and the inevitable diversity of patients, diseases and conditioning regimens across different centres has produced a range of results in the incidence of GvHD reported. A number of

factors must be taken into account when comparing results from different centres and these include the age of the patients (because the risk of GvHD increases with age) and technical differences in the use of the product (Apperley *et al.*, 1986).

In a group of patients at this centre with AML in first complete remission having HLA-matched sibling donor transplants there was no evidence of severe acute GvHD, three cases of grade 1 acute and one incident of limited chronic GvHD. The use of Campath IgM alone was associated with a relapse risk of only 14%, but the concurrent use of *in vivo* Campath IgG increased the relapse risk to 50%. This was attributed to additional *in vivo* TCD by residual Campath 1G at the time of the transplant and it would suggest that there is an optimum level of TCD which, if exceeded, results in relapse (Lowdell *et al.*, 1997).

The use of immunosuppressive drugs such as methotrexate and cyclosporine A (CsA) has been a major arm of the prophylaxis of GvHD. Bacigalupo and co-workers (1991) performed a study of two patient groups who received low dose (1 mg/kg per day) versus high dose (5 mg/kg day) CsA. By day 10 those patients on the higher dose had sustained significant nephrotoxicity and their dose was reduced to 2.5 mg/kg per day . The key message from this study was that the long term results showed that the dose received by the patients up until day 10 had a bearing on their subsequent risk of relapse. The inhibition of the GvL components had already taken place by day 10.

Although CsA is effective in preventing acute GvHD, in high doses it is a cause of multi-organ toxicity and results in a high incidence of leukaemic relapse (Bacigalupo *et al.*, 1991). One way to alleviate these problems is to use lower

doses and taper the treatment with CsA aiming to stop treatment by day + 100. This appears to allow for early GvHD prophylaxis and yet not completely abrogate the GvL effect (Shaw and Afify, 1998).

What has emerged from the development of BMT over the last 15 years is that there is a fine balance that clinicians must find for an individual between encouraging the GvL response and abrogating the GvHD response. TCD, donor leucocyte infusion (DLI) and immunosuppression are the tools at their disposal and the interplay of all three can result in a positive or negative outcome for the patient depending on how carefully they are managed.

1.5 Donor leukocyte infusion

The value of adoptive immunotherapy in the BMT strategy for leukaemia relapse post allogeneic BMT was first highlighted by Slavin and colleagues (1988), and it was a breakthrough in terms of a possible cure after relapse post-BMT. Before the introduction of DLI a patient with leukaemia who relapsed was faced with the option of a second bone marrow transplant, with the mortality and the treatment related morbidity that would incur, in the knowledge that further relapses are not uncommon. DLI involves infusing immunocompetent cells from the original donor into the recipient if they relapse post transplant. This approach obviates the need for a second transplant and the complications that can involve.

Slavin's first reported case was an ALL patient in third relapse. The infusion of donor peripheral blood lymphocytes (PBL) induced mild GvHD which allowed the patient to remain disease free eight years after the procedure (Slavin *et al.*,

1995). However the evidence from a comprehensive review of DLI for acute leukaemia suggests that the long-term outlook for ALL patients is not good despite an encouraging initial response (Slavin *et al.*, 1995). The long-term benefits of this treatment appear to be much more favourable with myeloid disease. The first peer-reviewed publication of DLI was by Kolb and co-workers and since then the greatest successes have been found with CML, some with AML and few with ALL (Kolb *et al.*, 1990, Kolb *et al.*, 1995). The treatment is less effective in CML in accelerated phase, and the efficacy of the treatment against aggressive disease or disease with high tumour burden is questionable, which makes timing of the DLI of great importance (Johnson and Truitt, 1995).

One point of interest is that GvHD is less severe when the infusion of donor cells is delayed, and that larger cell numbers can be infused as a DLI than at the time of BMT, without GvHD developing. It has been shown that early transfusion of leucocytes results in unmanageably severe acute GvHD and yet if the DLI is delayed until graft versus host tolerance has been established then the beneficial effects can be seen with only mild or no GvHD (Kolb *et al.*, 1995).

Although the reason for this is not fully understood, it seems likely that immediately post-transplant the inflammatory cytokines unleashed by the conditioning regimen make an unwelcome environment for the DLI. Although graft versus host tolerance is desirable it may be detrimental to patients with residual leukaemia where anti-leukaemic reactivity is required. It may be that GvHD regularly occurs with this treatment but remains subclinical (Johnson and Truitt, 1995).

Mackinnon and colleagues (1995) took on the challenge of trying to eliminate the high rates of GvHD seen in the initial trials of DLI by using escalating doses of donor lymphoytes to determine the optimum dosage achieving GvL without GvHD. They observed that remission could be obtained in CML with as few as $1x10^7$/kg T cells in the DLI, with little or no GvHD. They suggest that the safest form of treatment for CML patients may be a TCD transplant followed by regular polymerase chain reaction (PCR) tests for BCR/ABL transcripts to detect residual CML. If the patient became PCR positive, which in this case means that BCR/ABL is detected indicating disease, a DLI could be administered to achieve molecular remission. This protocol should reduce the incidence of GvHD whilst maintaining the benefits of GvL in CML patients. It is of more limited application in AML where the response to DLI is less predictable and molecular markers of disease are less commonly available.

1.6 Mechanisms of GvL

1.6.1 Minor histocompatibility antigens

Even if the donor is a fully matched sibling this does not exclude the possibility of immune responsiveness to minor histocompatibility antigens (mHA), and in this situation they are the likely source of the GvHD and the GvL reaction. Minor HA are presented by HLA Class I and Class II antigens, and unlike MHC which are found in a single chromosomal region, mHA are scattered all over the genome. Initially in solid organ transplant, mismatches at MHC loci lead to rapid graft rejection, whilst mHA mismatches lead to slower rejection. Acute rejection is now treatable and most transplants are not rejected, but these early

observations led to the concept that mHA are less immunostimulatory than MHC, and was why the term 'minor' was employed. However, it has since been noted that graft rejection and acute GvHD in HLA-identical sibling donor allografts seem to suggest that some mHA are at least as immunostimulatory as MHC antigens.

A single mHA may represent a single amino acid difference from the recipient's corresponding protein. The result is that only a few peptide-MHC molecule assemblages will be generated and this will stimulate only a few T cell clones. This results in a weak response, however, if there are many discrepancies this will multiply the number of peptide-MHC complexes formed and therefore a much greater T cell response will occur.

The targeting of myeloid-restricted mHA is one immunotherapeutic approach that has been pursued. It has been shown that two mHA (HA-1 and HA-2) are restricted to lymphoid and myeloid cells (de Buerger *et al.*, 1993). Leukaemic cells have also been found to express HA-1 through to HA-5 and H-Y so that mHA-specific cytotoxic T lymphocytes (CTLs) are capable of HLA class I restricted antigen-specific lysis of leukaemic cells. However, they are less susceptible to T cell mediated lysis by the HA-2 mHA-specific CTL. This may be because of lack of expression of the LFA-1 adhesion molecule (van der Harst *et al.*, 1994). Since the finding that HA-2 is specific to haematopoietic cells, there is the potential for a therapy which targets these cells, there is also the possibility that a mHA exclusive to leukaemia cells will be found, which raises new ideas for treatment. As yet this work has not come to fruition.

1.6.2 Leukaemia specific antigens

Identification of leukaemia specific antigens could allow the separation of the GvL from the GvHD effect. The GvL response may arise due to the reaction of cytotoxic T cells against leukaemia specific antigens although evidence for this has not yet transpired. A number of chromosomal translocations exist in leukaemias which are potential sources of leukaemia antigens; the most common of which are t(15;17) in AML M3, t(8;21) in AML M2, t(12;21) in ALL, t(9;22) in CML. Also ras mutations occur in the DNA in myeloid leukaemias which may be a source of leukaemia antigens.

There has been much research done on a candidate leukaemia specific antigen in the BCR/ABL fusion protein resulting from the t(9;22) chromosomal translocation (which is also known as the Philadelphia positive translocation), identified in cases of CML and ALL. This fusion gene encodes for a 210, 190 or 230-kDa chimeric protein (reviewed in Melo, 1996). T cell responses against the 210-kDa protein have been documented (Chen *et al.*, 1992). *In vitro* T cell stimulation with BCR-ABL$^+$ leukaemia cells has been used to derive leukaemia-specific CD4 cells which were restricted to peptides of the BCR-ABL fusion product. In a single report Sosman *et al* (1990) were able to isolate a small fraction of T cells with the phenotype CD3$^+$/CD4$^+$/$\alpha\beta$TCR$^+$ which appeared to be capable of specifically lysing leukaemic targets. As yet no further work has been published on this and to date allogeneic leukaemia specific cytotoxicity has not been found in the peripheral blood of BMT and DLI recipients (Kolb *et al.*, 1995). However, both class I and class II restricted peptides have been described and this makes the concept of BCR-ABL specific immune responses a possibility (Cullis *et al*, 1994, ten Bosch *et al.*, 1996).

Ten Bosch observed that the product of the BCR/ABL fusion gene can be processed by MHC class II molecules on the surface of leukaemia cells (ten Bosch *et al.*, 1996). The importance of CD4 cells in the GvL response in the production of interleukin-2 and interleukin-12 (IL-12) is already known, and the recognition of class I presented peptides by appropriate $CD8^+$ T cells would be central to effective clearance of minimal residual disease.

1.7 Separation of GvHD from GvL

This is the Holy Grail that all involved in the cure of leukaemia have been searching for and it has proved to be extremely difficult to find. T and NK cells are known to be involved in both responses but whether subsets of these cells can perform different functions pertinent to the two different phenomena has yet to be fully understood (Pattengale *et al.*, 1983). One possibility is that GvHD activates anti-leukaemic effector mechanisms which operate independently of the GvHD process. NK cells, through cytokine stimulation, can be directly lytic to leukaemia cells. T cells may destroy leukaemia cells non-specifically as they recognise mHA on normal host tissue, or specifically through tumour-specific antigens expressed on leukaemia cells. The anti-leukaemic response is not necessarily associated with acute GvHD but the problem remains how is it possible to separate the two.

It is known that both $CD4^+$ and $CD8^+$ T cells are important in GvHD, and they probably recruit NK cells via cytokines (Antin *et al.*, 1992). Animal studies implicate both CD4 and CD8 T cells, and in a human study specific depletion of $CD8^+$ cells from the donor graft prevented GvHD (Champlin *et al.*, 1990). Other

studies using CTL clones have shown *in vitro* that T cells alone are responsible for the GvL and GvHD effect, some clones were leukaemia specific and others also targeted normal and neoplastic cells (de Buerger *et al.*, 1993). Although this is evidence for the role of T cells it does not necessarily rule out NK cells, as using clones in isolation will not give a true picture of the *in vivo* mechanisms.

The killing mechanisms in GvL do not appear to narrow the search for the cells responsible for the effect. Direct killing of leukaemia cells by perforin and granzyme invasion by cytotoxic lymphocytes ($CD8^+$ and NK cells), apoptotic death through the fas/fas ligand connection ($CD4^+$ and $CD8^+$) and cytokine mediated death or control of proliferation ($CD4^+$) have all been found (reviewed in Barrett and Malkovska, 1996). This demonstrates that it is likely that all these cells play a role, and yet it may be possible to find subsets of these cells that are specifically responsible.

A possible T cell subset which may prove to be a key to GvL is the $\gamma\delta^+$ T cell. It has been shown that $\gamma\delta^+$ T cells can mediate *in vivo* anti-lymphoma activity, significantly improving survival in a severe combined immune deficiency (SCID) mouse model of human leukaemia (Malkovska *et al.*, 1992). In a later human study a group of patients received peripheral blood stem cell transplants (PBSCT) which had been TCD with an anti-TCR$\alpha\beta$ monoclonal antibody and complement. Those who developed an increased percentage and absolute number of $\gamma\delta^+$ T cells 60-270 days post- BMT had a significant survival advantage over those patients whose $\gamma\delta^+$ T cells were at normal donor levels

(Lamb *et al.*, 1996). The role of $\gamma\delta^+$ T cells is currently under investigation in $\alpha\beta$TCR-depleted transplants.

Irrespective of the degree of MHC and mHA compatibility between the donor and the recipient is the immunogenicity of the leukaemic cell. One reason that leukaemic blasts are less immunogenic than their normal haematopoietic counterparts is due to decreased relative expression of the co-stimulatory molecules essential to the initiation of primary immune responses (Hirano *et al.*, 1997). One method being used to increase the immunogenicity of the leukaemia target cells is to upregulate immunostimulatory cell surface antigens to make the cell more visible to the cytotoxic cells. T cells require at least two signals for activation. Interaction between the T cell receptor and the antigen/MHC complex provides one stimulus and another occurs between a costimulatory molecule and its receptor on the T cell. Once both signals have been received the T cell can clonally expand. It has been proposed that tumours do not express appropriate co-stimulatory molecules and so escape immune surveillance (Guinan *et al.*, 1994).

CD80 and CD86 also known as B7.1 and B7.2 respectively bind to the T cell antigens CD28 and CTLA 4. CD86 is commonly found on AML cells but at subnormal levels (Hirano *et al.*, 1997). It has been shown however that these co-stimulatory molecules can be upregulated by a combination of GM-CSF and IL-4 (Coleman *et al.*, 1997). Another approach to increase the immunogenicity of the leukaemia cells has been to transfect AML cells with a combined gene transfer of CD80 and IL-12 (Anderson *et al.*, 1997). With gene transfer of

cytokines to leukaemic blasts the proteins are secreted locally *in vivo* and therefore there is less systemic toxicity (Lowdell *et al.*, 1997).

Practical methods exist and are under review which will separate GvHD from GvL. The delayed add-back of T cells post-BMT shows that the timing of delivery of cells can influence the response and the effect achieved. It may also be possible to remove the host-activated T cells by *in vitro* stimulation by the host of donor T cells. This could be done by use of antibodies to CD25 or CD69 activation antigens coupled to immunomagnetic beads (reviewed in Barrett and Malkovska, 1996).

NK cells are peripheral blood mononuclear cells which have the morphology of large granular lymphocytes, a distinct surface phenotype, cytokine profile and are able to mediate spontaneous cytotoxicity (Silla *et al.*, 1995). It is now accepted that NK cells may be at least in part responsible for the GvL effect (Mackinnon *et al.*, 1990). It is known that they can lyse K562 which is a cell line derived from a CML patient and that they return more rapidly than other lymphocytes after BMT and in higher numbers than in normal donors (Reittie *et al.*, 1989; Lowdell *et al.*, 1997).

Jaing and co-workers (1993) found a GvL effect which reversed relapse in CML patients, was exerted by MHC-restricted and non-MHC restricted cells contained in the buffy coats given to these patients after their TCD BMT. This is in accordance with the findings of Kos and Engelman (1995) that there is an inter-dependant relationship between NK cells and CD8 cytotoxic T cells, in that NK cells play a crucial role in the generation of MHC class I restricted $CD8^+$ T

cells. This would explain the findings of Jaing and colleagues (1993) that possibly NK and T cells work in tandem to create antigen specific T cells.

Over the past decade evidence has been gathered attesting to the importance of these cells. Previously, research has concentrated on the role of the T cell, which has captured the minds of researchers more than the NK cell. Recently this has changed and the value of NK cells, working in conjunction with T cells, has become clear although there is still a lot of research needed to fully understand their role.

1.8 Autologous anti-leukaemia

1.8.1 Autologous BMT

70% of patients with AML do not have a matched sibling donor and therefore are reliant on chemotherapy alone, a matched unrelated donor or an autologous transplant for a cure (Talbot *et al.*, 1990). In autologous transplantation the bone marrow is taken from the patient and then they undergo high dose chemotherapy and radiotherapy before the re-infusion of their previously stored bone marrow. The procedure is much less toxic than the conditioning required for an allogeneic transplant. The patient does not receive any cells that would be seen as foreign; the conditioning regimen is therefore less severe and can be performed on older patients. It also has the advantage of readily available material for grafts, and no inherent risks of GvHD.

1.8.2 'Autologous GvHD' induced by cyclosporin

The anti-leukaemic effect of autologous grafts is presumably less than that of allogeneic transplants, because these grafts are lacking the immunogenic stimulus provided by alloantigens. Given the association between GvL and GvHD, some groups have tried to break immune tolerance to self-antigens in order to provoke 'autologous GvHD'.

It has been shown that an acute GvHD-like condition could be induced by cyclosporin in AML patients who had received autologous BMT, but the results in terms of disease-free survival have been disappointing (Talbot *et al.*, 1990). In rat studies the administration of cyclosporin resulted in auto-cytotoxic T cells which recognised self class II MHC antigens, and showed a reduction in T helper cells (Hess *et al.*, 1985). In a small patient study auto-GvHD was induced in 50% of recipients, but they subsequently suffered a prolonged period of aplasia (Garin *et al.*, 1996). This route of research although potentially very exciting has not proved as fruitful as at first hoped.

1.8.3 The use of LAK therapy

Lymphokine activated killer (LAK) cells are mainly MHC non-restricted and are defined as cells which are capable of killing NK-resistant targets after incubation with IL-2. In a study of patients with CML, LAK cells were generated by incubating peripheral blood mononuclear cells with recombinant IL-2 for up to seven days (Mackinnon *et al.*, 1990). The predominant phenotype of these cells was $CD56^{+}/CD8^{+}/CD4^{-}$ with a variable number of cells co-expressing CD3 [and hence termed NKT cells]. LAK cells were able to inhibit CML colony

forming units-granulocyte-macrophage (CFU-GM) proliferation, but did not inhibit donor marrow CFU-GM to the same degree, which suggests a GvL effect. Although the LAK population did include NKT cells, killing of recipient CML cells was maintained in this study even after depletion of $CD3^+$ cells (Mackinnon *et al.*, 1990).

This work demonstrated a role for LAK cells *in vitro*, and diminished the importance of the $CD3^+$ T cell. From this stemmed the idea that the GvL effect could be enhanced by the use of IL-2, without incurring GvHD. The trials that ensued covered the use of IL-2 *in vivo* with or without BMT, the use of IL-2 *in vitro* to stimulate NK/LAK responses and the subsequent infusion of activated cells, and the combination of *in vivo* IL-2 with NK/LAK cell therapy. This treatment was particularly popular with autologous BMT due to the higher relapse rate as compared with allogeneic BMT, and indeed a study from this centre showed that it could reduce the relapse risk from 54% to 17% (Hamon *et al.*, 1993).

The trials varied the administration of IL-2 by continuous intravenous infusion or bolus infusion (Higuchi *et al.*, 1991, Soiffer *et al.*, 1992) also the length and the timing of the treatment was altered. Initially the treatment seemed to be well tolerated and in all cases resulted in an increased number of $CD56^+/CD16^+/CD3^-$ cells. However, problems resulted because early treatment post-autologous BMT with an IL-2 infusion was required to prevent relapse. Although IL-2 is followed by rebound lymphocytosis which is valuable in the generation of LAK cells, apheresis was associated with unacceptable

thrombocytopenia, also administration of IL-2 itself results in thrombocytopenia which is not desirable early after allogeneic BMT (Benyunes *et al.*, 1993).

In a larger study conducted by Hauch et al the primary effector cells post transplant against host-derived and fully allogeneic CML targets were found to be $CD56^{+}/CD16^{+}/CD3^{-}$, these cells had been cultured in medium containing IL-2 (Hauch et *al.*, 1990). Also in this study those patients who failed to generate lytic activity against host CML cells were much more likely to relapse (72%) when compared with those whose LAK cells did lyse host CML cells (7%, p=0.002).

The best results achieved with LAK cell therapy have been reported from the Seattle group (Benyunes *et al.*, 1993). In 14 patients with AML in relapse or CR 2 the administration of IL-2 +/- LAK cells after ABMT gave a 4 year survival probability of 71%, suggesting that the benefit of IL-2 therapy may require the presence of minimal residual disease to generate a leukaemia-specific immune response.

NK cells have the advantage over T cells in that they do not require priming and they are MHC non-restricted. It appears however that the activation by IL-2 *in vivo* or *in vitro* is crucial to maximise the NK cell's potential and without that their ability to lyse leukaemic targets is limited. However, despite the obvious benefits of IL-2 as a means of stimulating cells the side effects have made effective therapy difficult. The number of clinical studies therefore has reduced dramatically since the early 1990s, due to treatment toxicity and the

thrombocytopenia frequently seen early on after BMT which negates the effect of an IL-2 infusion. It is possible though that another cytokine which shares the ability possessed by IL-2 to activate cells, could be used in its place if found to be less toxic. One candidate for this role is IL-15 which is central to this study.

This project was designed to give a comprehensive overview of immune reconstitution, after T-cell depleted and non-depleted allogeneic and autologous grafts. This was done in an attempt to uncover the likely effector cells in allogeneic GvL, and to compare the role of these cells in autologous transplant recipients and patients treated with chemotherapy alone. Autologous leukaemia reactive cytotoxic cells were identified and culture conditions investigated for *in vitro* maintenance and proliferation by using combinations of cytokines.

2. Immune reconstitution after bone marrow transplantation

2.1 Introduction

An important factor in the long-term recovery of the patient after high dose chemotherapy and BMT is that the immune system is reconstituted and a competent immune repertoire is re-established. Numerical recovery of the components of the bone marrow is just one aspect; functional recovery of the cellular interactions of those components is also vital. Various factors may affect how well the immune system recovers. The ablation of the host immune system during conditioning, the manipulation of the donor graft, the sustained transfer of donor-antigen specific immunity and the age of the patient are all significant.

It has traditionally been accepted that the patient's ability to recover a fully diverse functioning immune system depends on age, and the number of T cells returned to the patient in the graft. A child has a functioning thymus, but with age, post-BMT immunosuppression and GvHD the ability of the thymus to function diminishes (reviewed in Parkman and Weinberg, 1997). Recently it has been proposed that a relatively high degree of thymic activity does survive into adult life. It is suggested that through late middle-age adults retain thymic activity though it would be several log folds lower than a child (Douek *et al.*, 1998). Therefore a relatively diverse immune repertoire may eventually be attainable, in the post-BMT patient.

The possibility may also exist for adults to expand their immune repertoire by extra-thymic T-cell differentiation. The gut mucosa and liver have been suggested as possible sites of T-cell differentiation (Rocha *et al.*, 1995) although other tissues, including skin, may be shown to be important.

A major complication in BMT is the risk of life-threatening infections occurring in the weeks post-BMT. A naïve or dysfunctional immune system resulting from chemotherapy and BMT conditioning leaves the patient at risk of bacterial, fungal and viral, infections which can be fatal (Kook *et al.*, 1996, Parkman and Weinberg, 1997, Kruger *et al.*, 1999). BMT can produce prolonged, profound and rapid neutropenia, an ideal environment for bacterial infections such as Pseudomonas spp. to flourish. The advent of recombinant GCSF (Granulocyte colony stimulatory factor) has reduced the average period of post-transplant neutropenia to less than 15 days (the median time to $0.5x10^9$neutrophils/l is over 20 days (Hale *et al.*, 1998)).

Fungal infections may also be a major cause of mortality. In a recent report Aspergillus spp. and Candida spp. accounted for over half of all fatal infections (Kruger *et al.*, 1999). As resistance to fungal infections is chiefly mediated by T cell immunity T cell depletion can reduce the body's ability to control fungal infections (Klein and Horejsi, 1997).

The lack of antigen-specific T lymphocytes also leaves the body vulnerable to viral infections (reviewed in Parkman and Weinberg, 1997). One of the most common such infections seen in immunocompromised patients and recipients of bone marrow transplants is cytomegalovirus (CMV) (Prentice *et al.*, 1998).

There are a number of ways in which a transplant recipient can be infected. A seronegative bone marrow transplant patient may be infected by blood products, or by the marrow itself. If the patient was already seropositive they may be re-infected by another strain of CMV from blood products or marrow, or by their own endogenous strain of CMV which was latent but becomes reactivated in the immunocompromised patient (Prentice *et al.*, 1998). Other viral infections that can result in serious complications include hepatitis B, hepatitis C and Epstein-Barr virus (EBV). The latter is of particular importance in patients who receive TCD bone marrow transplants (Westmoreland, 1998).

Investigators have noted the rapid recovery of NK cells post-BMT (Reittie *et al.*, 1989; Lowdell *et al.,* 1997). These are largely "true" NK cells rather than NKT cells, particularly in patients such as those studied here who have received a T cell depleted donor graft. They may have an important role, possibly fulfilling the function of T cells prior to their recovery. Functional tests have shown that NK cells recover their activity not only against K562 (a traditional NK target) but also are active against T and B cell targets (HSB2 and Daudi or EBV-transformed B cells respectively)(Rooney *et al.*, 1986). This can occur as early as 4-6 weeks after transplantation, during the period when T cells are recovering slowly, particularly after a TCD BMT.

It is known that NK cells from normal donors can react to T and B cell targets if given the appropriate stimulation (*in vitro*, on exposure of NK cells to IFN-γ). In BMT patients high levels of IFN-γ have been identified in the body soon after bone marrow transplant (Antin *et al.*, 1992). In this group of BMT patients NK

cells were active against T and B cell targets without the possible side effects of T cell activity which could include GvHD, CMV or apparent graft rejection (Rooney *et al.*, 1986).

NK cells have also been shown to play an important role against certain strains of CMV. One group have demonstrated that cell surface LFA-3 (lymphocyte function-associated antigen-3) expression is crucial to sensitivity or resistance to NK cell lysis. This group used fibroblasts infected with different stains of CMV, and those that up-regulated LFA-3 were susceptible to lysis and those that down-regulated LFA-3 were resistant. This change in LFA-3 expression was mediated by CMV immediate early or early CMV genes (Fletcher *et al.*, 1999).

The type of graft used (allogeneic or autologous) can present the immune system with different problems. Prior to the use of peripheral blood stem cell transplants (PBSCT), transplants were performed either with autologous bone marrow taken from the patient in CR, or with bone marrow from an HLA-identical sibling or volunteer unrelated donor. PBSC transplants have become increasingly popular, and this form of transplant may determine a different outcome for the immune system of the patient than BMT due to larger numbers of T cells in the graft and the possible effects of GCSF used in the mobilisation of stem cells (Tayebi *et al.*, 2001, Volpi *et al.*, 2001). In the studies presented here all transplanted patients received bone marrow.

As described above, GvHD is a severe complication of allogeneic BMT and it is central to the issue of post-transplant immune reconstitution. Acute GvHD

targets the thymus and dendritic cells in the skin, liver and spleen and leads to severe reduction in the rate of reconstitution of $CD4^+$ T cells. In contrast, TCD for the prevention of GvHD leads to reduction in the T cell pool from which peripheral T cell expansion can occur – also leading to delayed T cell restoration. Treatment for severe GvHD may have similar effects.

With an autologous transplant the conditioning regimen is designed to eliminate residual tumour load. The immune system does not require complete ablation because the incoming graft is clearly seen as 'self' and therefore there is no risk of rejection. Similarly TCD is not required since there is no risk of GvHD. This also obviates the need for immunosuppressive drugs, which may interfere with immune recovery. The autologous setting therefore provides an opportunity to study the re-emergence of the immune system after chemotherapy and radiotherapy without other factors obscuring the picture (although all transplant recipients receive prophylactic treatment against infections, which may also have a bearing on immune recovery).

In this study patients receiving four different types of bone marrow transplant; manipulated, partially manipulated and unmanipulated allogeneic transplants and autologous transplants were followed up. A panel of antibodies (as detailed in Table 2.1) was used to study lymphocyte differentiation antigens to give an indication of the dynamics of immune system recovery, and the effects of TCD. CD4 and CD8 are surface molecules expressed on subsets of T cells and CD45RA and CD45RO were used to identify naïve and mature cells respectively and appear in conjunction with both CD4 and CD8.

The CD56 antigen is expressed on 95% of natural killer cells. CD56 and CD16 are the most commonly used and most informative molecules denoting NK cells with over 95% of such cells expressing CD56 (Robertson and Ritz, 1990). A small proportion of NK cells in normal donors lack CD56 but express CD16. In preliminary studies this subset $CD56^-/CD16^+$ represented <2% of $CD3^-/CD19^-$ cells in normal donors and transplant recipients and so CD56 alone was used. Data from previous studies in the department (Lowdell *et al.,* 1995) had shown that NKT cells were uncommon in transplant recipients which further supported the use of CD56 alone as a molecule denoting NK cells. The analysis of the $CD8^+$ subset of NK cells was aided by the fact that $CD56^+/CD3^-$ NK cells express only the CD8α chain as a homodimer and appear as $CD56^+/CD8^{wk+}$ cells when analysed by flow cytometry. In contrast, the subset of NKT cells which expresses CD8 (approximately 60%) shows levels of CD8 which are equivalent to T cells and also co-express CD57 (Ortaldo *et al.,* 1991).

A recent study into individuals that had received TCD-marrow from HLA-identical siblings (TCD by Campath 1G) found that there was a significantly reduced mean lymphocyte count of cells expressing CD3 at 8 weeks post transplant ($p=0.05$), but that populations expressing CD8 and CD56 remained within the normal range throughout the study. Normalisation of cell numbers of $CD3^+$ cells did not take place until 52 weeks post-transplant (Davison *et al.*, 2000). Also a study from 1997 into repopulation of circulating T, B and NK cells post autologous and allogeneic BMT and PBSCT without TCD, found that whilst that $CD56^+$ cell compartment showed a faster recovery than the T or B subsets

in all groups, the $CD56^+/CD3^+$ subsets did not recover until between 9-12 months post-transplant (Parrado *et al.*, 1997).

HLA-DR was used to denote activation of T cells and CD28 is the ligand for CD80 required for costimulation of T cells.

2.2 Materials and Methods

2.2.1 Peripheral blood and bone marrow cells

All peripheral blood (PB) and bone marrow (BM) samples were diluted in complete medium (CM) (RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2.5 mM L-glutamine and 100 units/ml penicillin and 10,000 μg/ml streptomycin)(all Gibco, Paisley Scotland). The dilute sample was layered over an equal volume of Lymphoprep (Nycomed, Pharma, Norway), and centrifuged with the brake off at 400 g for twenty minutes, in a MSE Mistral 2000 centrifuge. The mononuclear fraction was recovered and washed in CM. The cells recovered were then resuspended in an appropriate volume of CM and counted using a Neubauer haemocytometer. Where appropriate the cells were cryopreserved in RPMI 1640 /50% FCS /10% DMSO (Sigma) in the vapour phase of liquid nitrogen for 24 hours prior to freezing.

2.2.2 Phenotyping of patient samples

Patients were monitored at least three times a week after transplant with respect to white blood cell count and leukocyte differential to assess engraftment. At three weeks the post-BMT peripheral blood EDTA-preserved

samples were assayed for lymphocyte phenotypes, by a lysed whole blood technique and three colour flow cytometry. From then on the patient samples were phenotyped at each routine visit to the clinic, with the aim of monitoring each patient once a week during the first three months post-BMT, and then monthly for up to one year. All leukocyte counts and differentials were performed within 6 hours of venepuncture whilst immunophenotyping was performed usually within 4 hours and always within 24 hours. The combinations of monoclonal antibodies (mAbs) used are listed in table 2.1.

Table 2.1 Panel of monoclonal antibodies.

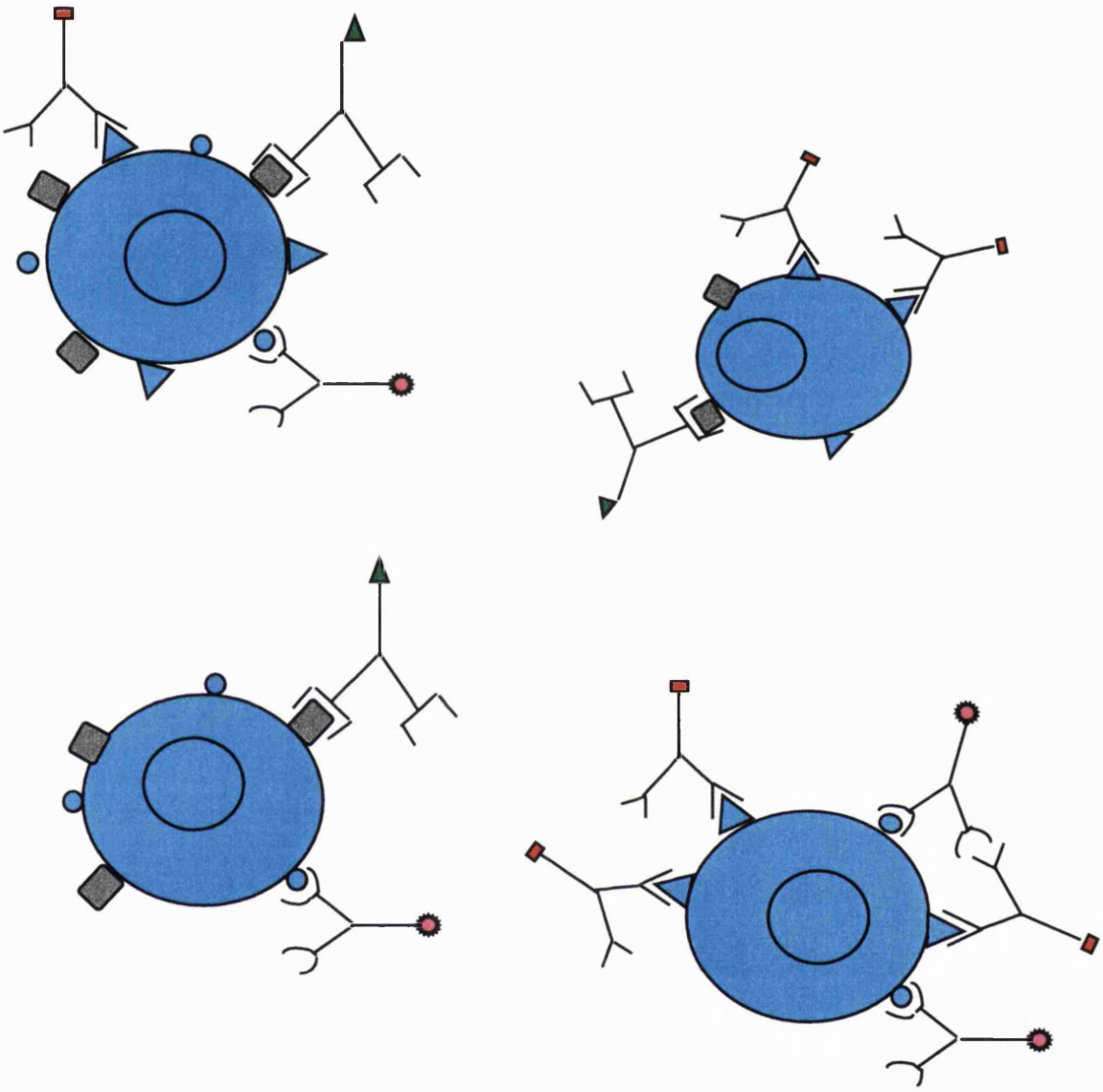
Tubes	FITC	PE	PerCP
1	CD45RA	CD45RO	CD4
2	CD45RA	CD45RO	CD8
3	CD57	CD28	CD8
4	CD57	CD56	CD8
5	CD57	HLA-DR	CD8
6	CD57	CD28	CD4
7	HLA-DR	CD56	CD8

All were supplied by Becton Dickinson Immunocytometry Systems (Oxford UK). The cells were labelled in polystyrene Falcon tubes with a proprietary red cell lysis buffer (FACSlyse - Becton Dickinson). 100µl of the peripheral blood sample was incubated with 10µl of the relevant fluorochrome conjugated monoclonal antibody. The monoclonal antibody had already been titrated in the laboratory and 10µl was used as standard practise. Three different fluorochromes were used fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlororphyll protein (PerCP) all of which are excited by a 488 nm argon laser. 2ml of lysis buffer was added to the tubes and left for 10 minutes at room temperature. The cells were then washed once in phosphate buffered

saline (PBS)(Microgen Bioproducts Ltd., Surrey) and resuspended in 0.5ml PBS (Figure 2.1).

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 5000 events were collected within a lymphocyte gate as list mode data (Lysis II - Becton Dickinson Immunocytometry Systems). Phenotyping data were analysed in an interelational 3-colour system (Paint-a-Gate Plus - Becton Dickinson Immunocytometry Systems). The instrument photomultiplier gain and compensation for three colour work was determined using peripheral blood mononuclear cells (PBMC) labelled with one or two fluorochromes.

Figure 2.1 Three-colour flow cytometry



The diagram depicts monoclonal antibodies conjugated to fluorochromes, in this instance FITC, PE and PerCp. The monoclonal antibodies attach to the antigen counterpart on the cell surface. The fluorochromes are excited by a 488nm argon laser and the resulting emission is recorded by the flow cytometer.

2.2.3 T-cell depletion

T cell depletion (TCD) was performed on the grafts *ex vivo* with Campath 1M (Therapeutic Antibody Centre, Oxford) and autologous serum, by members of the Stem Cell Processing Laboratory. The bulk of the graft was fully T-cell depleted. Campath 1 antibodies recognise the CD52 antigen which is a small lipid-anchored glycoprotein expressed on T, B and NK cells and monocytes and macrophages (Hale *et al.*, 1998).

2.2.3.1 TCD by Campath 1 M

Mononuclear cell suspensions were prepared from donor bone marrow by density gradient centrifugation (Lymphoprep; Nycomed, Pharma, Norway) in a "closed" system (Cobe 2991). TCD was achieved by complement-mediated lysis. The proportion of T cells in the donor marrow was determined by flow cytometry using anti-CD3-FITC Mab (Becton Dickinson, UK) and absolute mononuclear cell counts were measured by a particle counter (Coulter Z-1, Luton, UK.). The absolute T cell content of each donor bone marrow was calculated by combining the two measurements. The volume of the bone marrow that contained the appropriate T cell dose for re-infusion was removed from each harvest and the residue incubated with Campath 1M (final concentration 100μg/ml) at room temperature for 15 minutes. Donor serum was used as the source of complement at a final concentration of 25% and incubated with the residue of the bone marrow for 45 minutes at 37°C.

T cell lysis in the Campath 1M-treated bone marrow was monitored in a sample of the treated harvest by flow cytometry. One millilitre aliquots of Campath 1M-treated bone marrow were incubated with anti-CD3-FITC monoclonal antibody (Becton Dickinson, Oxford, UK) for 15 minutes at 21°C, washed once and resuspended in propidium iodide/PBS (5μg/ml)(Sigma, Poole, Dorset). Propidium iodide was used at 10μg/ml in PBS, and 50μl of this solution was added to the cells in 500μl suspension. The proportion of live $CD3^+$ T cells was determined by propidium iodide (PI) exclusion. PI^{-ve} cells in the samples were simultaneously assessed for residual Campath 1M binding to $CD3^+$ cells using anti-rat IgM PE. T cells which remained viable after *ex vivo* Campath 1M but which continued to bind the rat Mab were considered to be part of the depleted fraction since lysis was likely to occur *in vivo* following re-infusion.

The final product was a combination of the non-depleted and depleted fractions, adjusted to achieve the desired T cell content. The degree of T cell depletion was a clinical decision made with respect to the HLA (human leucocyte antigen) compatibility of the donor: recipient pair, the type of disease, disease status at transplant and the age of the patient.

Table 2.2 T-cell numbers re-infused after full and partial TCD

Type of manipulation	**Number of T cells infused**
Full TCD	$<0.03x10^6$/kg
Partial TCD	Between $0.3x10^6$/kg - $0.75x10^6$/kg

2.2.4 Patients

All patient samples came from the Haematology Departments of the Royal Free Hospital and Colchester General Hospital. The patient group was comprised of those with acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), or chronic myeloid leukaemia (CML). The samples were obtained at routine visits to the clinics after informed consent. Control samples were obtained from healthy volunteers, in the Department of Haematology at the Royal Free Hospital.

All patients were monitored for 12 months post-BMT with regard to reconstitution of T and NK cell subsets. The monitoring started at three weeks post transplant and continued weekly for the first 3 months and then monthly until month 12. Thirty-five patients were included in the study, 28 of whom received allogeneic transplants and 7 autologous transplants. 11 patients received non-TCD grafts, 12 received partial-TCD grafts and 5 received fully-TCD grafts. However after the 6 month time point no patients in the fully-TCD category remained in the study, they had either died or moved back to their referring hospital, and were lost to follow-up.

The details of patients studied are given in Table 2.3. Those who had an allogeneic transplant received pre-transplant conditioning with cyclophosphamide (60mg/kg body weight per day for 2 days) and total body irradiation (750 Gy, fast rate 15cGy/min, single fraction). Those who received unrelated donor marrows also received Campath 1G (Therapeutic Antibody

Centre, Oxford) (20mg/day; 5 days commencing day -12). Donor bone marrow was processed to a mononuclear cell fraction in a closed system (Cobe 2991) and either infused immediately or T-cell depleted to the pre-determined level of T cell content and infused within 6 hours. The patients were monitored from three weeks post-transplant. Peripheral blood was obtained in EDTA-preserved samples and these were analysed for lymphocyte phenotypes by a lysed whole blood technique and three-colour flow cytometry (as described in 2.2.2).

Table 2.3 Patient demographics

Name	Sex	Disease	Age at BMT	Type of graft	TCD	$CD3^+$ cells infused
MAB	M	AML	40	allogeneic	Partial	$0.59x10^6$/kg
NAR	F	AML	31	allogeneic	Non	
NB	F	AML	18	allogeneic	Non	
GK	F	AML	8	allogeneic	Non	
AZ	M	AML	44	allogeneic	Partial	$0.53x10^6$/kg
PM	M	AML	33	allogeneic	Non	
ET	F	AML	16	allogeneic	Partial	$0.75x10^6$/kg
HAM	M	AML-M3	19	allogeneic	Partial	$0.4x10^6$/kg
AT	M	AML-M3	26	allogeneic	Non	
AA	F	AML-M4	29	allogeneic	Partial	$0.53x10^6$/kg
WAM	F	AML-M4	28	allogeneic	Partial	$0.94x10^6$/kg
GC	F	AML-M4	15	allogeneic	Full	$0.02x10^6$/kg
RAM	M	AML-M5	35	allogeneic	Non	
NS	F	AML-M5	47	allogeneic	Partial	$0.31x10^6$/kg
MAD	M	T-ALL	28	allogeneic	Full	$0.11x10^6$/kg
FE	M	ALL	14	allogeneic	Partial	$0.3x10^6$/kg
MGO	F	ALL	13	allogeneic	Partial	$0.52x10^6$/kg
MG	F	ALL	36	allogeneic	Partial	$0.57x10^6$/kg
QH	M	ALL	29	allogeneic	Partial	$0.3x10^6$/kg
TJ	M	ALL	12	allogeneic	Non	
AK	F	ALL	8	allogeneic	Non	
KAJ	M	ALL	10	allogeneic	Non	
KE	M	ALL	30	allogeneic	Non	
RS	M	ALL	7	allogeneic	Non	
GS	M	CML	32	allogeneic	Partial	$0.57x10^6$/kg
MT	M	CML	27	allogeneic	Full	$0.006x10^6$/kg
AAN	F	CML	34	allogeneic	Full	$0.03x10^6$/kg
JC	F	CML	30	allogeneic	Full	$0.08x10^6$/kg
GA	F	ALL	16	autologous		
MAD	M	T-ALL	27	autologous		
LA	M	AML	37	autologous		
AG	F	AML	30	autologous		
AH	F	AML-M6	47	autologous		
RH	F	AML-M4	50	autologous		
GT	M	AML	49	autologous		

2.2.5 Normal donors

Samples were taken from 22 normal healthy donors (11 female, 11 male) covering the age range of the patient cohort to provide a point of comparison for the BMT transplant recipients. There were no significant differences between the normal donors, and the patient cohort with regard to median age range or sex. The normal donors were analysed by the same panel of monoclonal antibodies as the patients.

A minimum of 11 and a maximum of 123 samples were used to calculate the means at each time point.

2.2.6 Statistical analysis of lymphocyte subset recovery

Statistical analysis was performed on the means and standard deviations of each comparable distribution by non-paired Student t Test after initial analysis of variance by F test. Homoscedastic distributions were compared by conventional Student t Test while those of unequal variance were compared by Snedecor's modified t Test (Snedecor, 1956).

2.3 Results

The analysis has been restricted to the relative proportions of individual cell subsets rather than their absolute number. The matched lymphocyte counts were frequently not available for the specific time points at which the samples

were analysed, and in the samples taken in the first 2 months from post-TCD allogeneic transplant recipients were frequently below the reliable performance range of the haematology analysers used. Manual counts would also have been unreliable, for such small samples.

2.3.1 Comparison of autologous and allogeneic immune reconstitution

2.3.1.1 T cell reconstitution

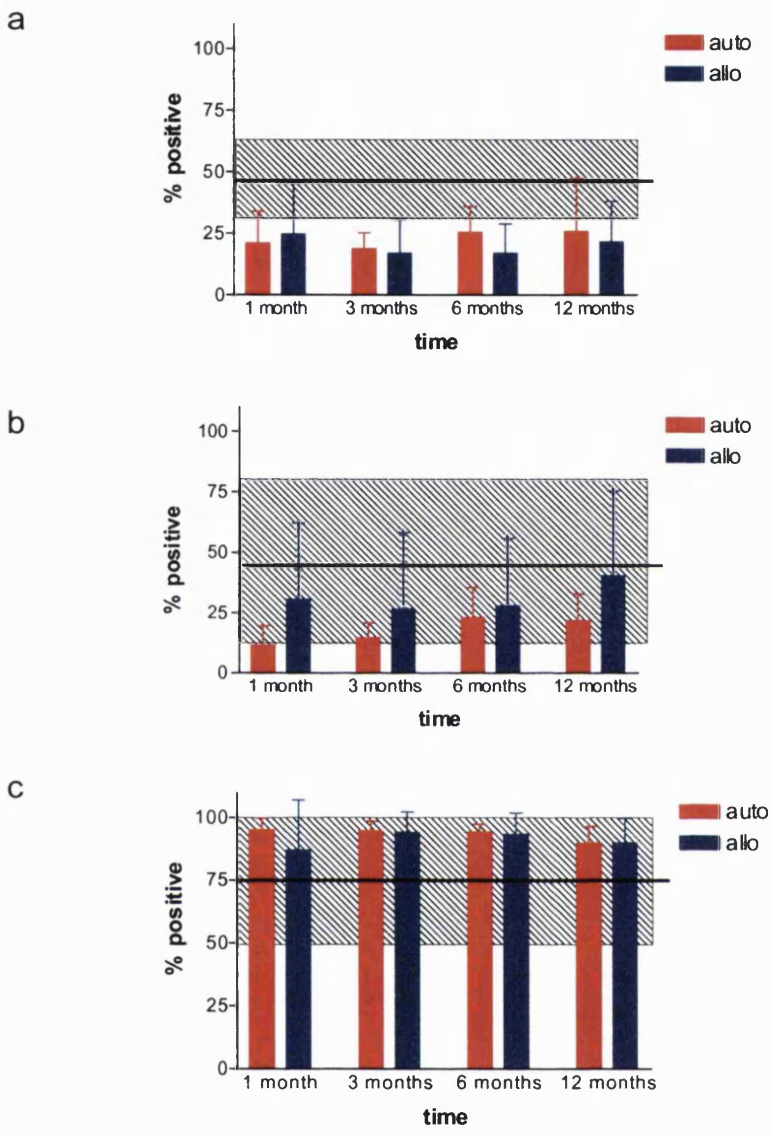
2.3.1.1.1 CD4

At six months (Figure 2.2a) the CD4 cell proportion (25.5%; s.d. 10.6) for autologous BMT recipients was significantly greater than in the recipients of allogeneic transplants (17%; s.d. 11.7) ($p=<0.002$). For both allogeneic recipients (21.7%; s.d. 16.3) and autologous recipients (26.0%; s.d. 21.6) the percentage of CD4 levels remained well below normal (46.4%; s.d. 7.7), for 12 months post-transplant.

The levels of naïve and mature CD4 and CD8 cells were determined by expression of CD45RA and CD45RO surface markers respectively. Naïve cells were determined on the basis of $CD45RO^-/CD45RA^+$ expression since RO^+/RA^+ and RO^+/RA^- are thought to be antigen primed and memory cells respectively. For purposes of clarity, cells referred to as "$CD45RA^+/CD4^+$ T cells" are $CD45RA^+/RO^-/CD4^+$ cells. $CD45RA^+/RO^+/CD4^+$ cells are included in the $CD45RO^+/CD4^+$ fraction. (The same nomenclature system is used to describe the $CD8^+$ T cell subsets in section 2.3.1.1.2 below). From one month into the

study allogeneic transplant recipients had a mean level of 30.6% (s.d. 31.4) $CD4^+/CD45RA^+$ expression (Figure 2.2b) and those with autologous transplants a mean of 11.5% (s.d. 8.1) (p=0.01). At 3 months this reduced to 26.5% (s.d. 31.7) for allogeneic recipients versus 14.5% (s.d. 6.3) (p=0.001) for autologous recipients, and by 12 months 40.4% (s.d. 35.1) for allogeneic recipients versus 21.8% (s.d. 11.1) (p=0.005) for autologous recipients.

Figure 2.2 CD4 T-cell recovery for autologous and allogeneic transplants



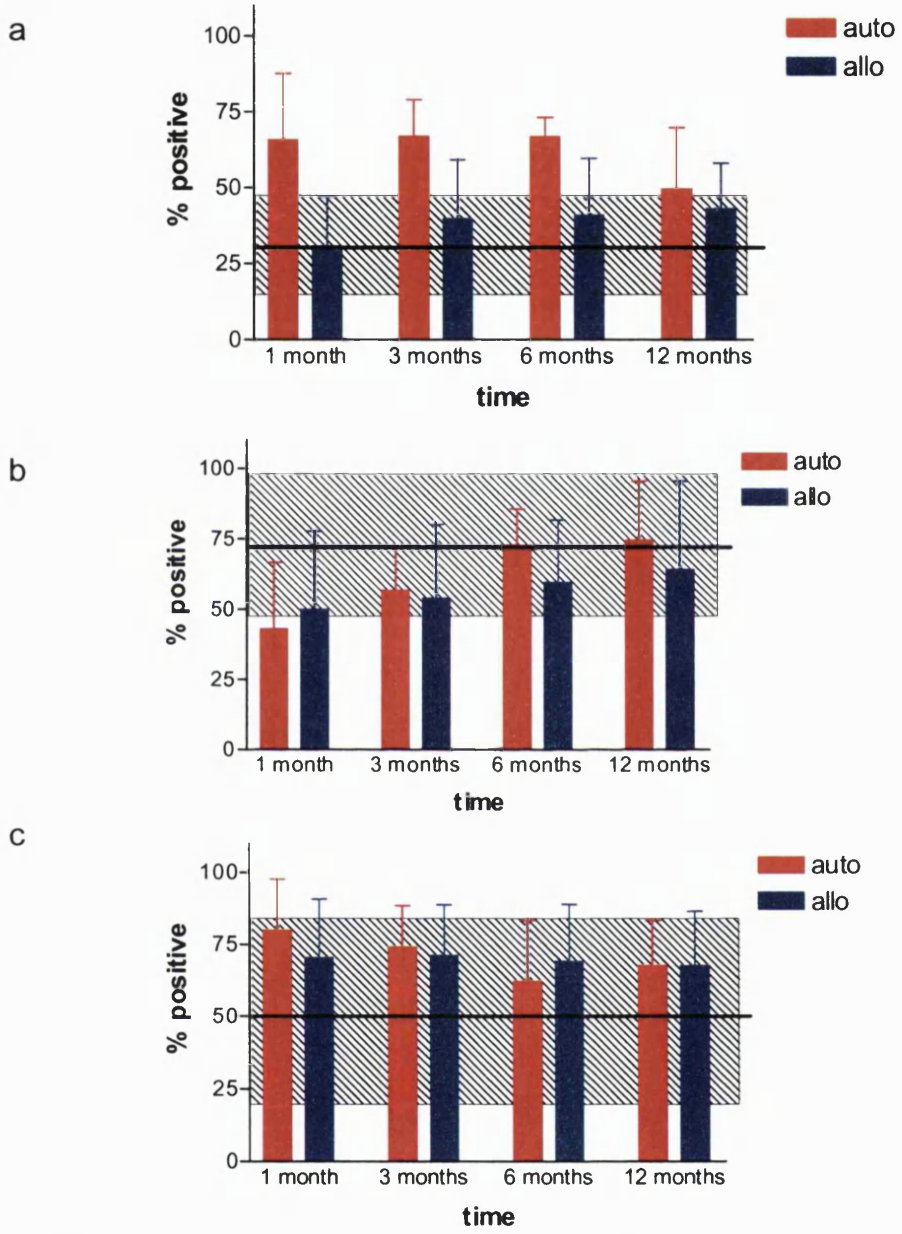
(**a**) $CD4^+$ T cell recovery. The bars depict arithmetic means values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**b**) $CD45RA^+$ expression on CD4 cells as a proportion of total CD4 cells. The bars depict arithmetic means values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**c**) $CD45RO^+$ expression on CD4 cells as a proportion of total CD4 cells. The bars depict arithmetic means values with the error bars showing standard deviations. The horizontal line depicts the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors.

2.3.1.1.2 CD8

The recovery of CD8 cells presented a very different picture to that of CD4 cells (Figure 2.3a). At one month the percentages of CD8 cells in recipients of autologous transplants were double those of normal donors (65.9%; s.d. 21.8) and recipients of allogeneic transplants (31.6%; s.d. 8.0). There was a significant difference between autologous and allogeneic transplant recipients for the first three time points (p=0.001 at each time point) with the percentage of CD8 cells in autologous BMT recipients always remaining higher than in allogeneic recipients. By 12 months autologous BMT recipients (49.8%, s.d. 20.2) and allogeneic BMT recipients (43.2%, s.d. 15.0) still had percentages of CD8 cells which were well above normal subject levels (31.6%) but the gap between the two patient groups had narrowed, they were no longer significantly different from each other and they were almost within the upper regions of the normal range.

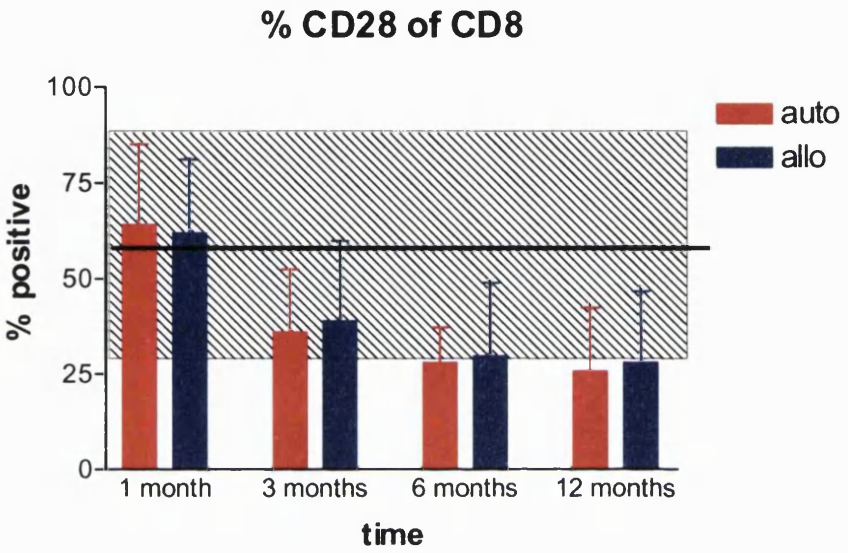
The expression of CD45RA on $CD8^+$ cells increased over each time point for both autologous and allogeneic BMT recipients (Figure 2.3b). By 6 months percentage of $CD45RA^+/CD8^+$ in autologous BMT recipients had already reached normal levels (73.2%; s.d. 11.8) and this level was significantly greater than the $CD45RA^+/CD8^+$ level of expression for those who had received allogeneic transplants (59.5%; s.d. 22.2) (p=0.001). The pattern of $CD45RO^+/CD8^+$ recovery was very similar to that of $CD45RO^+/CD4^+$ recovery with both groups above the normal mean (51.8%; s.d.16.5) but tending towards this level over the 12 month study period (Figure 2.3c).

Figure 2.3 CD8 T-cell recovery for autologous and allogeneic transplants



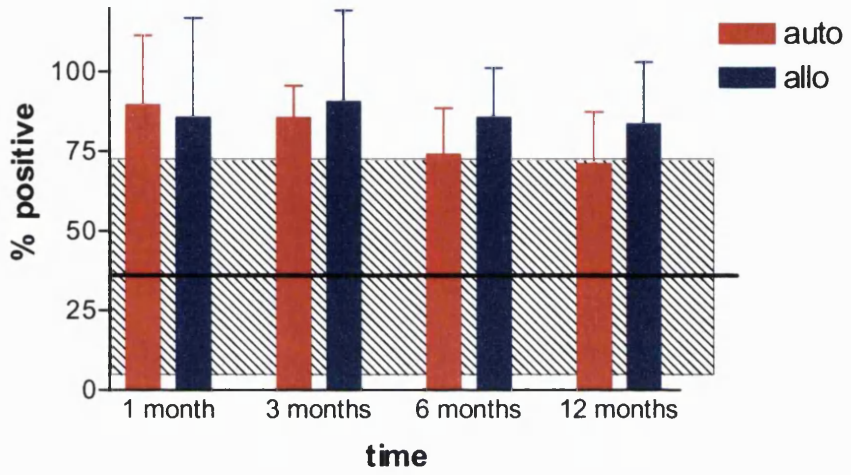
(**a**) $CD8^+$ cell recovery. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**b**) CD45RA expression on CD8 cells as a proportion of total CD8 cells. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**c**) CD45RO expression on CD8 cells as a proportion of total CD8 cells. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors.

Figure 2.4 %CD28/CD8 recovery for autologous and allogeneic transplants



The graph depicts CD28 expression on CD8 cells as a proportion of total CD8 cells following autologous and allogeneic BMT. The bars depict arithmetic mean values with the error bars showing standard deviation. The horizontal line represents the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors.

Figure 2.5 HLA-DR/CD8 in autologous and allogeneic transplantation

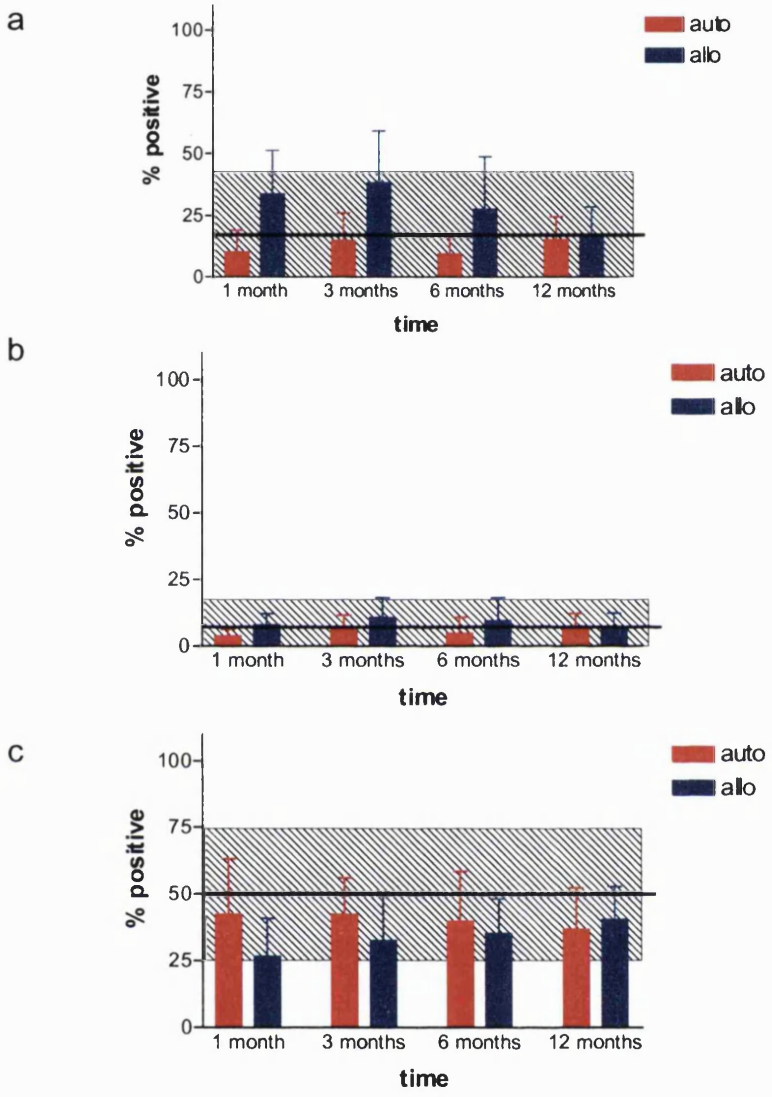


The graph depicts HLA-DR expression on CD8 cells as a proportion of total CD8 cells following autologous and allogeneic BMT. The bars depict arithmetic mean values with the error bars showing standard deviation. The horizontal line represents the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors.

2.3.1.1.3 The expression of CD28 on CD8 cells and the expression of HLA-DR on CD8 cells

The expression of CD28 (which is the ligand for CD80) on CD8 cells was never significantly different between the two groups. Although expression started at approximately normal levels (58.5%; s.d. 15.2) in the first month for both types of BMT recipients (Figure 2.4) it fell consistently from then on until by 12 months it was only at half the initial levels. In contrast the percentage of HLA-DR of CD8 cells remained very high for the entire study (Figure 2.5). The lowest it fell to in either group was 71.1% (s.d. 16.3) for autologous recipients and for both types of BMT recipient the level was above the normal range. No significant differences were seen with respect to activated CD8 cells between the two patient groups at any time during the first 12 months, although levels of expression fell in autologous patients whilst remaining stable in recipients of allogeneic transplants.

Figure 2.6 CD56, CD8/CD56 and CD8%/CD56 recovery after autologous and allogeneic transplantation



(**a**)CD56$^+$ cell recovery. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**b**) CD8/CD56$^+$ cells. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**c**) The percentage of CD56 cells expressing CD8. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors.

2.3.1.1.4 Natural killer cell reconstitution

From 1 to 12 months (Figure 2.6a) recipients of allogeneic BMT had far higher percentages of $CD56^+$ expressing cells than their normal counterparts whereas autologous recipient NK cell levels hovered just under normal mean which was 17.5% (s.d. 12.6) with a marked dip to 9.6% (s.d. 7.9) at 6 months. The two groups were significantly different at every time point up to 12 months ($p=0.001$).

Cells co-expressing CD56 and CD8 but lacking CD57 were also monitored (Figure 2.6b) (NB – for clarity these cells are referred to henceforth as "$CD56^+/CD8^{wk+}$" to distinguish them from an NKT cell subset whilst the NK-restricted CD8 antigen is described as CD8α since it lacks the ß chain). The

levels of percentage positive $CD56^+/CD8^{wk+}$ cells in the two groups were significantly different at each time point up to 12 months ($p=0.005$ at 1 month, $p=0.001$ at 3 months and $p=0.005$ at 6 months). The percentages of these co-expressing cells ($CD56^+/CD8^{wk+}$) peaked at 3 months in allogeneic recipients (10.8%; s.d. 7.5), but by 12 months had reached 7.0% (s.d. 5.4) which approached the normal figure of 8.5% (s.d. 4.9). For autologous transplant recipients the levels of $CD56^+/CD8^{wk+}$ expression remained below normal until 12 months when it reached 6.3% (s.d. 5.9).

The proportion of CD56 cells expressing CD8α within the NK population is shown in Figure 2.6c. With the panel of normal volunteers half of the CD56 positive cells expressed CD8α, but the figures were much less for both the groups studied. In the first 3 months the autologous recipients and allogeneic

recipients were significantly different at one month p=0.015 and at 3 months p=0.001.

2.3.2 Comparison of full, partial and non-T cell depleted grafts

The data were also used to compare immune reconstitution after T-cell depletion (TCD). The patients were divided into three groups depending on the extent of manipulation, if any, of their graft as described in 2.2.3.1.

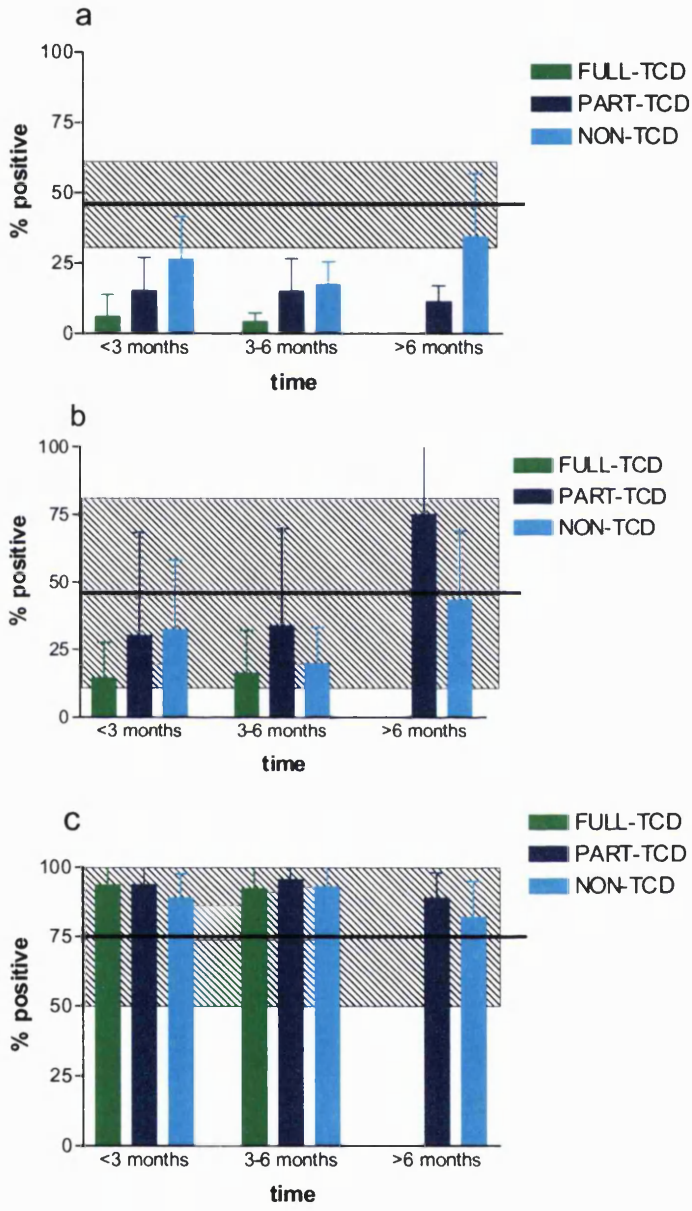
2.3.2.1 T cell reconstitution

2.3.2.1.1 CD4

The slowest recovery across all the study groups was seen with the expression of CD4. Throughout the study period levels of CD4 expression were higher in the non-TCD group than either of the groups who received manipulated grafts. Yet even by 12 months the non-TCD group who showed the best recovery had only reached 34.5% (s.d. 22.5) against the normal mean level of 46.4% (s.d. 7.7) (Figure 2.7a). By 12 months there was significant difference between the non-TCD (17.6%; s.d. 8.0) and the partially-TCD groups (11.4%; s.d. 5.7) (p=0.001).

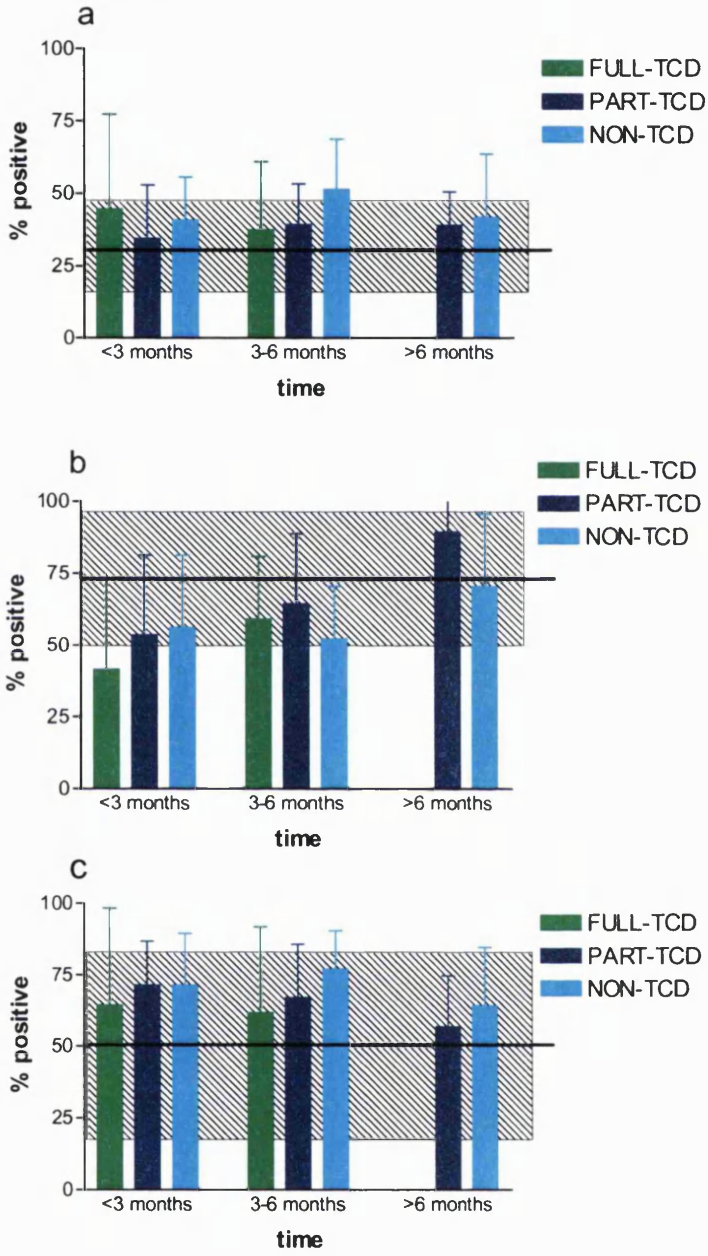
The percentage of $CD45RA^+/CD4^+$ cells remained below the normal mean in all groups up until 12 months (Figure 2.7b). There was a rise in the first 6 months in the fully and partially depleted groups but after six months, whilst the non-TCD almost reached normal levels (46.4%; s.d. 17.3) at 43.3% (s.d. 25.7) the partially depleted groups far exceeded the norm by reaching 75% (s.d. 31.6.). Three patients in particular consistently had approximately 90% double positive $CD45RA^+/CD45R0^+$ $CD4^+$ cells.

Figure 2.7 CD4 T-cell recovery after full-, partial-, and non-TCD allogeneic transplantation



(**a**) $CD4^+$ T cell recovery. The bars depict arithmetic means values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**b**) $CD45RA^+$ expression on CD4 cells as a proportion of total CD4 cells. The bars depict arithmetic means values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**c**) $CD45RO^+$ expression on CD4 cells as a proportion of total CD4 cells. The bars depict arithmetic means values with the error bars showing standard deviations. The horizontal line depicts the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. No full-TCD data was available at 6 months for evaluation.

Figure 2.8 CD8 T cell recovery after full-, partial- and non-TCD allogeneic transplantation



(**a**) $CD8^+$ cell recovery. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**b**) $CD45RA^+$ expression on CD8 cells as a proportion of total CD8 cells. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (**c**) $CD45RO^+$ expression on CD8 cells as a proportion of total CD8 cells. The bars depict the arithmetic mean values with the error bars showing standard deviations. The horizontal line shows the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donor. No full-TCD data was available at 6 months for evaluation.

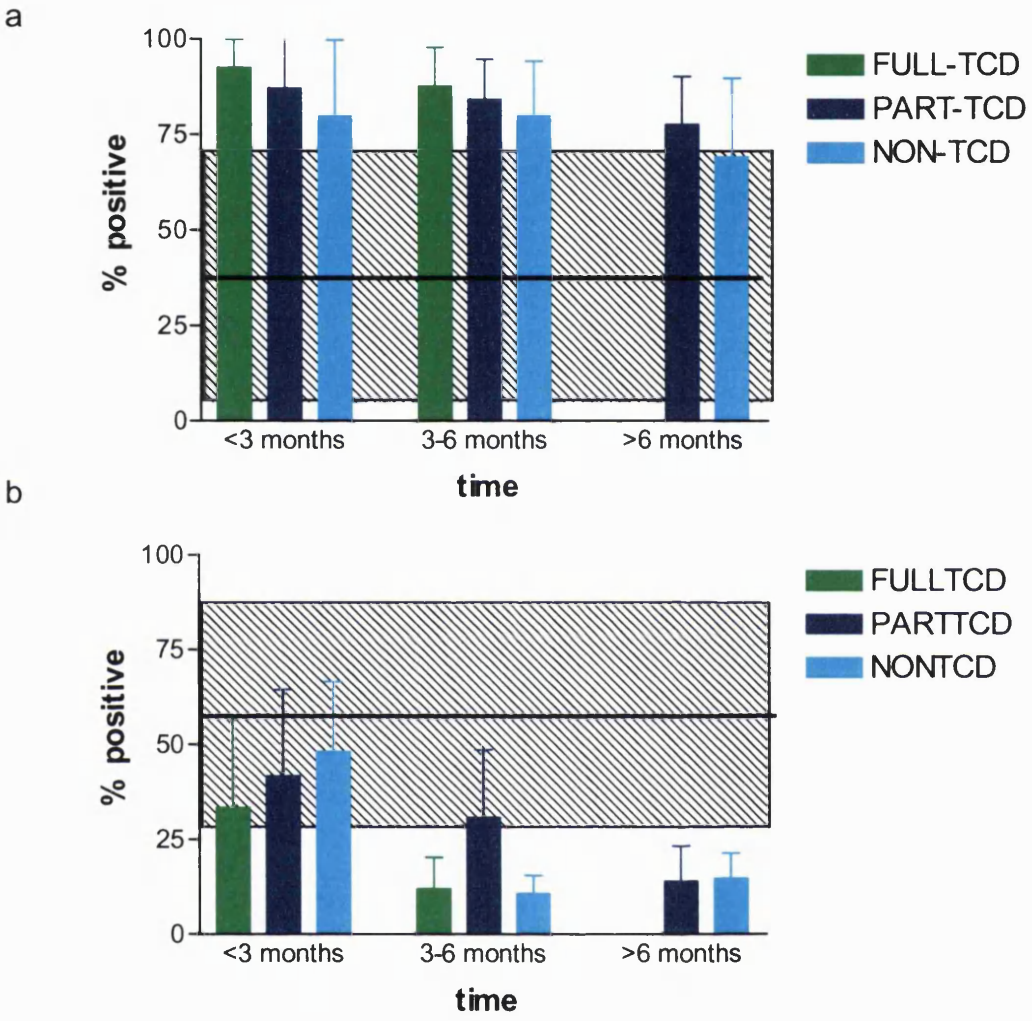
2.3.2.1.2 CD8

CD8 cells were above the normal mean level of 31.6% (s.d. 8.0) by the end of the first 3 months in all patient groups (Figure 2.8a). However, in the 6 months post BMT the percentages of $CD8^+$ cells recovered faster in non-TCD (51.4%; s.d. 17.4) recipients than in their fully (40.6%; s.d. 23.7, p=0.001) and partially TCD counterparts (39.2%; s.d. 14.1, p=0.026). With the $CD45RA^+/CD8^+$ cells (Figure 2.8b), as with the $CD45RA^+/CD4^+$ cells, the partially TCD group (89.4%; s.d. 21.7) exceeded the normal mean level (73.2%; s.d. 11.8) after 6 months, whereas the non-TCD only just reached normal levels (70.4%; s.d. 25.1) though was within the normal range. At the 6 month time point the non-TCD was significantly different from the partially TCD recipients (p=0.01) and after the 6 month time point the difference was p=0.012.

2.3.2.1.3 HLA-DR expression on CD8 cells and CD28 expression on CD8 cells.

The levels of activated $CD8^+$ cells (Figure 2.9a) highlighted by the HLA-DR surface marker were high throughout the study in all three study groups. By 12 months the remaining two groups were still well above normal levels of expression (38.3%; s.d. 16.6) with partially TCD at 77.6% (s.d. 12.6) and non-TCD at 69.1% (s.d. 20.7). The proportions of $CD8^+$ cells co-expressing CD28 (Figure 2.9b) were at the lower end of the normal mean (58.5%, s.d. 15.2) at 3 months. By the 12 month mark partially-TCD recipients had levels of 14.0% (s.d. 9.3) and non-TCD recipients 14.8% (s.d. 6.6) which was well below the normal range.

Figure 2.9 HLA-DR/CD8, and CD28/CD8 after full-, partial- and non-TCD allogeneic transplantation



(a) The graph depicts HLA-DR expression on CD8 cells as a proportion of total CD8 cells following TCD- BMT. The bars depict arithmetic mean values with the error bars showing standard deviation. The horizontal line represents the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors.(b) The graph depicts %$CD28^+$ expression on $CD8^+$ cells following TCD-BMT. The bars depict arithmetic mean values with the error bars showing standard deviation. The horizontal line represents the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. No full-TCD data was available at 6 months for evaluation.

2.3.2.2 Natural killer cell reconstitution

2.3.2.2.1 CD56^{+}

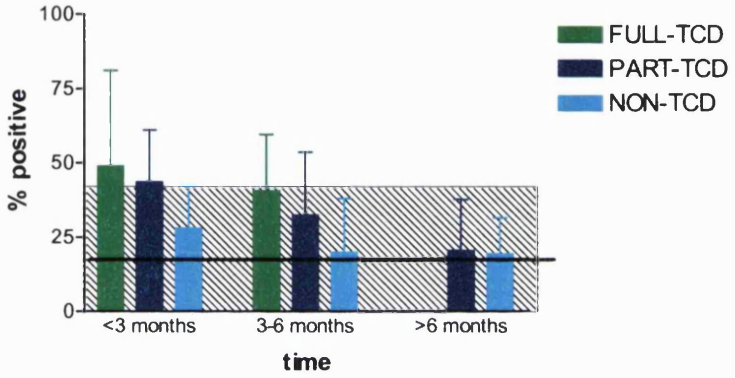
By 3 months NK cell percentages from those patients receiving fully T cell depleted grafts exceeded the normal range. The percentage of CD56^{+} cells (Figure 2.10a) in all three groups reached normal levels (17.5%; s.d. 12.6) at the 12 month time point. As was expected NK cells recovered faster than their T cell counterparts and in the fully T cell depleted group provided a larger share of the lymphocytes than in the non-manipulated group. The difference between the fully-TCD (49.6%; s.d. 30.1) and non-TCD (28.2%; s.d. 13.9) groups was significant at 3 months (p=0.01) and at 6 months (fully-TCD 44.9%; s.d. 22.6, non-TCD 20.1%; s.d. 18.0, p=0.001).

2.3.2.2.2 CD56^{+} cells which were CD8^{+}

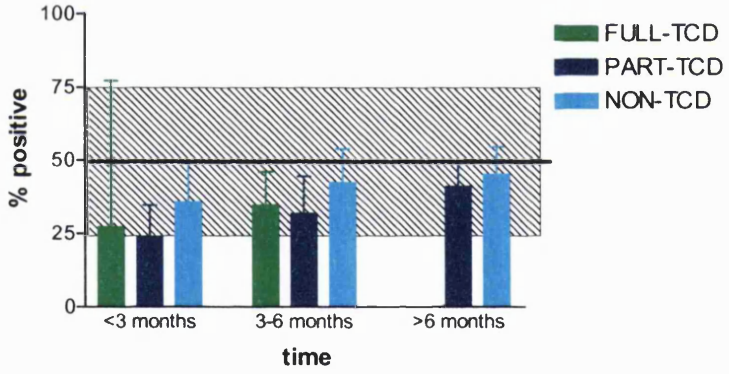
By contrast the percentage of CD56 cells which were CD8^{wk+} was below the normal mean and at the lower end of the normal range but by 12 months was nearing the normal mean level (50.2%; s.d. 13.1)(Figure 2.10b). The trend was the same across all three groups.

Figure 2.10 CD56 recovery after full-, partial- and non-TCD allogeneic transplantation

a



b



(a) The graph depicts $CD56^+$ expression following TCD- BMT. The bars depict arithmetic mean values with the error bars showing standard deviation. The horizontal line represents the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. (b) The graph depicts $CD8^+$ expression on $CD56^+$ cells as a proportion of total $CD56^+$ cells following TCD- BMT. The bars depict arithmetic mean values with the error bars showing standard deviation. The horizontal line represents the normal adult mean from 22 normal donors. The shaded area represents the normal adult range (mean ± 2 s.d.) from 22 normal donors. No full-TCD data was available at 6 months for evaluation.

2.4 Discussion

The data presented here encompass T and NK cell reconstitution following different types of transplant, manipulation of graft and disease and this study therefore presents a comprehensive analysis of cell re-emergence after BMT. Unfortunately none of the patients receiving fully–TCD grafts was available for evaluation after 6 months which restricted the analysis. What must also be taken into account when comparing results of recovery of immune reconstitution from this study with that of earlier TCD studies, and TCD programmes from different groups, is that the method of TCD and the pre-transplant conditioning regimen used will have a bearing on the speed and completeness of recovery.

Some of the procedures are particularly relevant to this study because groups whose work is discussed here used them. For a number of years at this centre the Royal Free T cell depletion protocol was used (TCD by anti-CD6, anti-CD8 monoclonal antibodies and rabbit complement) (Prentice *et al.*, 1984). In other centres TCD by fractionation of donor bone marrow with the lectin soybean agglutinin followed by removal of rosette-forming cells with sheep erythrocytes has been used (Keever *et al.*, 1993). The procedure for the patients in this study used Campath 1M which uses CD52 commonly found on T, B and NK cells (Hale *et al.*, 1998). These different approaches will affect which subsets of cells are removed and will therefore have an overall bearing on immune recovery.

The patient groups were divided by autologous or allogeneic transplant and the level of expression of $CD45RA^{+}/CD4^{+}$ in the autologous group when analysed

was significantly below normal and that of the allogeneic group. In the non-TCD and TCD study the levels of $CD45RA^+/CD4^+$ were at or above normal levels. The CD45RA marker is used to denote immature cells (Akbar *et al.*, 1988), and the partially depleted group had a much higher percentage of these immature CD4 cells than normal. This finding was in contrast to that of Storek and co-workers (1995) who found high percentages of $CD4^+/CD45RA^+$ in neonates but subnormal levels in adults 1 year post-BMT.

The slow recovery of immature CD4 cells is therefore peculiar to autologous transplant recipients and does not occur as a result of T cell manipulation of the graft. Indeed TCD appears to lead to an expansion of $CD45RA^+/CD4^+$. This is possibly due to mature T cells being removed from the graft.

Other investigators have found little or no naïve $CD4^+$ cells post BMT in an adult patient group (Small *et al.*, 1999). They, in common with another group, observed adult recipients of unrelated TCD BMT suffered long and severe deficiencies of $CD3^+$, $CD4^+$ and $CD8^+$ cells compared to their paediatric counterparts (Dumont-Girard *et al.*, 1998). This resulted in a far higher incidence of opportunistic infections, which reduced with an increase in $CD4^+$ cell numbers. There was no significant difference in the T cell repopulation of the paediatric group whether or not their grafts were manipulated.

There are a number of reasons why the results may vary between groups. Storek's results are based on non-TCD transplant recipients and this may be the crucial difference. Our findings in keeping with Roux and colleagues (1996) would suggest that patients who receive manipulated grafts can experience rapid T cell recovery. Also our partially-TCD group at 6 months was

predominantly a young patient profile, undoubtedly these patients have higher thymic activity than older patients do. It seems likely that the thymic activity reduces from eighteen years onwards on a sliding scale and that age is the crucial factor, not the type of transplant performed.

The percentages of mature cells were well above normal in all patient groups and this was true for $CD4^+$ and $CD8^+$ T cells. This expansion is common in patients in whom the T cell population expands with no functioning thymus. In one study it was found that there was little difference between the fully and the non-TCD groups. The cells found very early on even in the fully-TCD groups were mature, and it is possible that these were residual host cells which escaped the pre-conditioning regimen which was less intensive than that used at RFH. The effect of the mature T cells in the graft is weakened by TCD with fewer cell numbers and the recipient T cells can survive (Roux *et al.*, 1996). If this were the case however, it is surprising that they did not reject the incoming graft. It therefore seems more likely that they are donor cells that have undergone rapid expansion.

The finding that supranormal levels of mature $CD4^+$ and $CD8^+$ cells are found post transplant is in agreement with the literature. However in this study double positive $CD45RA^+/CD45RO^+$ $CD4^+$ T cells were found which again is not in accord with Storek and colleagues (1995). It is known though that on activation CD4 cells become $CD45RA^-/RO^+$ but may re-express CD45RA later (Dumont-Girard *et al.*, 1998) and there is a circulating population of $CD4^+CD45RA^+/RO^+$ present in normal donors (Hamann *et al.*, 1996). Memory $CD8^+$ T cells may

lose the expression of CD45RO completely (Hamann *et al.*, 1997) but this has not been seen in $CD4^+$ cells.

In all patient groups $CD8^+$ levels were above the normal mean, and were significantly higher in non-TCD graft recipients as against partially depleted grafts. This was in contrast to the re-emergence of $CD4^+$ cells post-transplant. However, it is known that clonal expansion is possible with $CD8^+$ cells and is not seen in the case of $CD4^+$ cells. Roux and colleagues (1996) suggest one reason for the predominance of $CD8^+$ cells is that most of the antigens encountered post BMT are MHC class I restricted and therefore stimulate CD8 T cell expansion.

Significant differences in percentages of $CD8^+$cells were found between the allogeneic and autologous groups up to 12 months. It is notable that the autologous group had higher than average levels of $CD8^+$ cells, but lower than normal levels of $CD56^+$ cells and that was not seen in the allogeneic group. Perhaps the increase in numbers of $CD8^+$ cells in the autologous setting inhibited the natural killer cell expansion seen in the allogeneic setting.

Percentages of $CD8^+/CD28^+$ cells were observed that at one month were normal but by 12 months were well below the normal mean and below the start of the normal range. Another study has suggested that T cells in older BMT recipients with chronic GvHD lose expression of CD28 (Storek *et al.*, 1995). However in this study it was also found it to be the case in autologous transplant recipients, who do not suffer from GvHD. Also as mentioned above CD28 is not found on T cells from an extra-thymic pathway which may account for the reduced levels in older BMT recipients (Rocha *et al.*, 1995).

The T cell repertoire post BMT must stem either from T cells that survive the conditioning, or from the donor T cells infused. In a TCD transplant this would inevitably lead to quite a restricted T cell repertoire in adult patients with little thymic activity. After BMT conditioning the thymus can become involuted, damaged by radiation, cytotoxic drugs and GvHD, all of which may restrict T cell regeneration (Storek *et al.*, 1995). It is known that resistance to disease declines with age in normal individuals which is perhaps surprising given that the immune system should build up a good repertoire of memory T cells over a lifetime. This is presumably due to the longevity of the memory T cell and the relative lack of thymic activity in later life.

It may be that the thymus is more important for the development of CD4 cells than CD8 cells. This theory is supported by the fact that very few CD4 cells are generated in athymic mice, whereas extrathymic development of CD8 T cells is common (Storek *et al.*, 1995, Rocha *et al.*, 1995). It is possible therefore that there is a way of T cells regenerating but Storek would argue that for $CD4^+$ cells this ability declines with age and is only seen in the young. It is of note that a higher than normal percentage of $CD4^+/CD45RA^+$ cells was observed in the partially depleted group in this study at 6 months but this may be in keeping with the fact that they were comprised of a young patient profile.

Recipients of a manipulated or unmanipulated bone marrow graft do recover their T cell numbers and clinically maintain near normal resistance to infection `from two years post transplant. Much of this immune recovery must come from the donor-derived, mature T cells (Mackall *et al.,* 1996), but particularly for

recipients of TCD-BMT this would lead to a limited repertoire. If not via the thymus then the development of naïve T lymphocytes must emerge from another pathway.

Sites for extra-thymic development, as discussed earlier, include the gut and the liver where the epithelium is of endodermic origin as in the thymus (Rocha *et al.*, 1995). Thymic-independent lymphocytes lack CD5 and CD28 and LFA-1 seen on most thymic-dependent lymphocytes. These cells are activated and express CD69 on the cell surface. *In vitro* they have been shown to express Fas-ligand and are able to kill Fas-bearing targets (Rocha *et al.*, 1995). Much more work is required to understand the capabilities and the role of thymic-independent T cells and whether they can fully compensate for their thymic counterparts.

NK cells are the first line of defence against diseased and tumourigenic host cells. Unlike T cells they have no memory and do not require specific priming but perform a key role in the efficiency of the immune system (van den Broek *et al.*, 1998). NK cells appear to be able to assess the MHC class I status of the cells that they come across and eliminate those which have down-regulated or lost their MHC class I (Ljunggren and Karre, 1990, Vales-Gomez *et al.*, 1998). Inhibition must occur rapidly and yet be transient to allow the NK cells to move on (Colonna *et al.*, 1990). At the same time NK cells produce cytokines that create favourable conditions for the generation of CTLs (Cytotoxic T lymphocytes)(Scott and Trinchieri, 1995).

NK cells are the first of the lymphocytes to recover post-BMT (Hercend *et al.*, 1986, Keever *et al.*, 1993, Lowdell *et al.*, 1998). They may therefore provide a first line defence against infection and disease relapse whilst T cell reconstitution occurs. Although the NK cells are crucial, it is important that T cells recover as fast as possible to provide adequate defence against infection, and this means a method of transplantation which favours the early return of T cells would be extremely beneficial. However the role of the pre-transplant conditioning is to eradicate the host immune system and that includes the peripheral T cell pool which contains the T memory cells central to a robust immune system. If these T memory cells are also eradicated from the donor graft then the recipient is extremely vulnerable to infection and the cost of reduction of GvHD has to be weighed against the paucity of T cells left to replenish the recipient.

T and B cells provide specific responses to a pathogen. For this to happen selective expansion of a high-affinity antigen-specific clone must take place (Tay *et al.*, 1998). Whilst T and B cells are clonally expanding NK cells reduce the virus load by removing those cells which have already undergone manipulation of their MHC class I (Ljunggren and Karre, 1990). This is essential as otherwise a virus could overwhelm the body before the T and B cells became effective (Tay *et al.*, 1998).

Many groups have found that $CD56^+$ cells had recovered to normal or increased cell numbers by 1 month post-transplant (Hercend *et al.*, 1986, Kook *et al.*, 1996, Small *et al.*, 1999). It is known that NK cell function is particularly

resistant to irradiation (Cudkowicz and Hochman, 1979) and it has previously been thought this might be responsible for the rapid return of the NK cells as studied in the allogeneic setting (Rooney *et al.*, 1986). They suggested that the NK cells found shortly after BMT are of both recipient and donor origin.

The poor recovery of $CD56^+$ cells in autologous recipients may be explained by a number of factors. The degree of immunosuppressive conditioning required in allogeneic transplantation leads to an inflammatory cytokine response, not seen in the autologous setting. This results in a rapid increase in cytokine messages, which activate cells and inevitably lead to an increase in $CD56^+$ cell numbers. GvHD seen in allogeneic transplants also leads to cell activation and proliferation and this trigger is lacking in the autologous setting.

The percentages of $CD56^+$ cells recover much faster in recipients of fully-TCD grafts than non-TCD grafts. It is possible that the rapid return compensates for the lack of T cells present in the fully-TCD immune system post-BMT. These data also show that the percentages of cells that are both $CD56^+$ and $CD8^{wk+}$ are quickly back to normal in all patient groups. This is of note because of the subsequent work conducted in this study with the $CD56^+/CD8^{wk+}$ subset (Lowdell *et al.*, 1997).

Rooney and colleagues (1986) found a higher level of activation of NK cells in the BMT patients than the normal donors, possibly this activation was triggered by viral infections such as cytomegalovirus or EBV. Post-transplant lymphoproliferative disease is common in some TCD transplant centres. It

appears to be related to the degree of TCD or of post-transplant immunosuppression for GvHD or rejection.

HLA-DR expression on $CD8^+$ T cells appears to be maintained in the allogeneic group over the 12 months and to diminish in the autologous group. The immune recovery of autologous BMT recipients is faster than that of their allogeneic counterparts and therefore one would expect their levels of CD8 activated cells to return to normal quicker than the allogeneic group. When the allogeneic group is broken down into the manipulated versus the unmanipulated grafts it shows that each group shows a reduction in activation levels over each time point. Although slower than the autologous group it shows a trend towards normal.

3. Anti-leukaemic activity and identification of effectors by CD69

3.1 Introduction

All of the research performed on the separation of GvL from GvHD has been carried out in the allogeneic setting. Attempts to find the key cells in the allogeneic setting have included depleting CD4 cells (Gallardo *et al.*, 1997) and also isolating $CD4^+$ and $CD8^+$ cells that on incubation with high dose IL-2 proliferate, become cytotoxic and secrete cytokines (Coleman *et al.*, 1996). Different groups working in murine models and humans and both the allogeneic and the autologous setting have between them implicated all the major lymphoid cell subsets ($CD4^+$, $CD8^+$ and NK cells) in the GvL response (reviewed in Barrett, 1997).

Traditionally individual lymphoid subsets responsible for specific immune responses have been identified by selective cell depletion or enrichment experiments. However positive selection has the potential to activate the cell and leave antibody on the cell surface which may interfere with subsequent experiments. Negative selection on the other hand does not result in such a pure population and this means one cannot eliminate the role of the other remaining subsets. It also means that the function is studied in isolation thereby

losing any cellular interactions that may play a crucial role in assisting the function of the subset involved. The presence of NK cells has been shown to be important in the generation of a typical T cell mixed lymphocyte response to alloantigens and there will be other similar interactions that are eliminated by the use of cells in isolation (Kos and Engelman, 1995).

The use of molecule which can identify early activation may allow the detection of a reactive cell subset within a mixed cell population without removing the cells from the cellular and cytokine network in which they exist. As early as 1988 researchers became interested in CD69 as an early activation molecule on T and NK cells (Lanier *et al.*, 1988, Ziegler *et al.*, 1994). This molecule is a surface homodimer formed by the association of 28kDa and 32kDa chains which are glycosylated to different extents, held together by disulphide bridges. It is a type II integral membrane protein belonging to the family of C-type lectin receptors (Testi *et al.*, 1994). CD69 has been shown to function as a co-stimulatory molecule in conjunction with either phorbol-12-myristate-13-acetate (PMA), or with anti-CD3 antibody yet its precise biological role is unknown (Ziegler *et al.*, 1994).

It has been shown that CD69 can appear on the cell surface as early as 1- 2 hours after activation in T cells (Yokoyama *et al.*, 1988). Surface expression peaks in activated NK and T cells after 12 hours (Craston *et al.*, 1997) and upregulation of the CD69 antigen on T lymphocytes may be followed by proliferation and an increase in secretion of IL-2 and IFN-γ (Cebrian *et al.*, 1988).

This study uses three-colour flow cytometry to analyse time-dependent expression of CD69 in T and NK cell subsets following a variety of stimuli. Responses to the mitogenic lectin phytohaemaglutinnin (PHA), phorbol myristate acetate (PMA), cytokine stimulation by interleukin-12 (IL-12) and the erythroleukaemic cell line K562 are all assessed. The resulting data give a comprehensive analysis of the temporal dynamics of CD69 (Craston *et al.*, 1997) which have been valuable in the assessment of cell subsets responding to patient derived leukaemia cells.

The history of GvL research has centred on the analysis of recipients of allogeneic transplants as reviewed above. However, if it were possible to identify anti-leukaemic activity in patients following autologous transplant the response could not be due to differences in MHC or mHA and must be leukaemia restricted. In this study we looked for anti-leukaemic activity in patients in complete remission (CR) post autologous and allogeneic BMT and post-chemotherapy.

3.2 Materials and Methods

3.2.1 Cell culture

The erythroblastoid leukaemic cell line, K562, was used in the natural killer (NK) cell assays. This cell line was isolated from a Caucasion female with CML in terminal blast crisis (ECACC No. 89121407- Porton Down) and is highly sensitive to NK cell-mediated lysis in an *in vitro* assay. The vials of K562 were

thawed from frozen into CM (RPMI 1640 supplemented with 10% foetal calf serum, 2.5mM L-glutamine and 100 units/ml penicillin and 10,000 µg/ml streptomycin) and washed. They were then counted by haemocytometer and placed into tissue culture flasks either $25cm^3$ or $80cm^3$ (Nunc) depending on the number of cells thawed, and placed at 37°C/5%CO_2. The optimum suspension for growth was between $1x10^5$/ml and $1x10^6$/ml. The cells were passaged every other day with CM, by 50% volume exchange.

3.2.2 Cytotoxicity assays

Presentation samples collected from patients contained at least 50% leukaemic blasts (median 89%; range 50-99%) and autologous remission bone marrow. In cases where <90% of the cells in the "target" cell sample were leukaemic blasts as determined by flow cytometry, the assessment of killing in the assay was restricted to those cells with blast morphology as determined by forward scatter (FSC) and side angle light scatter (SSC) signals. Mononuclear cells were isolated as described in section 2.2.1. and cryopreserved. After chemotherapy with or without ABMT heparinised blood samples were collected at regular intervals when the patient was in complete remission (CR) and tested immediately or cryopreserved as above for future analysis. Before testing for anti-leukaemia activity all patient samples were tested against the erythroleukaemic cell line K562 to confirm NK cell function.

3.2.3 Labelling of cells

Cell-mediated cytotoxicity was determined by a dye-exclusion assay (Slezak and Horan, 1989, Hatam *et al.*, 1994). Autologous leukaemic and normal bone marrow were labelled with the red dye PKH-26 (Sigma, Poole, Dorset). To label

with PKH26, target cells were thawed rapidly, washed once in CM, to remove the DMSO, and resuspended in Hanks balanced salt solution (HBSS) (without magnesium, calcium and without phenol red) (Life Technologies). This was used because the technique for labelling required the cells to be in a serum free buffer.

The cells were then washed in HBSS and any residual supernatant was removed by pipette. The cells were resuspended in the proprietary PKH labelling buffer (Sigma) and mixed with an equal volume of 4μM PKH-26 for two minutes at 21°C. The labelling reaction was stopped by the addition of an equal volume of neat FCS for one minute and the cells were washed twice in CM by centrifugation at 6000 rpm for thirty seconds in a microfuge.

Despite the fact that the target and effector cell populations have overlapping FSC and SSC light scatter characteristics they could be resolved from each other in a mixed assay system by PKH-26 labelling. This is because the labelled target cells showed high levels of orange fluorescence and could easily be differentiated from non-labelled effector cells by virtue of their FL2 signal (Figure 3.1). The membrane dye was very stable and there was no transfer to non-labelled effector cells during the assay.

Post-transplant peripheral blood mononuclear cells were suspended in CM and were monocyte-depleted on tissue culture plastic for 2 hours at 37°C/5%CO_2. The non-adherent fraction was recovered and incubated in triplicate with labelled targets at a ratio of 10:1 for 4hrs. After the incubation period the cells

were resuspended in a solution of propidium iodide (Sigma) in PBS (1μg/ml) and analysed by flow cytometry (FACScan or FACS Vantage) (Figure 3.2).

Briefly, with compensation circuits turned off, non-labelled effector cells without propidium iodide were used initially to set the FL2 and FL3 photomultiplier tube (PMT) voltages such that the non-labelled cells appeared in the 1st log decade for each parameter on a 2-D dot plot of FL2 versus FL3. Next, PKH-26 labelled target cells without propidium iodide were analysed and the FL3-FL2 compensation circuit increased until the FL2^{+} events were within the 1st log decade of the FL3 parameter. 100μl of the propidium iodide solution was then added to the PKH-labelled target cells and they were re-analysed. This allowed the FL3-FL2 compensation circuit to be micro-adjusted such that the PI^{-}/PKH-26^{+} cells fell in the 1st log decade of the FL3 (Figure 3.2).

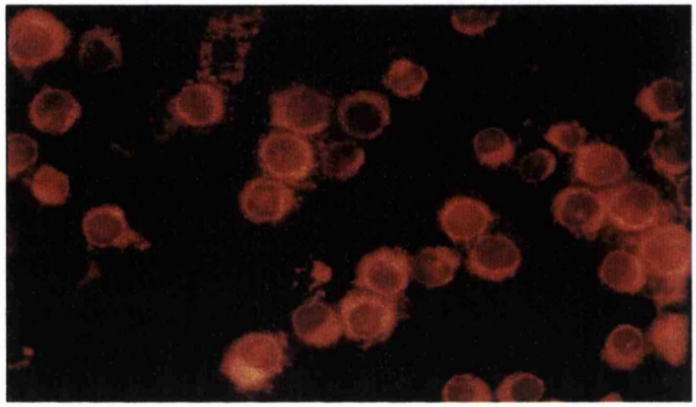
The effector cell sample was then incubated with the PI suspension and analysed to allow the FL2-FL3 compensation circuit to be set such that the dead effector cells (PI^{+} /PKH26^{-}) fell within the 1st log decade of the FL2 parameter. Having set the PMT voltages and the compensation circuits, at least 10,000 target cells were acquired with 1024 channel resolution after electronic gating on FL3, and the proportion of those absorbing propidium iodide was calculated. The mean was determined from the triplicate samples, and background target cell death was taken from cells incubated in the absence of effector cells. Cell-mediated cytotoxicity was reported as percentage killing over background cell death averaged from the three samples:

Mean (% necrotic, including apoptotic in test - % background necrosis)

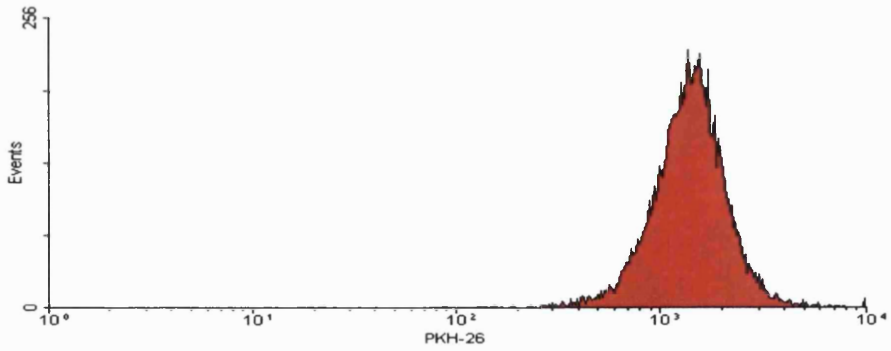
This was calculated for both leukaemic and normal autologous target cells which were used as negative controls to confirm leukaemia-specificity. Leukaemia-specific killing was determined by subtracting the percent cytolysis of remission normal BMMC from the percent lysis of leukaemic blasts.

Figure 3.1 Labelling of targets by PKH26 membrane dye.

A

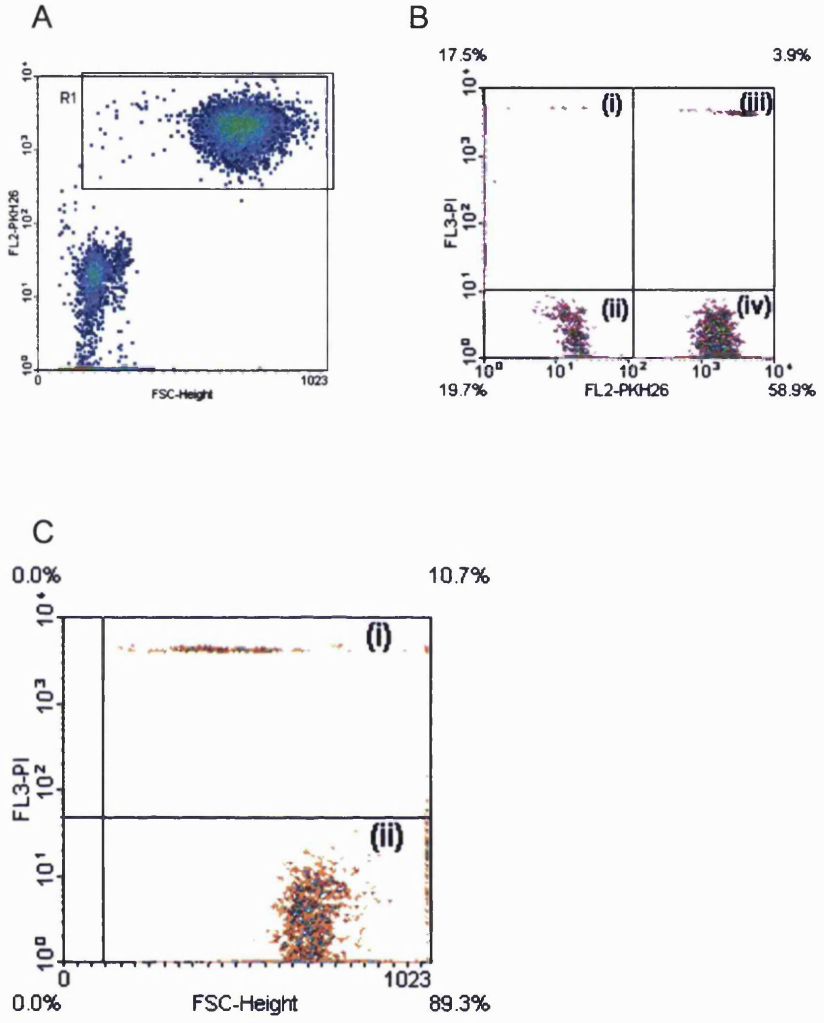


B



A – Photomicrograph of PKH-labelled K562 cells.
B – Flow cytometric histogram of the PKH-26 labelled cells shown in "A" above.

Figure 3.2 Example of data analysis of cytotoxicity experiments



Plot A shows a typical mixture of PKH-26 labelled K562 target cells (region 1) and unlabelled effector NK cells. The concomitant use of FSC and PKH-26 allows easy resolution of the two cell populations.

Plot B presents the ungated data and shows how the PKH-26$^+$ target cells are easily resolved from the unlabelled effector cells. "Dead" effector NK cells are in quadrant (i), "live" effector cells in (ii). "Dead" target cells are in quadrant (iii) and "live" target cells in quadrant (iv). Despite the close emission spectra of PKH-26 and PI it is possible to resolve the overlap although the cells in quadrant (i) are largely confined to the first channel.

Gating on region 1 as shown in panel C, allows analysis of the degree of target cell lysis with PI$^+$ "dead" cells in quadrant (i) and PI$^-$ "live" cells in quadrant (ii). In this example, 10.7% of the target cells are dead.

3.2.4 CD69 stimulation

As the time-dependent expression of the CD69 molecule on lymphoid cells had not been studied in depth initial work was required to provide a background of data. A number of experiments were performed with different types of stimuli to establish a base line response from which to analyse subsequent experiments with leukaemic cells. Three different methods to stimulate cells were used: mitogen, cytokine and NK-sensitive target cells and their response was assessed by CD69 upregulation.

3.2.4.1 Mitogen stimulation by Phytohaemaglutinnin (PHA)

2x 10^6 cells were placed in polystyrene tubes (Falcon - Becton Dickinson) and incubated with and without PHA-P in HBSS (2µg/10^6 cells)(Sigma, Poole, Dorset). Cells were analysed at 1, 2, 3, and 24 hours and phenotyped for CD69, CD56, CD3 and CD8 expression using fluorochrome conjugated Mabs. Three different combinations were used as listed in Table 3.1.

Table 3.1 Monoclonal antibodies used to label cells

COMBINATIONS	FITC	PE	PerCP
1	CD69	CD56	CD3
2	CD69	CD4	CD8
3	CD69	CD56	CD8
4	CD69	CD28	CD8

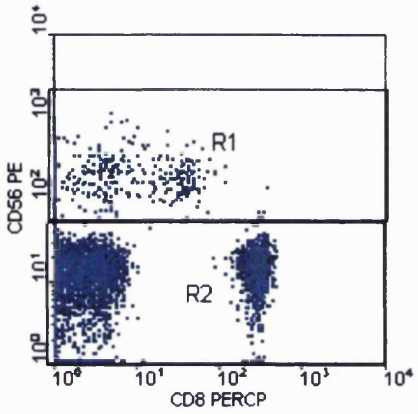
Cells were incubated with Mabs at 21°C for 15 minutes and washed twice with PBS before analysis by flow cytometry (FACScan with Lysis II software – Becton Dickinson, Oxford, UK.). 10,000 events were collected as list mode

data in Lysis II after live gating of lymphocytes and lymphoblasts by forward scatter (FSC) and side angle light scatter (SSC) signals on linear amplification.

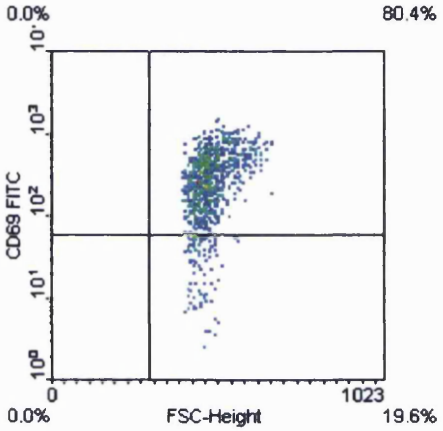
Using logarithmic amplification the natural killer cells were electronically gated on the basis of CD56 PE expression (Figure 3.3a). Region 1 was drawn up around the $CD56^{+ve}$ cells and region 2 around the $CD56^{-ve}$ cells. Each region was then analysed on a separate dot plot by linear amplification which displayed CD69 expression by placing CD69 on the y axis and FSC on the x axis (Figure 3.3b and c). Quadrant statistics were then used to determine the relative proportions of $CD8^+/CD69^-$, $CD8^+/CD69^+$, $CD8^-/CD69^-$ and $CD8^-/CD69^+$ subpopulations of NK cells. $CD8^+$ T cells were analysed on the same basis after electronic gating. The $CD8wk^+$ cells were assumed to be NK cells (because no cell population was found during the study that was $CD3^+$ and $CD8wk^+/CD56^+$).

Figure 3.3 Examples of dot-plots to show the gating of the CD69 experiments

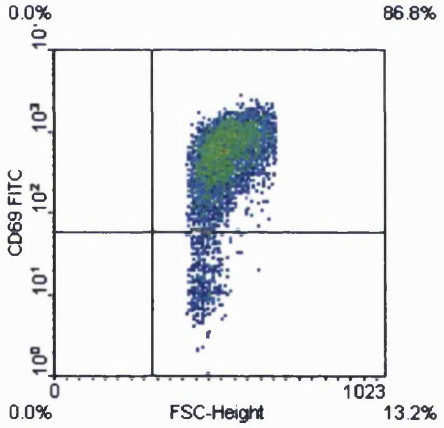
A



B



C



Dot plot A, region 1 was drawn up around the $CD56^{+}/CD8^{wk+}$ cells. Region 2 was drawn up around the $CD56^{-}$ cells and the $CD8^{+}$ cells.
Dot plot B shows the events from region 1 displayed by CD69 expression on the y axis and FSC on the x axis. In this example 80.4% of events (ie $CD56^{+}/CD8^{wk+}$) are $CD69^{+}$.
Dot plot C shows the events from region 2 displayed by CD69 expression on the y axis and FSC on the x axis. In this example 86.8% of events (ie $CD56^{-}/CD8^{+}$) are $CD69^{+}$.

3.2.5 Cytokine stimulation by Interleukin-12 (IL-12)

$1x10^6$ PBMCs were suspended in serum free medium (X-Vivo 10, Biowhittaker, Walkersville, Maryland) supplemented with 100 units/ml penicillin and streptomycin. They were placed in 24 well plates (Nunclon, Nunc, Denmark) and incubated with and without IL-12 (Roche) at a final concentration of 25ng/ml. Serum free medium was used to ensure any activation was as a result of the cytokine and not a component of the medium. The cells were analysed at 0, 24, 48 and 72 hours to assess IL-12-induced activation. The cells were labelled (as in 3.2.4.1.) with CD69 FITC, CD56PE and CD3PerCP (Becton Dickinson, Oxford, UK). 10,000 events were collected as list mode data in Lysis II after live gating of lymphocytes and lymphoblasts on linear amplification by forward scatter (FSC) and side angle light scatter (SSC) signals.

A dot plot was drawn up encompassing the lymphocytes and lymphoblasts on the basis of FSC and SSC on linear amplification as described above. On a subsequent graph PE expression was arrayed on the x axis and PerCp on the y axis on logarithmic amplification using the events gated on the previous graph. The cells expressing CD56 PE were electronically gated and so were the cells expressing CD3 PerCp (excluding any cells which were jointly $CD3^+$ and $CD56^+$ ($CD3^+/CD56^+$)(for example see Fig 3.3). These populations were further analysed by dot plots showing CD69 expression on the y axis against forward scatter light signals on the x axis. Quadrant statistics were used to determine the proportions of true NK and T cells that expressed CD69.

3.2.6 K562 stimulation of NK cells

Exponentially growing K562 cells were recovered and washed once in CM. The cell density of the K562 was adjusted to 10^6/ml in CM and PBMC were added in 1:1 ratio. Parallel cultures were set up of PBMC alone. Aliquots were labelled with fluorochrome conjugated Mabs CD69FITC/CD56PE/CD8PerCP at 1,2,3 and 4 hours. 20,000 events were acquired and stored as list mode data (Lysis II, Becton Dickinson).

K562 stimulator cells were excluded from the NK cell analysis by electronic gating. The K562 cells are of a different morphology to the lymphoid cells and can therefore be easily distinguished. NK cells were selected on the basis of CD56 expression from within the lymphoid gate. The $CD56^+$ cells were then arrayed on a two-dimensional dot plot on the basis of CD69 and CD8 expression. The relative proportions and absolute numbers of cells in each quadrant were recorded (for example see Fig 3.3).

3.2.7 Anti-leukaemic activity experiments

Three patient groups were tested in the anti-leukaemic cytotoxicity experiments. Recipients of allogeneic transplants, recipients of autologous transplants and those who had received chemotherapy were all tested by the assay. Recipients of allogeneic and autologous grafts received pre-transplant conditioning with cyclophosphamide (60mg/kg body weight per day for two days), and total body irradiation (750 Gy, fast rate 15 cGy/min, single fraction). Initially presentation leukaemic blasts were collected and then samples were collected at regular intervals post-BMT and either cryopreserved for future use or tested

immediately. Either bone marrow or peripheral blood was used in these assays but peripheral blood was favoured if both types of samples were available. A typical presentation sample would contain leukaemic blasts and normal cells in the peripheral blood, and therefore it was possible by gating with forward scatter and side angle light scatter on the flow cytometer to distinguish the blasts cells from the normal cells. The leukaemic samples were phenotyped to confirm large numbers of malignant cells. In this series of experiments the anti-leukaemic effect of either bone marrow or peripheral blood from patients in remission was always tested against their presentation samples.

Mononuclear cells were separated by density gradient separation (for method see section 2.2.1). The cytotoxicity experiments were carried out as detailed in 3.2.2 (Figure 3.2). The incubation and analysis of cells is detailed in 3.2.2. Cell mediated toxicity was calculated as in 3.2.2 for both leukaemic and normal autologous target cells which were used as negative controls to confirm leukaemic-specificity. Leukaemia-specific killing was determined as follows:

Leukaemia specific killing = % lysis of leukaemic blasts - % lysis of remission BMMC

The phenotype of the effector cell population was determined by expression of the CD69 molecule. Target cells were labelled with PKH-26 (see 3.2.3)(Figure 3.1) and incubated with unlabelled effector cells in CM at a ratio of 1:1. Positive control assays were set up in which PHA (200ng/ml) was added to the effector cells in the absence of target cells. The control was used to establish that the effector cells were capable of responding despite having been frozen. After 3-4 hours incubation at 37°C/5%CO_2 the samples were labelled with a combination

of CD69 FITC, CD56 PE and CD4 or CD8 PerCP (Becton Dickinson, Oxford, UK.).

Other control samples used were effector cells incubated with remission bone marrow mononuclear cells (BMMC). The level of CD69 expression after incubation with remission BMMC did not in any experiment exceed that of the effector cells incubated alone. All the samples were set up in triplicate. The cells were analysed by flow cytometry and electronic gating was used to exclude the target cells that had been labelled with PKH-26. PKH-26 fluorescence was detected by FL2 on the FACScan along with the signal from $CD56^+$ PE cells, but was extremely bright by comparison and this allowed the two populations to be separated on the screen. The mean proportion of CD69 positive cells in each cell subset was recorded.

3.2.8 Measurement of autologous cytotoxicity to presentation leukaemic blasts

Having established the active subset of NK cells by the use of CD69 Mab, highly purified NK cell subsets were isolated for functional testing against presentation leukaemic blasts and remission BMMC. This was done by MACS sorting on CD56 expression, followed by FACSVantage sorting for CD8 expression, to purities of >98%. The leukaemic samples were incubated in the 4 hour cytotoxicity experiment with three different targets: unfractionated NK cells, $CD56^+/CD8wk^+$ and $CD56^+/CD8^-$ subsets.

Preliminary experiments were conducted on allogeneic BMT recipients and three autologous BMT recipients, and once they had been completed more patients who had either received autologous BMT or chemotherapy alone were monitored for anti-leukaemic activity. The patients' details are listed in Table 3.2 and Table 3.3. These patients were all tested by the flow cytometric cytotoxicity assay for specific anti-leukaemic activity as detailed in section 3.2.2.

Table 3.2 Patient characteristics: autologous BMT or chemotherapy alone

Patient ID	Age at treatment	Diagnosis	Treatment	Current status
GG	40	AML	Baltimore x2	CR
RY	72	AML	Ara C & Mitoxantrone x3	CR
SS	47	AML-M1	Bu/Cy aBMT	R/D
SF	27	AML-M2	Baltimore x2; FLAG/Ida x2	CR
RH	50	AML-M4 Eo	Bu/Cy aBMT	CR
AGD	69	AML M4	Ara C & Mitoxantrone x2	CR
LA	35	AML-M4	Bu/Cy aBMT	CR
RR	72	AML M4 PH+	Baltimore x2	R/D
JW	18	AML-M5	Cy/TBI aBMT	CR
VH	72	AML/MDS	Ara C & Mitoxantrone x3	R/D
MAd	27	T-ALL	Bu/Cy aBMT	R/D
MAb	40	T-ALL	aBMT	R/D
SH	8	Pre-B-ALL	UKALL; FLAG/Ida x2	R/D
EP	56	B-ALL	MEGA III x3	R/D

The different treatment regimens refer to the conditioning the patients received:

Baltimore= modified timed sequential therapy (Vaughan *et al.*, 1980)

Ara-C=Cytosine arabinoside

Bu/Cy=Busulphan/ Cytarabine

FLAG/Ida=Fludarabine, Ara-C, G-CSF, Idarubicin (Deane *et al.*, 1998)

Cy/TBI=cyclophosophomide/total body irradiation

UKALL=MRC acute lymphoblastic leukaemia trials protocol

MEGA III=cyclophosphomide, Vincristine, Bleomycin, methotrexate, prednisilone

Current status refers to the condition of the patient at the end of the study:

CR =complete remission

R/D= relapsed/deceased

AML=acute myeloid leukaemia

ALL=acute lymphoblastic leukaemia

ABMT=autologous bone marrow transplant

Table 3.3 Patient characteristics: allogeneic BMT patients

Patient ID	Age at treatment	Diagnosis	Treatment	Current status
GC	15	AML-M4	TCD-BMT	R/D
JL	47	AML-M4	TCD-BMT	CR
AB	37	AML-M4	BMT	CR
MA	40	T-ALL	TCD-BMT	R/D
JC	30	CGL	TCD-BMT	TRM/D

TCD-BMT= T cell depleted-bone marrow transplant refers to the conditioning regimen.

Current status refers to the condition of the patient at the end of the study:

R/D= relapsed/deceased

CR= complete remission

TRM/D= transplant related mortality/deceased.

3.2.9 Anti-leukaemic activity with interferon α

Having relapsed at 9 months post-BMT, patient RH was treated with α-interferon therapy (1 & 2 megaUnits alternate days, sc.) (Schering-Plough). A further patient (SF – AML M2) was studied post-chemotherapy and α-interferon treatment. This 27-year-old woman presented at this hospital for consolidation chemotherapy and subsequently a second course of chemotherapy consisting of Cytarabine (1.33g/m^2/day; days 1-3); Daunorubicin (45mg/ m^2/day; days 1-3) and Etoposide (400mg/ m^2/day; days 8-10). The following year the patient relapsed and received Fludarabine (30mg/ m^2/day; days 1-5); Ara-C (2g/ m^2/day; days 1-5); Idarubicin (5mg/ m^2/day; days 1-5) and G-CSF (600μg/day, days 1-5).

This patient relapsed from 2nd CR, refused both autologous and allogeneic stem cell transplantation, and three months later commenced α-interferon (α-IFN) treatment (Schering-Plough). The patient is maintained on an α-IFN dose of 5 megaunits 5 times per week.

Both these patients were tested post α-interferon therapy for specific anti-leukaemic activity using the cytotoxicity assay.

3.3 Results

3.3.1 CD69 expression

3.3.1.1 PHA stimulation

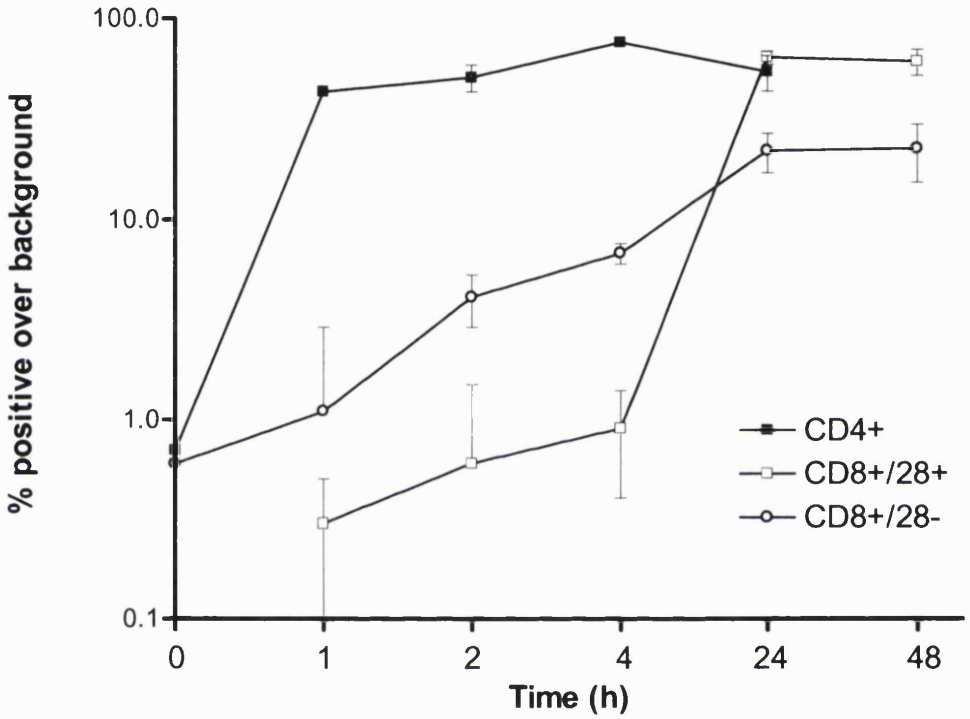
In 4/4 experiments PHA stimulation resulted in an increase in CD69 expression on $CD4^+$ cells, over the non-stimulated controls, within 1 hour (Figure 3.4). To achieve the same level of expression $CD8^+$ cells required 24 hours of stimulation. Both $CD8^+/CD28^+$ and $CD8^+/CD28^-$ cells expressed CD69 on mitogen stimulation.

$CD56^+$ cells showed a rapid upregulation of CD69 expression in response to the mitogen, but there was no difference in response between the $CD8^-$ and $CD8wk^+$ subsets (Figure 3.5). Although the total percentage of $CD69^+/CD56^+$ cells did not change at 24 hours there was a fall in the proportion of $CD8wk^+$ cells expressing CD69 and an increase in the numbers of $CD8^-$ cells expressing CD69. This could be due to shedding of the CD8 α-chain upon activation.

3.3.1.2 IL-12

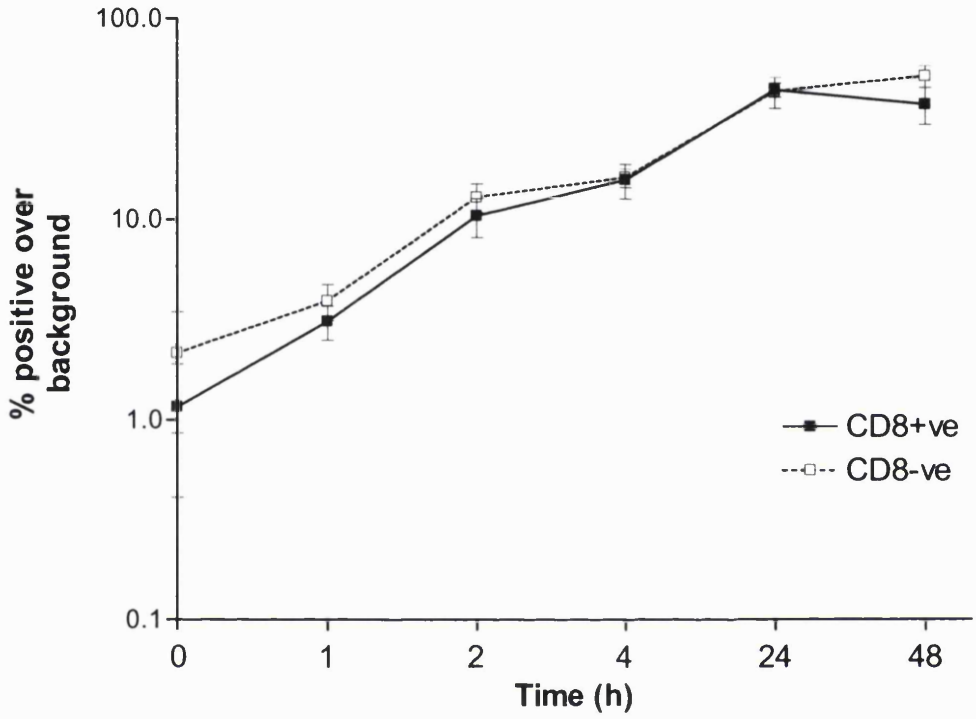
IL-12 produced a marked increase in CD69 expression on $CD56^+$ cells at 24 hours after incubation. This response peaked at 48 hours. The T cells did not respond to cytokine stimulation over 48 hours (Figure 3.6).

Figure 3.4 Temporal expression of CD69 on $CD4^+$ and $CD8^+$ lymphocytes after PHA stimulation.



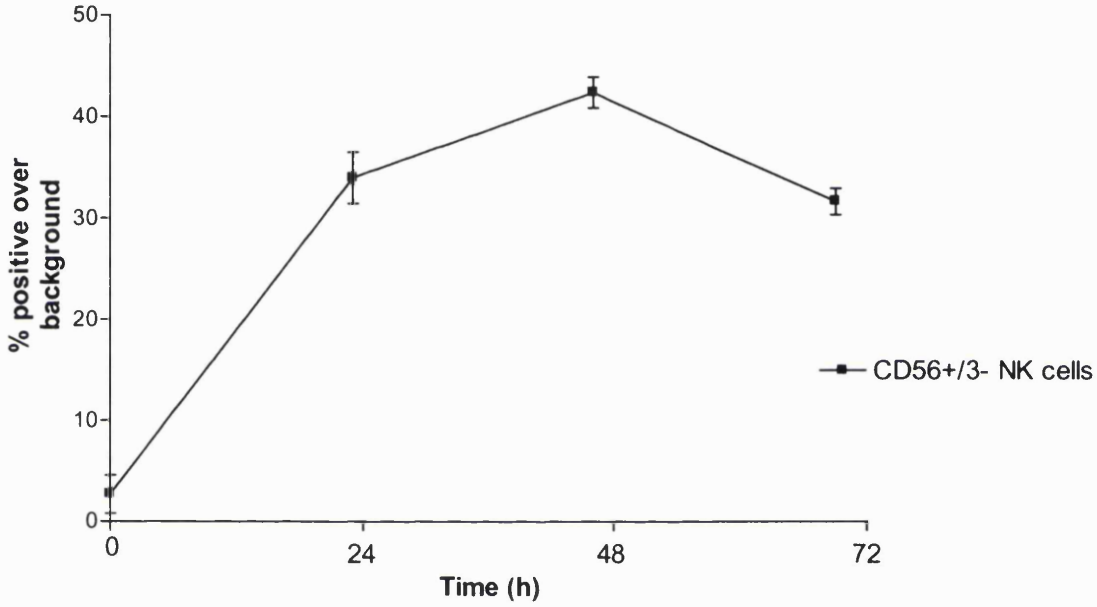
Taken from four experiments. Data are presented as means (± 2 SD). Where no error bars appear this is due to the similarity of results at a given time point.

Figure 3.5 Temporal expression of CD69 on CD56$^+$ lymphocytes after PHA stimulation.



The results are taken from four experiments and show the increased expression of CD69 over time on CD56$^+$/CD8^{wk+}/CD3$^-$ cells and CD56$^+$/CD8$^-$ after co-incubation with PHA. Data are presented as means (± 2 SD).

Figure 3.6 Temporal expression of CD69 on NK cells in response to IL-12.

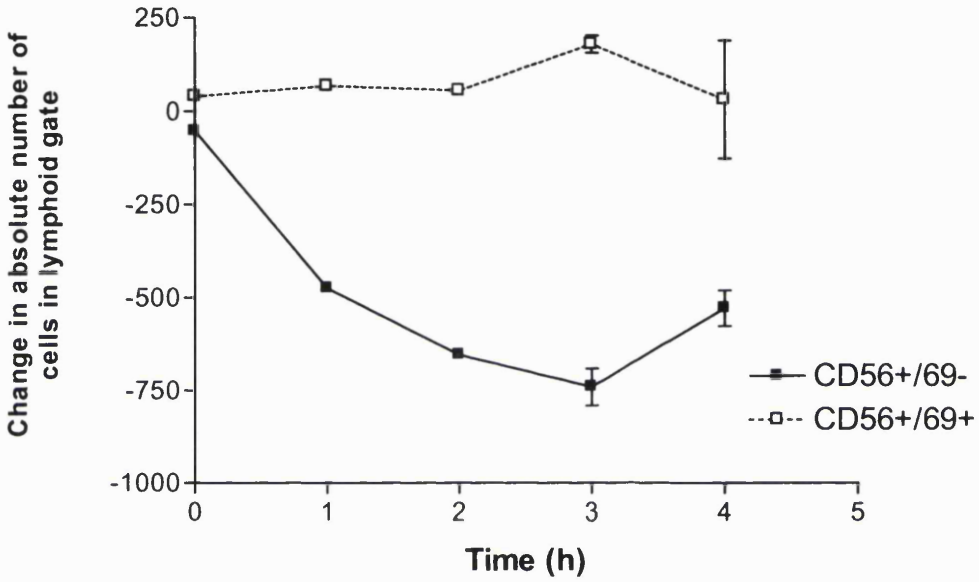


Data are presented as means (± 2 SD). Three experiments were performed in triplicate.

3.3.1.3 K562 stimulation

CD69 upregulation was more difficult to monitor in those cells stimulated with K562. After 2 hours incubation with K562 there was an increase in the $CD69^+$ NK cell fraction, but the total NK cell population within the lymphoid gate decreased. The reduction was consistently within the $CD56^+/CD69^-$ population leading to an indirect increase in the $CD56^+/CD69^+$ population. There was no apparent increase in the $CD56^+/CD69^+$ cell population within 4 hours (Figure 3.7).

Figure 3.7 NK cell subset response to K562.



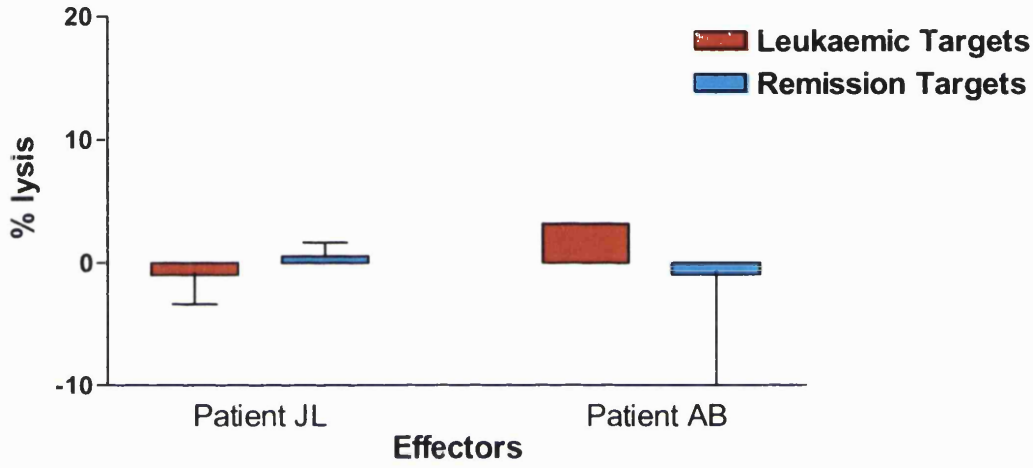
Data are presented as means (± 2 SD). Ten experiments were performed in triplicate. The results were so consistent at one and two hours that no error bars appear on the graph.

3.3.2 Specific anti-leukaemia killing

3.3.2.1 Allogeneic BMT patients

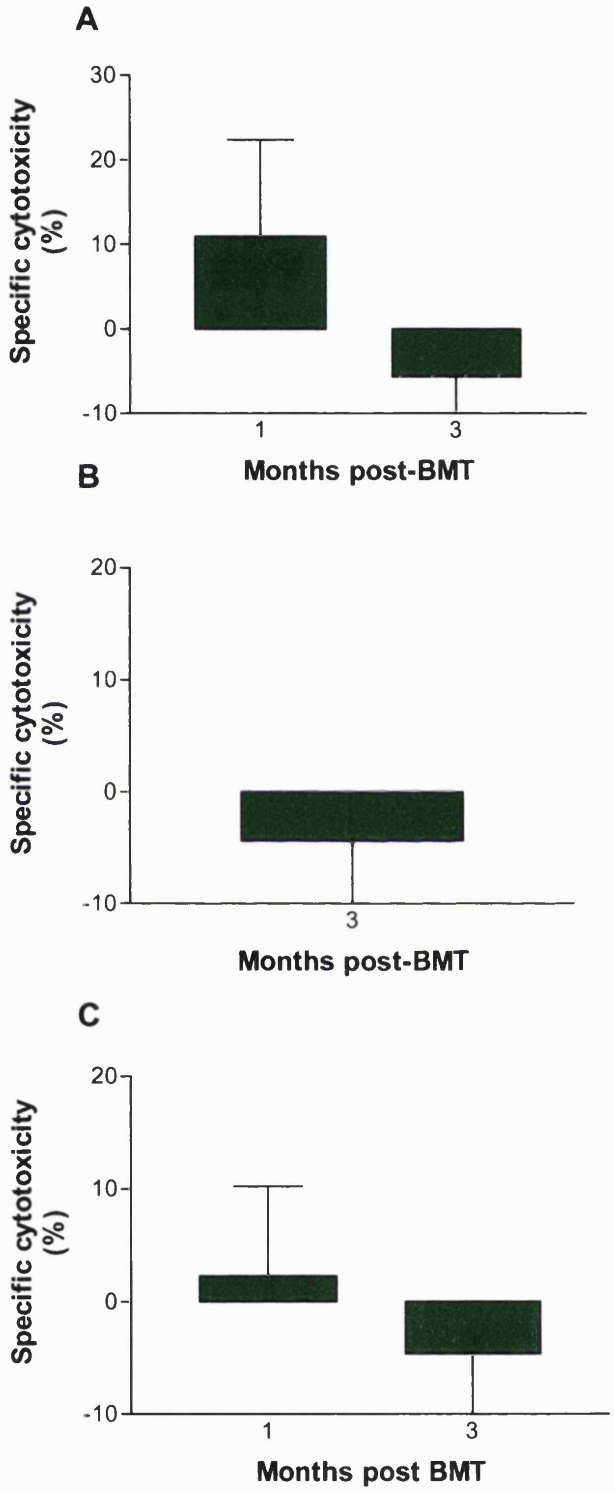
Preliminary experiments were performed with samples from allogeneic and autologous BMT recipients. The responses in the allogeneic BMT patients were indistinguishable between the remission BMMC and the leukaemic blasts. There appeared to be an overall GvHD response which made it impossible to dissect out a GvL response (Figure 3.8 – 3.10). It became apparent that there was a response in some of the autologous BMT patients against leukaemic blasts, but no response against their remission BMMC. Further experiments were therefore conducted on the autologous BMT patients, and patients who had received chemotherapy but no transplant.

Figure 3.8 Response to remission and leukaemic cells in allogeneic transplant patients



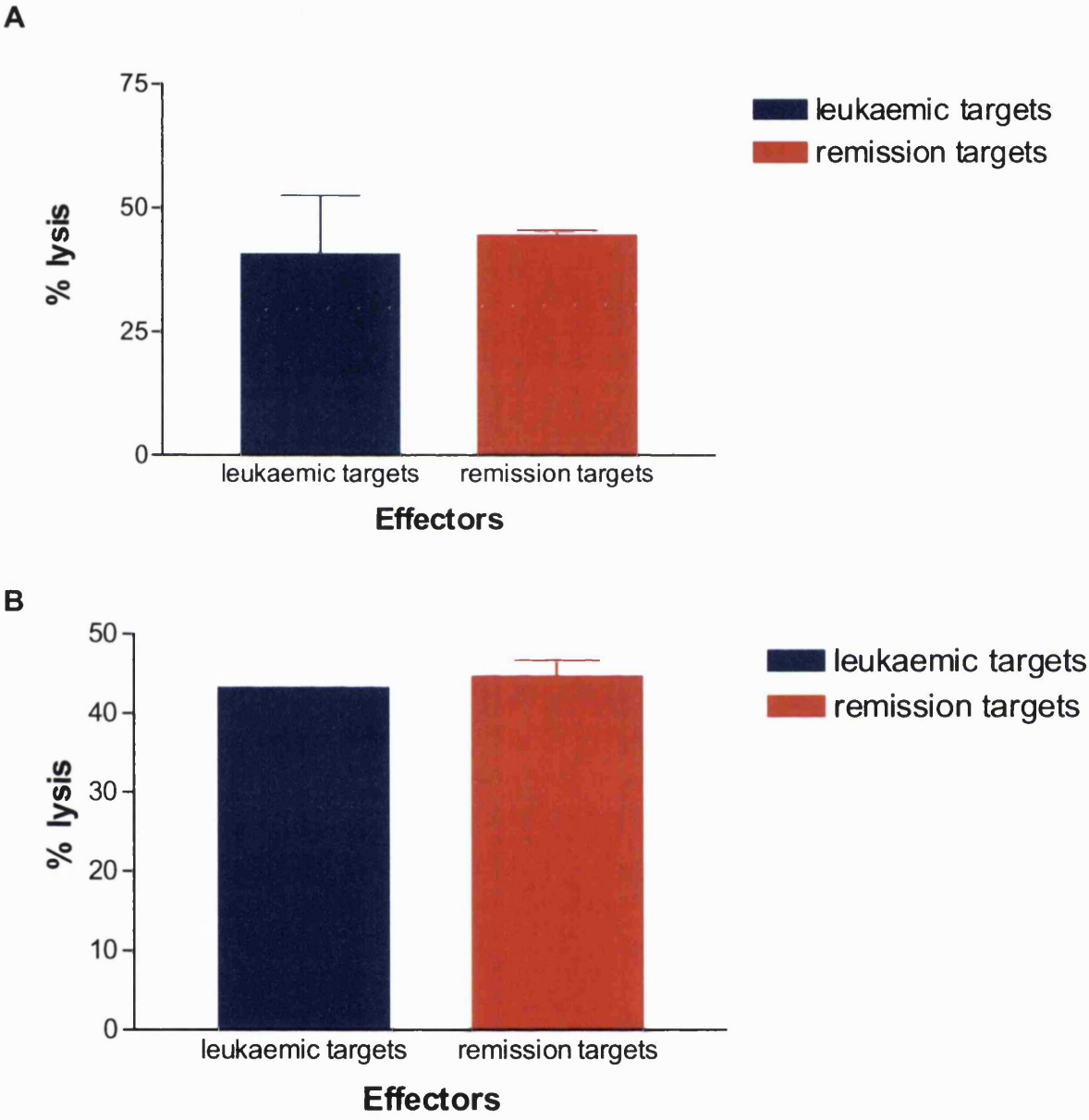
Two long term survivors of an allogeneic BMT, the samples were tested for cytolytic activity four years post-BMT. The data are presented as means and standard deviations. Three experiments were performed in the case of JL, but only one in the case of AB as there was limited leukaemic target material available.

Figure 3.9 Anti-leukaemic activity in three allogeneic BMT recipients



The experiments were conducted in those cases where there was sufficient material. Figure A depicts patient AB, B depicts patient JC (sample for the study unavailable at 1 month) and C depicts patient GC – all three patients were tested within 3 months of BMT and relapsed within 6 months. Specific cytotoxicity=%lysis of leukaemic blasts – lysis of remission BMMC.

Figure 3.10 Anti-leukaemic activity in allogeneic patients

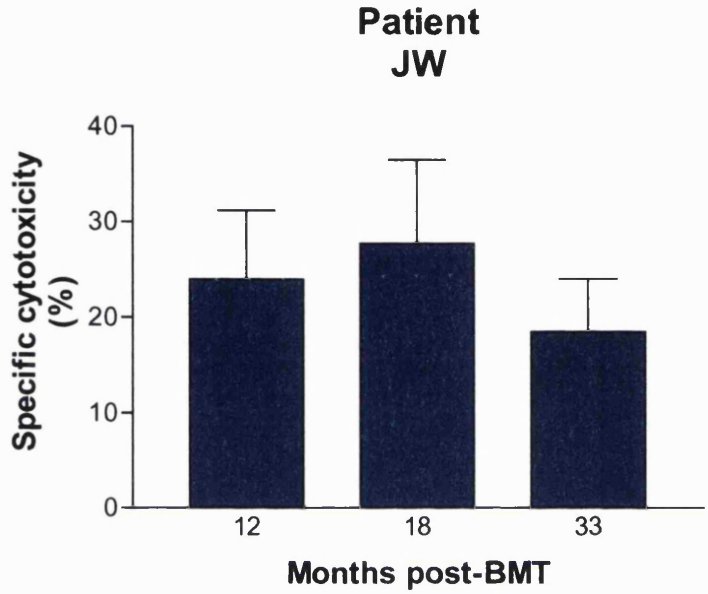


This graph depicts cytolytic activity post allogeneic BMT, in two patients. They are presented in a separate graph to those in Fig 3.8 because they were only tested once at different time points. In figure A patient GC was tested at one month post-BMT and in figure B patient JL was tested at 4 years post-BMT (there is no error bar for leukaemic targets because the results were so consistent). The data are presented as means and standard deviations.

3.3.2.2 Autologous BMT patients

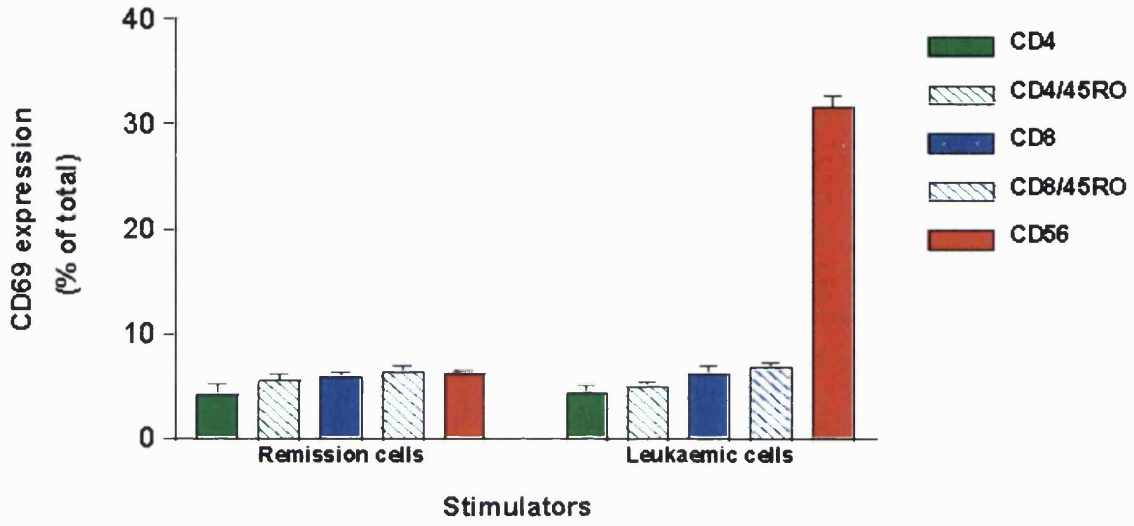
The first patient studied was an 18 year old woman (JW) in CR after autologous BMT for AML (M5). The first sample was taken 12 months post-BMT, and at this time analysis revealed 24% specific anti-leukaemic activity. Testing at 18 and 33 months post-BMT also showed anti-leukaemic cytotoxicity with no lysis of remission BMMC (Figure 3.11). When the cellular responders were analysed by CD69 expression they were found to be $CD56^+$ (Figure 3.12). No increase in activation was found in CD4 or CD8 T cell subsets, also there was no activation of any cell subset in response to the remission autologous bone marrow, suggesting that the response was specifically anti-leukaemic.

Figure 3.11 Anti-leukaemic activity of patient JW



The graph shows specific lysis of patient JW at designated time points post-transplant. The error bars represent standard deviation.

Figure 3.12 JW cellular responders shown by CD69 expression

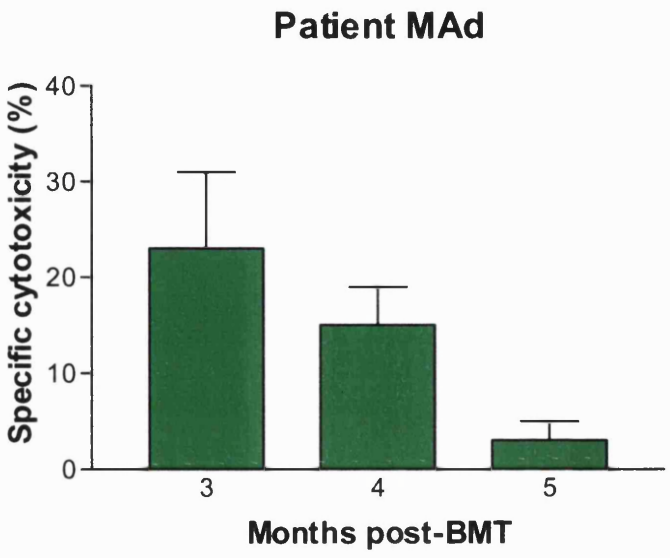


The graph shows CD69 expression (shown on the y axis) by PBL subsets at 12 months post-autologous BMT following stimulation with pre-transplant remission and autologous leukaemic BMMC. The data are represented by means and standard deviations.

The second subject in the study was a 27 year-old male with T-ALL who had also received an autologous transplant (MAd). Anti-leukaemic cytotoxic activity (specific lysis - 23%) was detected at 3 months post-autologous BMT; there was no lysis of autologous remission BMMC. Also no activity was found against two allogeneic leukaemic samples from other ALL patients. At 4 months post-BMT anti-leukaemic activity was detectable but much reduced (specific lysis – 15%). By 5 months it was undetectable but the patient remained in complete remission (Figure 3.13). By week 22 the patient had relapsed. The patient received an allogeneic BMT from an unrelated donor, and died within 12 months.

Figure 3.13 Anti-leukaemic activity exhibited by patient MAd.

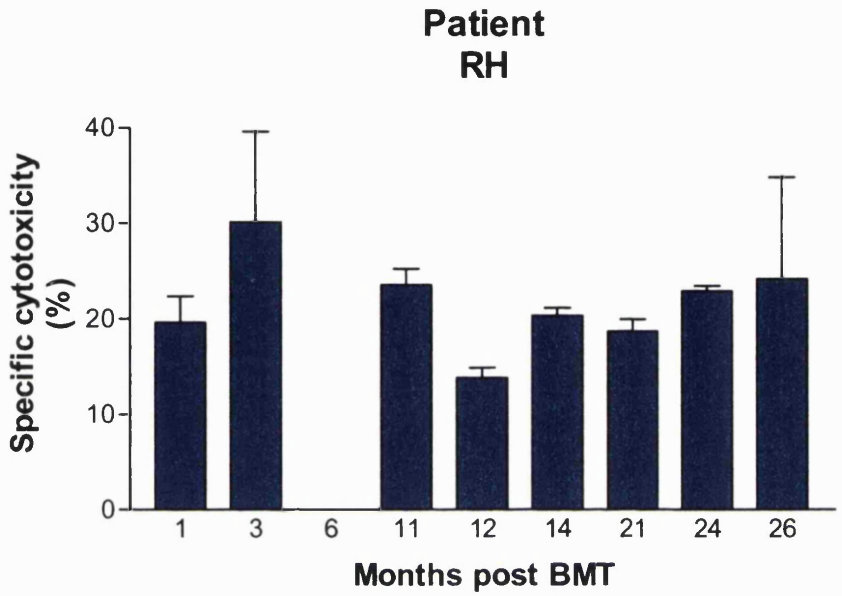
The graph depicts specific cytotoxicity of MAd at specific time points post-transplant. The error bars represent standard deviation.



The third autologous transplant recipient studied was a 50 year-old woman in 2nd CR with AML M4 Eo. Samples from this patient (RH) were received at one and three months post-BMT and they showed high levels of cytolytic activity against the leukaemic BMMC and some against her autologous remission BMMC (Figure 3.14). At six months no cytolytic activity was found against any targets (K562 included) and at this point there were no detectable numbers of NK cells in her peripheral blood. One week later the patient relapsed in her bone marrow. The patient received re-induction chemotherapy (FLAG/Idarubicin – Fludarabine 30mg/m^2 x5; Ara C 29mg/m^2 x5; Idarubicin 8mg/m^2 x3) and once in CR a further course of FLAG/Idarubicin was given as consolidation.

Three months after this relapse the patient was treated with α-interferon therapy (1 & 2 megaUnits alternate days, sc.) (Schering-Plough). This resulted in a recovery of her NK cell function. One month after the start of this treatment the patient recovered her leukaemia-specific cytolytic activity which has been maintained subsequently. The cells taken in second CR showed no non-specific lysis against remission BMMC stored prior to autologous BMT. This patient has now remained in complete molecular remission for more than 4 years.

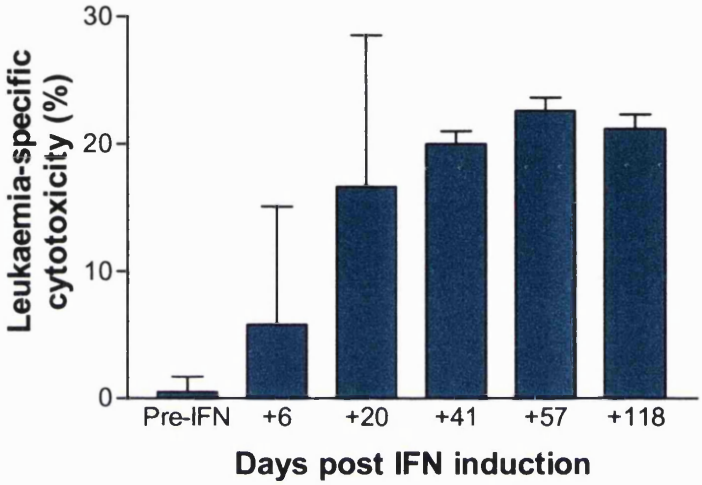
Figure 3.14 Anti-leukaemic activity of patient RH post-transplant



The graph depicts specific cytotoxicity of patient RH post-transplant. The effector cells were from PBMC taken from the patient at the specific time points shown. This patient commenced α-interferon therapy 9 months post-BMT. The targets were taken from samples of presentation leukaemic BMMC and autologous remission BMMC. The error bars represent standard deviation.

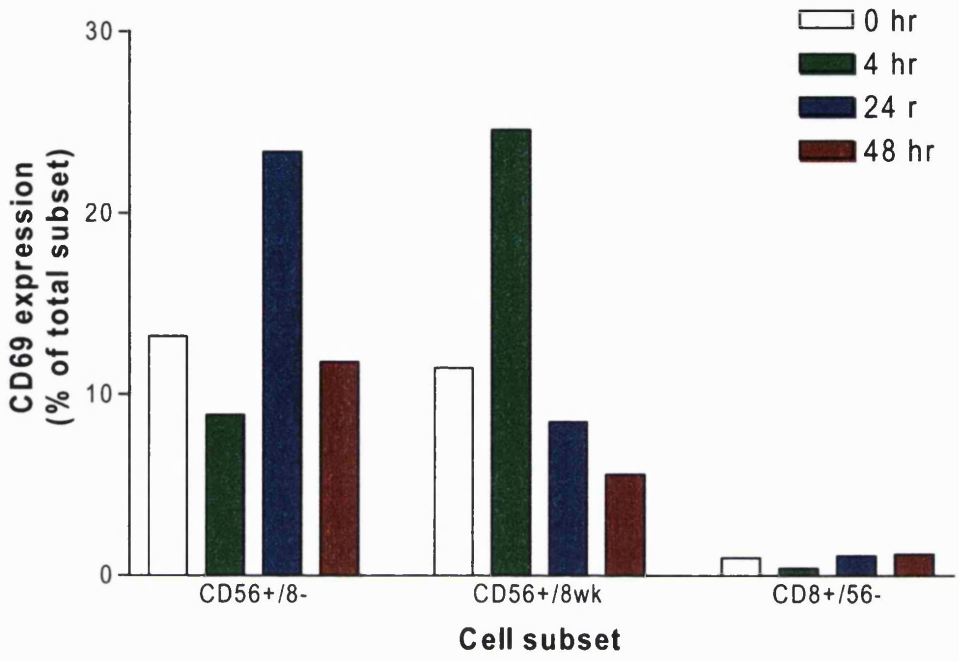
The impact of α-IFN on anti-leukaemic activity was investigated in another patient with AML M2 (SF). This 27 year-old patient also received α-IFN treatment post chemotherapy-induced 2nd CR. There was no detectable lysis of autologous leukaemic blasts in samples taken in CR after the chemotherapy treatment and prior to the start of α-IFN treatment. However after 6 days of treatment specific anti-leukaemic activity had developed and the levels increased over the next 50 days of treatment (Figure 3.15). Since that time levels of specific anti-leukaemic activity have been maintained and the patient remains well and in remission for the past 30 months. She has subsequently had a child.

Figure 3.15 SF Development of anti-leukaemia treatment post alpha-interferon treatment



The effect of αIFN treatment on immune response to leukaemia: leukaemia-specific cytotoxicity after four hours co-incubation of patient $CD56^{+}/CD3^{-}$ NK cells isolated from PBMC from samples taken on the days indicated, with cryopreserved autologous leukaemic blasts taken at disease presentation. The results are presented as arithmetic means of triplicate assays and the error bars represent standard deviation.

Figure 3.16 CD69 expression by post-transplant PBL on patient RH



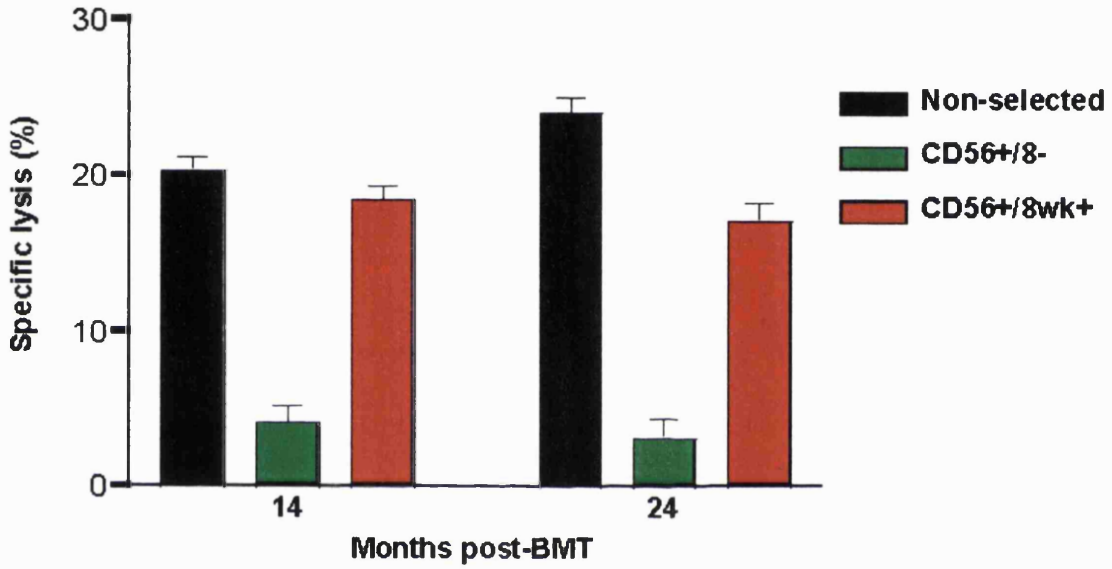
The graph shows CD69 expression by post-BMT PBL following stimulation with autologous leukaemic BMMC for 4, 24 and 48 hours. The $CD56^{+}/CD8wk^{+}$ cells are $CD3^{-}$ and the $CD56^{-}/CD8^{+}$ are $CD3^{+}$ as determined by flow cytometry. The cells were selected by labelling the samples with CD69 FITC, CD56PE and CD4 or CD8 PerCP (all from Becton Dickinson) after the cytotoxicity assay had been performed. The control samples consisted of effector cells incubated with remission BMMC (in all cases the level of CD69 expression after incubation with remission BMMC was no greater than that of effector cells incubated alone). All samples were set up in triplicate and the mean proportion of CD69 positive cells in each cell subset was recorded after analysis by flow cytometry.

3.3.2.3 Analysis of effector cells in anti-leukaemic killing

The use of CD69 expression as a tool to monitor activation of cells in the anti-leukaemic cytotoxicity experiments showed that most of the early activity resided in the $CD56^+/CD8wk^+$ cell population (Figure 3.16). At 4 hours there was over double the response in the $CD56^+/CD8wk^+$ group as against the $CD56^+/CD8^-$ group and there was no response from the $CD56^-/CD8^+$ group. Neither the $CD56^+$ nor the $CD8^+$ responded to the autologous remission BMMC over 4 hours. However by 24 hours the response in the $CD56^+/CD8wk^+$ population had fallen to below 10% and the level of expression in the $CD56^+/CD8^-$ subset had risen to nearly 25%.

The effector cells incubated with PHA produced a measured response that peaked at 24 hours and was much greater in $CD4^+$, $CD8^+$ and $CD56^+/CD8^-$ cells than the $CD56^+/CD8wk^+$ subset. In the group stimulated with K562 the majority of the activation resided in the $CD56^+/CD8^-$ population and continued to increase up to 48 hours. There was no response seen in the $CD8^+/CD56^-$ sub-group, even at 24 hours. Previous studies with mitogens suggested that $CD8^+$ cells not up-regulating CD69 at 24 hours post stimulation were unlikely to activate subsequently.

Figure 3.17 Anti-leukaemic activity in RH- comparison of $CD56^+/CD8^-$ and $CD56^+/CD8wk^+$ cells.



The graph shows cytolytic activity of the fractionated subsets of $CD56^+$ cells at 14 and 24 months post-BMT. The cytolytic activity stays consistently within the $CD56^+/CD8wk^+$ population. These data are presented as means and the error bars represent standard deviations.

In one patient highly purified NK cell subsets (95%) were sorted for functional testing and incubated with presentation leukaemic blasts and remission BMMC. This confirmed the $CD56^{+}/CD8wk^{+}$ subset as the population of interest because the results showed that killing lay almost exclusively within this population and not the $CD8^{-}$ subset (Figure 3.17).

3.3.2.4 Measurement of anti-leukaemic killing in autologous BMT patients and those receiving chemotherapy alone.

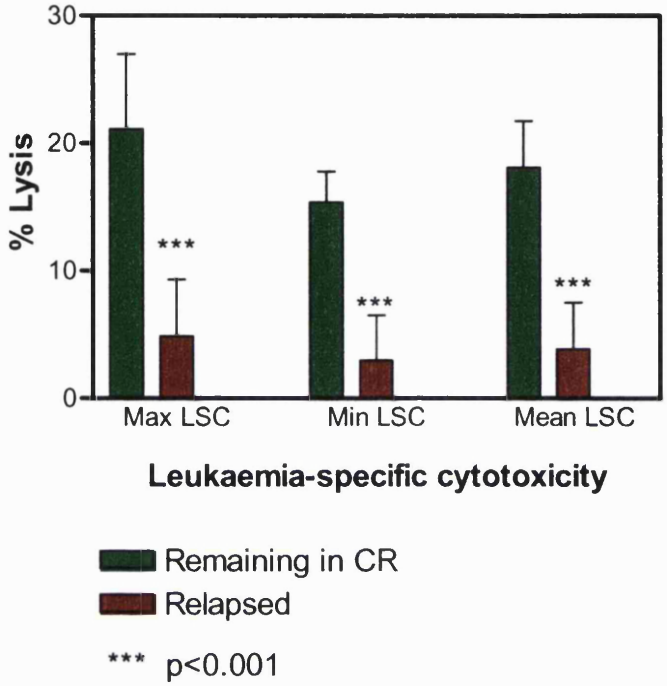
Once a relationship between anti-leukaemic activity and survival had been established in a small group, a further 10 patients who had either received an autologous transplant or chemotherapy alone were monitored. Out of 14 patients 7 relapsed during the study with a median CR duration of 8 months (range 2-21), the other 7 remain in CR with a duration of 31 months (range 24 - 71 months). The leukaemia specific cytotoxicity (LSC) was measured in all patients at several time points during CR. The maximum, minimum and mean LSC was determined for the patients remaining in CR and compared with those patients in the group that relapsed (Figure 3.18). The differences between the two were highly significant ($p=<0.001$).

To establish a threshold value for the test and assess sensitivity and specificity the lowest recorded LSC for each of the non-relapsing patients and the highest recorded LSC for the relapsing patients was analysed (Table 3.4). The mean of the minimum LSC in the non-relapsing patients was 15.36% (s.d. 2.45). This figure was used to establish a LSC lower limit of 10.46% (the mean minus 2x

the standard deviation). For all but two of the patients a level of LSC above this figure conferred freedom from relapse whilst this level of LSC was maintained.

Five of the patients who's maximum LSC was below 10.46% relapsed within 14 months of induction of CR. However two of the patients who relapsed each had LSC levels above the predictive value in one test sample, this gave the assay a sensitivity of prediction of relapse of 71.4%. All of the patients in the group who did not relapse had LSC levels above 10.46%, which gave the assay a specificity for prediction of relapse of 100%. There was no correlation between the mean of LSC and the duration of remission in those who relapsed suggesting that there was a threshold LSC required and there was no partial protection conferred below this point.

Figure 3.18 Leukaemia-specific cytotoxicity



The lowest LSC and the highest LSC were recorded for both the group that remained in remission and the group who relapsed. The LSC was measured on a number of occasions in all patients during CR after completion of treatment.

Table 3.4 Leukaemia specific cytotoxicity

Patient ID	Disease	Lowest LSC detected in patients remaining in CR (%)	Patient ID	Disease	Highest LSC detected in patients who relapsed (%)
RY	AML	11.3	SH	Pre-B-ALL	3.7
JW	AML-M5	18.5	EP	B-ALL	0.0
RH	AML-M4	13.8	RR	AML-M4	10.9
GG	AML	17.6	VH	AML	8.2
SF	AML-M2	16.6	SS	AML-M1	0.0
LA	AML-M4	15.2	MAd	T-ALL	6.4
AGD	AML	14.5			
Mean		**15.36**	**Mean**		**4.87**
SD		**2.45**	**SD**		**4.44**

LSC= leukaemia specific cytotoxicity
CR= complete remission

The LSC on 13 out of the 14 patients listed in Table 3.2 is shown in the table. The remaining patient relapsed before a sufficient number of samples could be tested for LSC.

3.4 Discussion

The results show that CD69 can appear very quickly on the cell surface given the appropriate stimulation. PHA provides a detectable increase in less than 1 hour with CD4 cells, which suggests that the molecule can reside in the cytoplasm and expression can occur without *de novo* protein synthesis. This confirmed the findings of Cebrian and colleagues (1988) who used PMA as the stimulus and despite pre-treatment of cells with inhibitors of protein or RNA synthesis could not hinder CD69 expression.

The addition of IL-12 produced an upregulation of CD69 expression on NK cells that peaked at 48 hours and showed that the response to the cytokine was much slower than the response to mitogens or target cells. Lanier and colleagues (1988) showed that the majority of NK cells will express CD69 after 6 hours incubation with IL-2 and all after 18 hours incubation. Both these cytokines therefore are extremely influential in the generation of NK cells, but less stimulatory than mitogens or target cells. These experiments determined the time course over which CD69 upregulation should be analysed following incubation of patient lymphocytes with presentation leukaemic cells.

Initially the early reactivity highlighted by CD69 expression was in the $CD56^+/CD8wk^+$ population but by 24 hours the $CD56^+/CD8^-$ population were $CD69^+$. This can be explained by the propensity of the CD8α chain to be shed upon activation. The fact that the $CD56^+/CD8^-$ subset seemed to gain $CD69^+$

expression in inverse proportion to the loss of CD69 on the $CD56^+/CD8wk^+$ subset suggested that the same subpopulation was being identified.

There is evidence in the literature to support the fact that CD8 can be shed on activation of a cell. An increase in soluble CD8 in plasma has been shown to be a marker of activation and it has also been demonstrated that the shedding of soluble CD8 *in vitro* takes place around 6 hours (Tomkinson *et al.*, 1989, Yoneyama *et al.*, 1995). If this is the case, the switch from $CD56^+/CD8wk^+$ to $CD56^+/CD8^-$ takes place shortly after the 4 hour time point, which is why there is such a clear distinction by 24 hours. With hindsight it would have been interesting to have an intermediate time point between 4 and 24 hours to see if it was possible to pinpoint the timing of the shedding of the CD8 molecule.

Although CD3 was not specifically phenotyped for in all of these experiments, it is unlikely that $CD3^+/CD56^+/CD8^+$ cells were responsible for the killing observed. By using $CD56^+/CD8^{wk+}$ cells $CD3^+$ cells were excluded. Also earlier work from this department on CD69 showed that CD8 T cells take at least 24 hours to respond to cell stimulus and express CD69 (Craston *et al.*, 1997). Cytotoxic T lymphocytes take much longer than NK cells to activate, and in order to kill require specific peptides presented by MHC class I molecules. If these cells were to kill within four hours they would need to have been primed already (van den Broek *et al.*, 1998).

The cells observed that express the activation marker at 4 hours are therefore likely to be NK cells, and this was supported by the subsequent experiments in

which $CD56^+$ cells were sorted to exclude $CD3^+$ cells. Finally it has also been shown that there is a correlation between expression of CD69 on NK cells and lytic activity (Jewett and Bonavida, 1995b). IL-2 and IFNα were used in this series of experiments to induce cytotoxicity in NK cells and this was accompanied by a concurrent increase in CD69 expression. This supports the theory stated here that CD69 expression is a possible indicator of cytotoxicity.

The cytotoxicity assay used in these experiments was favourable to a ^{51}CR release assay for a number of reasons. The target cell labelling time is short and the dye does not transfer to other cells. It does not require any radioactive probes, and there is no problem with non-specific release of the probe. Also it allows monitoring of cytotoxicity on a single-cell basis, which has made it possible to analyse the killing of leukaemic blast cells by normal cells (Hatam *et al.*, 1994). This is vital for the study of primary leukaemias in contrast to leukaemic cell lines since primary leukaemic cells do not take-up ^{51}Cr uniformly which is an essential requirement for a conventional chromium-release assay. It has also been shown recently that a microcytotoxicity assay, which is a similar method to the PKH-26 assay, detects apoptosis whereas the ^{51}Cr assay does not. This research shows that the ^{51}Cr assay works with some cell lines, but the microcytotoxicity assay is more widely applicable because it detects both necrotic and apoptotic killing pathways (Wahlberg *et al.*, 2001).

For the cytotoxicity experiments all patient samples were tested against K562 cells to confirm NK cell function. One group has reported that cultured cells were strongly cytotoxic against K562 target cells but had no anti-leukaemic

activity (Hercend *et al.*, 1986). It has also been observed that killing of K562 and LCL (an NK-resistant LAK-sensitive EBV-transformed B-lymphoblastoid cell line) did not predict killing of patients' pre-transplant leukaemia cells (Mackinnon *et al.*, 1990). These groups were both working in the allogeneic not the autologous setting but their work shows that too much emphasis can be put on the similarity of the response of target cell lines to leukaemic cells. The reaction of cell lines should only be used as a point of comparison but not as a guide as to how leukaemic blasts would necessarily respond.

The findings of both these studies also support the observations made here that anti-leukaemic activity is difficult to detect in the allogeneic setting. Hercend's work also highlights the heterogeneity of cells that have an NK-like cell function. Some of the clones he cultured from normal donors were unable to kill cryopreserved leukaemic blasts even though their phenotype and cytotoxicity for other NK targets was similar to that of the clones that could mediate anti-leukaemic activity (Hercend *et al.*, 1986). This would support the theory that an additional lymphocyte surface molecule, as yet undetermined, maybe the key to distinguishing the cells that can mediate anti-leukaemic activity and these cells have not so far been fully characterised.

It is not all NK cells that have the ability to kill leukaemic blasts but a subset of the population, and it may also be that the crucial subsets or possibly lineages are different for each type of leukaemia. This was demonstrated by the fact that only around 30% of all $CD56^+$ cells expressed CD69 in the 4 hour stimulation assay with autologous leukaemia and was confirmed by the dichotomy in

response shown by the $CD56^+/CD8^+$ sorted population. The observation that some patients make a leukaemia specific response after chemotherapy whilst others fail to do so may be explained by one of two hypotheses. The most hopeful hypothesis is that there is a deficiency in the effector cells of these patients which can be corrected. The alternative hypothesis is that some patients have a leukaemia specific clone which is resistant both to chemotherapy and NK-mediated lysis. This question is unresolved currently but is the subject of further study.

In a study of allogeneic NK cell lysis of primary AMLs, preliminary results indicate that some AML blasts resist NK-cell mediated lysis by prevention of binding of perforin to the leukaemic cell membrane. An assay of perforin binding may prove to be an indicator of resistant disease (Lehmann *et al.*; 1999).

From the cytotoxicity experiments shown here it seems probable that in *in vitro* studies AML target cells are more susceptible to lysis by $CD56^+/CD8wk^+$ cells than ALL blasts, certainly the patients (Table 3.4) who showed leukaemia specific cytotoxicity and remained in CR all had AML. It may therefore be something pertinent to the myeloid cell that makes it a better target than the lymphoid cell. An inducible *in vitro* GvL response with use of LAK cells has previously been shown in CML and the effect was largely mediated by $CD3^-$ NK cells (Mackinnon *et al.*, 1990). The explanation may lie in the differential expression of LFA-1 by target cells. AML blasts express high levels of LFA-1 in the majority of cases (>80%) and are good targets for NK cell lysis. Antibodies

to LFA-1 can block this NK mediated killing *in vitro*. In contrast, ALL cells which inherently lack LFA-1 are generally NK resistant (Lowdell *et al.*, 2001).

The existence of the $CD56^+/CD8^+/CD3^-$ subset was first noted in 1983 (Perussia *et al.*, 1983). It must have been highly unexpected to find a T cell antigen on an NK cell subset and as yet its function has not been ascertained. Also this finding means the timing of the division of lineages has to be reconsidered. Perhaps the importance of $CD56^+/CD8wk^+$ cells has now been uncovered, but further work is essential to discover the function of the CD8 molecule on an NK cell.

Autologous transplants are safer and more readily available than allogeneic transplants. The problem to date has been that they are less successful in the treatment of AML than allogeneic transplants and therefore it would be valuable to be able to highlight the cells which can mediate anti-leukaemic activity and generate them for use post-transplant. Further work needs to establish how to generate these cells and in the long term how to predict which patients will be responsive to this type of treatment.

One approach that may prove to be useful is the use of IFNα. Although the use of this cytokine in the management of CML is well established it would appear the first reported use of this cytokine therapy was as a result of the work conducted in this study (Lowdell *et al.*, 1999). IFNα-therapy has been successful in the treatment of two of the autologous patients who have exhibited leukaemia specific cytotoxicity. Some research has been done on the effect of

IFNα on NK cells *in vitro*, and it has shown that this cytokine can activate immature NK cells into cytotoxic cells and enhance the cytotoxic activity of mature NK cells, but it does not cause proliferation. Indeed the addition of IFNα to IL-2 treated NK cells has been shown to inhibit proliferation (Jewett and Bonavida, 1995b).

IFNα also causes upregulation of the CD2 antigen which is an adhesion molecule that binds to LFA3, this in turn activates protein kinase C and causes an increase in intracellular Ca^{2+} concentration. Therefore IFNα may indirectly serve as a triggering molecule on NK cells. This cytokine also suppressed TNFα secretion and upregulated IFNγ secretion by NK cells (Jewett and Bonavida, 1995b). The role of this cytokine appears to be contradictory and more research could usefully be done on its effect on NK cells, and more generally a better understanding is needed of the different activation pathways that NK cells may use when stimulated by different cytokines.

4. The influence of specific cytokines on $CD56^+$ cells

4.1 Introduction

4.1.1 Cytokines

The term cytokine denotes a messenger molecule that can deliver signals between cells. Cytokines are proteins or glycoproteins that are made by cells in order to influence the behaviour of other cells. They act over short distances on a number of cell types, and have a variety of different effects on the cells they target. They are produced on the stimulation of the cell but secretion is for a restricted period of hours or very occasionally days and the free cytokine remains biologically active for little longer. Their half-life is even shorter if introduced into the area artificially. Cytokines interact with the cell via specific cell surface receptors and this leads to activation of intracellular signal transduction pathways. This, in turn, results in specific genes in the cell nucleus being activated or inactivated.

At present six main cytokine families have been described; haematopoietins, interferons, immunoglobulin superfamily, tumour necrosis factor (TNF), transforming growth factor (TGF) and the chemokines. The cytokines in this study all belong to the haematopoietin family. They are grouped together

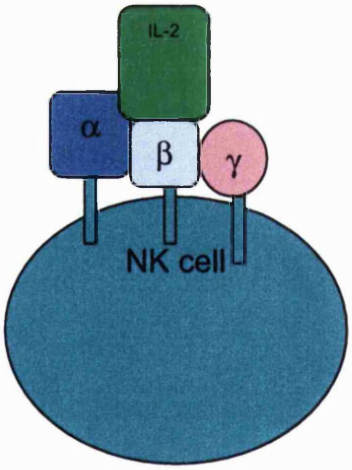
because they are involved in the growth and differentiation of haemopoietic cells (Klein and Horejsi, 1997). The receptors for the haematopoietins belong to the type 1 cytokine receptor family which means they share structural features in their extracellular domains, this includes a five amino acid motif tryptophan-serine-X-tryptophan-serine (WSXWS). The cytokines that bind to these receptors share a tertiary structure consisting of four alpha helices (Lichtman *et al.*, 1997).

Interleukins are so called because they are molecules that signal between leucocytes. Three cytokines were used in this study, interleukin 2 (IL-2), interleukin 7 (IL-7) and interleukin 15 (IL-15). They were chosen because it has already been reported that they may affect NK cell function, viability and replication or generate cytotoxic lymphocytes (Naume *et al.*, 1992, Cavazzana-Calvo *et al.*, 1996, Mingari *et al.*, 1997, Loubeau *et al.*, 1997). Also of interest was the possibility that these three cytokines might have a close relationship with each other. The receptors for these cytokines all belong to the type 1 cytokine family, and the γ chain is common to all their receptors (Lichtman *et al.*, 1997). IL-2 and IL-15 share the same IL-2Rβ and γ chain which has led to speculation that they may have similar functions (Grabstein *et al.*, 1994). There is some evidence to suggest that IL-2 and IL-7 may act synergistically to increase cytotoxicity in NK cells produced post autologous transplant (Pavletic *et al.*, 1993).

4.1.1.1 Interleukin-2

IL-2 is a 14- to17-kDa glycoprotein and is produced by CD4 cells and to a lesser extent CD8 cells (Lichtman *et al.*, 1997). The high affinity IL-2R consists of α, β and γ components (Figure 4.1). The α chain (CD25) is the low affinity component and the β chain (CD122) is the intermediate component and together with the γ chain they comprise the high affinity receptor for IL-2. The ligands for the high affinity receptor are IL-2 and IL-15 (Klein and Horejsi, 1997). The γ chain is required for signal transduction and is not involved in IL-2 binding. Stimulation by antigen upregulates IL-2Rα expression and this leads to the generation of the high affinity receptor.

Figure 4.1 The three components of the IL-2 receptor



The pleiotropic effects of IL-2 on NK cells have been known for more than a decade, and have led to its use in numerous clinical trials in oncology. The induction of cytokine secretion and enhancement of cytotoxic effector functions is thought to contribute to the anti-neoplastic effects of IL-2 therapy. IL-2 has

been shown to stimulate TNF-α (Dilloo *et al.*, 1994) and IFNγ (Heslop *et al.*, 1989) production, which is believed to be important in inhibiting haematopoiesis (reviewed in Barrett, 1997). Unlike T cells which require two signals for stimulation, NK cells can be rapidly activated and induced to proliferate by IL-2 alone and it is known that IL-2 induces NK cells to become activated killer cells capable of lysing autologous haematopoietic tumour cells (Reittie *et al.*, 1989).

Leukaemia patients have been treated with IL-2 but not without a number of side effects (Hamon *et al.*, 1993). Unless administered at low doses IL-2 has been found to be toxic, principally due to thrombocytopaenia and capillary leak syndrome. Miller suggests that given at tolerable doses IL-2 is insufficient to induce maximum activation of NK cells (Miller *et al.*, 1997) however work done by Warren and co-workers shows that submitogenic quantities of IL-2 can activate $CD56^{bright}$ cells *in vitro* (Warren *et al.*, 1996).

It is apparent that IL-2 may have an important role in the treatment of leukaemia, but optimal use of IL-2 has not yet been established. IL-15 has been shown in mice to be less toxic than IL-2 *in vivo* (Carson *et al.*, 1997). Therefore the use of IL-2, in conjunction with such a cytokine may be a way of improving treatment efficacy by using the two cytokines to produce a synergistic effect without incurring the same levels of toxicity.

4.1.1.2 Interleukin-7

IL-7 was used in this study because it has been reported that it can induce the generation of cytotoxic T lymphocytes (CTL) and lymphokine-activated killer

(LAK) cells in bulk cultures (Alderson *et al.*, 1990) and in PBMC from autologous BMT patients (Pavletic *et al.*, 1993). The cells generated by the addition of IL-7 alone in the bulk cultures, included $CD8^+$, $CD56^+$ and $\gamma\delta^+$ cells. It was therefore hypothesised that IL-7 might affect the production of cytotoxic cells from $CD56^+$ resting cells, and may be involved in the generation of $CD56^+/CD8^+$ cells from $CD56^+/CD8^-$ cells.

IL-7 alone has been reported to induce low proliferative activity in purified $CD56^+$ cells *in vitro* (Naume *et al.*, 1992) and can stimulate $CD56^+$ cells to produce TNF-α. It has been observed that anti-TNF-α monoclonal antibodies can inhibit the proliferative capacity, and IL-7-induced activity of LAK cells. Therefore endogenous TNF-α must regulate the activity of the cytokine *in vivo* (Naume *et al.*, 1992). IL-7 along with IL-2 has also been shown to prevent apoptosis in NK cells and to up-regulate bcl-2 expression (Armant *et al.*, 1995). The BCL-2 protein acts as an anti-apoptotic agent in cells when present above a given threshold.

4.1.1.3 Interleukin-15

IL-15 was first described in 1994 and was discovered whilst testing supernatants from a simian kidney epithelial cell line (Grabstein *et al.*, 1994). The human IL-15 gene was originally cloned from a human bone marrow stromal cell line and its transcript is widely expressed in a number of human tissues including placenta, skeletal muscle, kidney, lung, liver and pancreas. cDNA clones show that it is a 162-amino acid residue precursor polypeptide, containing a 48-amino acid residue leader sequence, which is cleaved at the

experimentally determined NH_2-terminus to generate the 114-amino acid mature IL-15 protein (Grabstein *et al.*, 1994).

IL-15 is produced by activated monocytes and bone marrow stromal cells (Carson *et al.*, 1997), and has been reported as a key player in the proliferation and differentiation of NK cell progenitors (Mrozek *et al.*, 1996, Leclercq *et al.*, 1996). This cytokine is known to be able to generate cytotoxic activity and cytokine production by human NK cells (Leclercq *et al.*, 1996).

IL-15 shares many common features with IL-2, one of which is that they are both members of the α-helix-bundle cytokine family. This means that the structure of the molecule is similar in that they have four boxed sequences connected by three loops. They both use the IL-2R$\beta\gamma$ common chain to activate lymphoid cells. IL-2Rβ and γ are crucial for signalling through the IL-2R and are constitutively expressed on NK cells. IL-2 and IL-15 also share functions. In relation to NK cells they both stimulate activation and proliferation, and they act in synergy with IL-12 in facilitating synthesis of IFNγ and TNFα.

However there are differences between the two. They do not share any sequence homology, and IL-15 is more abundant in tissue than IL-2. IL-15 has a specific α chain that is structurally similar to that of IL-2 but has little sequence similarity (Leclercq *et al.*, 1996). It has also been found that the addition of IL-15 to thymic precursors results in differentiation into NK cells, whereas the addition of IL-2 results in thymocytes becoming T cells. Mrozak and co-workers (1996) showed that the culture of $CD34^+$ progenitor cells in the presence of IL-

15 resulted in cells which expressed CD56, but not CD34, CD16 or CD2 (CD2 is expressed in the majority of NK cells found in the peripheral blood). However, this is the phenotype of cord blood NK cells (Han *et al.*, 1999). These cells had large granular lymphocyte (LGL) morphology, with high density surface expression of $CD56^{bright}$ and showed cytotoxic activity against K562 targets. IL-15 has also been found to be less toxic than IL-2 in mice which would make it a valuable tool in immunotherapy if this proved to be the case in humans as well (Loubeau *et al.*, 1997). A summary of the cytokines used appears in Table 4.1.

Table 4.1 Summary of the cytokines used in the project

Cytokine	Cell source	Targets	Effects
IL-2	activated T cells	T,B & NK cells, mononuclear phagocytes	stimulates growth and cytotoxicity of NK cells, enhances IFN-γ production by T & NK cells, T-cell growth factor, and susceptibility of T cells to activation induced cell death.
IL-7	bone marrow, thymic cells & spleen cells	T & NK cells	generate cytotoxic T cells and LAK cells
IL-15	activated monocytes & bone marrow stromal cells	T & NK cells	activates NK cytotoxicity via IL-2R

4.1.2 Cytokines and NK cells

The initial phenotyping studies presented earlier (Chapter 2) showed that NK cells are the first lymphocyte group to reappear in large numbers after autologous or allogeneic BMT, this has also been shown by a number of other observers (Reittie *et al.*, 1989, Jacobs *et al.*, 1992). CD56 is a known marker of NK cells, and was used to sort NK cells from PBMC. Within the CD56 subgroup there are $CD56^{dim}$ ($CD56^{+}$) cells and $CD56^{bright}$ ($CD56^{++}$) cells. Approximately 10% of the NK population are $CD56^{bright}$ and the rest $CD56^{dim}$ (Frey *et al.*, 1998). The $CD56^{bright}$ cells constitutively express the IL-2R$\alpha\beta\gamma$ and this allows the cells to respond to picomolar concentrations of IL-2 (Warren *et al.*, 1996). These cells would possibly therefore be targets for other cytokines sharing receptors with IL-2.

The cells that express $CD56^{+}$ can be further subdivided by other molecules. Of interest to this project were the $CD56^{+}/CD8^{wk+}$ cells, because of the observations made whilst analysing the NK cells involved in autologous anti-leukaemic cytotoxicity (Chapter 3). These cells have a lower density of CD8 antigen expression than is found on T cells and express the CD8α/CD8α homodimer rather than the CD8α/CD8β heterodimer found on most T cells (reviewed in Robertson and Ritz, 1990).

It has previously been demonstrated by this group that in some patients $CD56^{+}/CD8^{wk+}$ cells can exhibit early cytolytic ability against autologous

leukaemia cells (Lowdell *et al.*, 1997). For this reason it was decided to study the $CD56^+/CD8^+$ and $CD56^+/CD8^-$ subsets to see whether it was possible to stimulate $CD8^-$ cells to express CD8, and finally to compare both subsets in cytotoxicity assays.

Different mechanisms of cell death were also studied to try and evaluate whether the effector cells killed by apoptosis or necrosis. In apoptosis a number of morphological changes take place including cell shrinkage, condensation of the nuclear chromatin, fragmentation of the nucleus and blebbing of the cell surface (Wickremasinghe and Hoffbrand, 1999). With necrosis the integrity of the cell membrane is lost. The aim was to see if these cells had a preferred mechanism for killing.

4.2 Materials and Methods

4.2.1 Purification of NK cells

Initially peripheral blood was donated by volunteers and stored in preservative free heparin (PFH). Subsequently the number of $CD56^+$ cells required made this no longer feasible and it became necessary to purchase leucocyte preparations (single donor whole blood units) from the National Blood Authority. The samples were separated over Lymphoprep (section 2.2.1) to recover the mononuclear cells and the cells were put through a 100μm nylon cell strainer (Falcon, Becton Dickinson), to remove any clumps which might hinder the separation process. All samples were separated using Lymphoprep so any changes in the proportions of cell types that may have occurred due to the use

of Lymphoprep was the same for all samples. At this stage an aliquot was removed and phenotyped for $CD8^+$, $CD56^+$ and $CD3^+$ expression. The rest of the lymphocytes were sorted on the basis of $CD56^+$ expression using MACS CD56 Microbeads (Miltenyi Biotec, Germany).

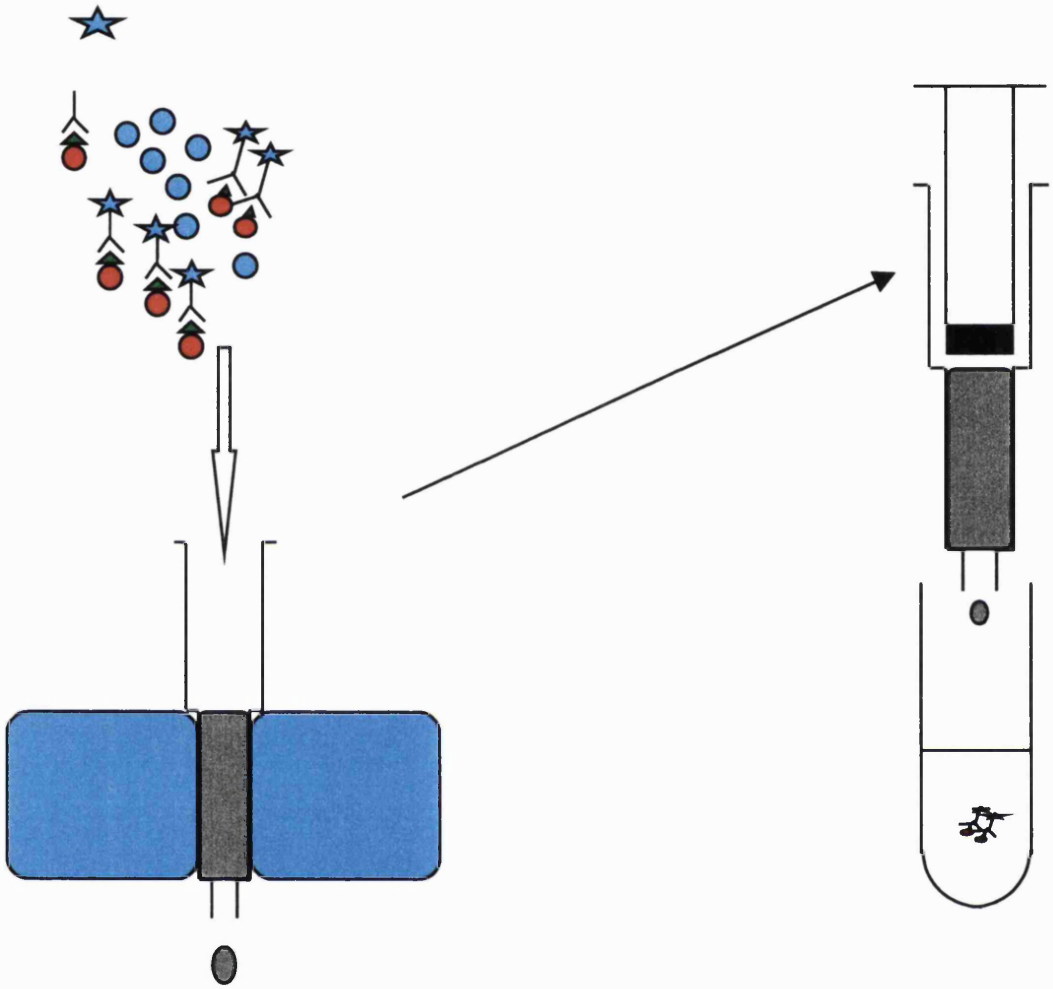
4.2.1.1 Sorting CD56 with microbeads

The mononuclear cell fraction was isolated by discontinuous density gradient centrifugation (section 2.2.1) and the recovered cells were resuspended in 80µl of buffer (MACS buffer - PBS supplemented with 2mM EDTA and 0.5% bovine serum albumin) per 10^7 total cells. 20µl of MACS CD56 Microbeads per 10^7 total cells were added. The suspension was mixed vigorously and placed at 5°C for 15 minutes. At this point CD56PE conjugated monoclonal antibody was added, and the cells were placed at 5°C for a further 15 minutes. The use of the conjugated monoclonal antibody which was specific for a different CD56 epitope than that to which the beads bound, enabled the sort to be monitored by flow cytometry as it progressed. After 30 minutes the cells were washed in 10 - 20 times the labelling volume of buffer. They were centrifuged at 300xg for 10 minutes. The supernatant was tipped off and any residual supernatant was removed by pipette. The cells were then resuspended in buffer (500µl of buffer per 10^8 total cells).

The MACS column (MS^+ for positive selection) was placed in the MACS separator (MiniMACS) (see Figure 4.2). It was prepared by applying 500µl of MACS buffer to the column and allowing it to run through. The effluent was

discarded. The cell suspension diluted in 500µl - 1 ml was added to the column and the CD56 negative cells were allowed to run through. It was possible at this stage to analyse the negative sample for any $CD56^+$ cells by flow cytometry, because the cells were PE labelled. The column was then rinsed with 4x500µl of MACS buffer, removed from the MiniMACS, placed over a collection tube and 1ml of buffer was used to flush the cells through.

Figure 4.2 Separation of CD56$^+$ cells by MACS



The cartoon shows the magnetic labelling of NK cells with MACS microbeads. The cells labelled with CD56 MACS microbeads are placed in the MACS separator. When the column is removed from the magnets and the plunger is pushed down, it pushes the magnetically labelled cells into the collection tube. In this way the CD56$^+$ cells are positively selected from PBMC.

When using leucocyte preparations the numbers of cells involved made it necessary to use the MACS VS^+ columns and the VarioMACS separator. The VS^+ column was for positive selection. To prepare the column 3mls of buffer were allowed to run through, and the effluent discarded. The cell suspension containing up to 10^8 positive cells in a maximum of $2x10^9$ cells was applied to the column and allowed to run through. The column was washed with 3x3mls of buffer, removed from the separator, and placed on a collection tube. The plunger was used to force 5mls of buffer through the column and release the cells.

4.2.1.2 Sorting CD8 cells by FACS Vantage

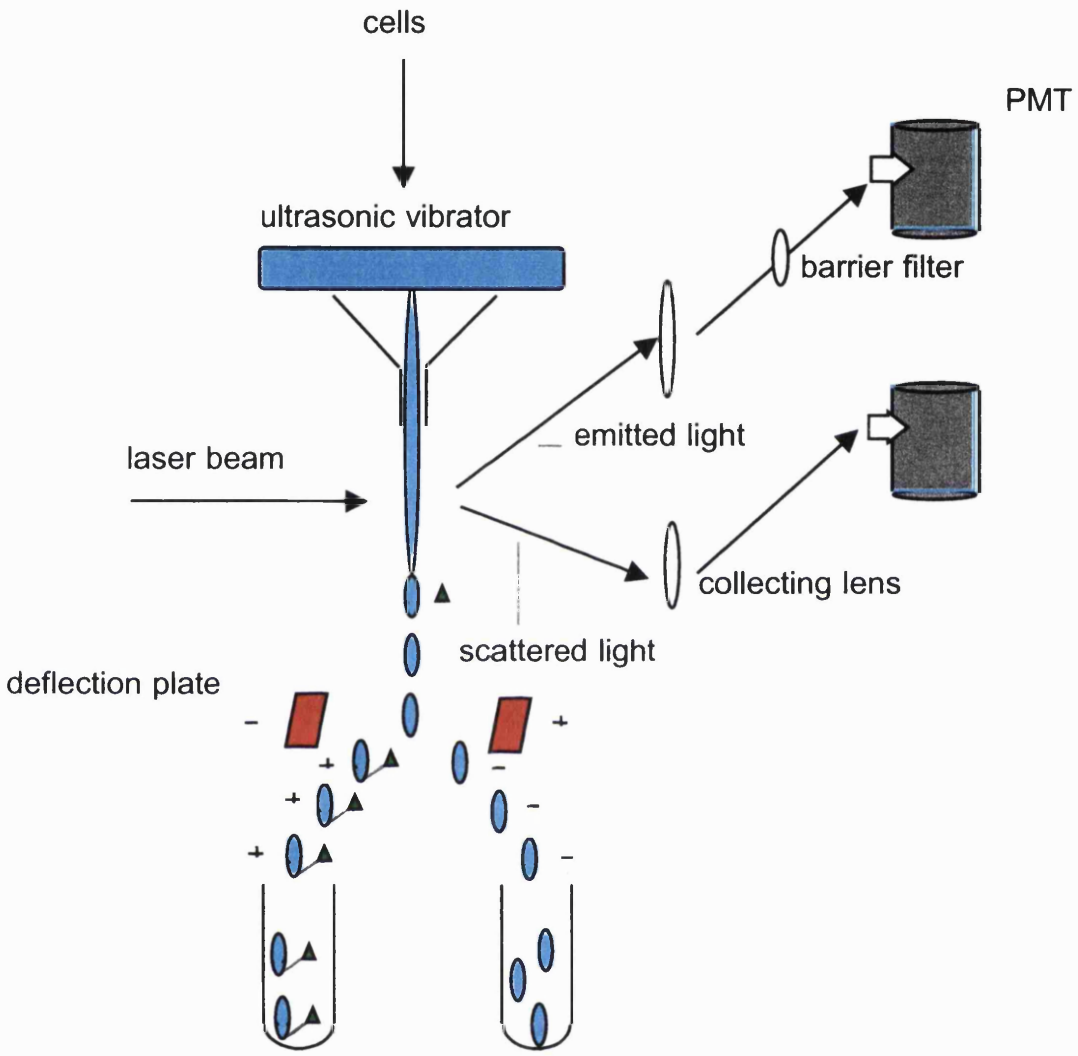
Initial experiments revealed that on average 30% of $CD56^+$ cells expressed CD8. For all the experiments both the $CD8^+$ and the $CD8^-$ populations were sorted on the FACSVantage, but in some instances the number of $CD8^+$ cells made any experimental work with that population impossible.

After the initial MACS sort on the basis of CD56 expression the cells were labelled with CD8 FITC and CD3 FITC and then left at 5°C for 10 minutes. They were then sorted on the FACS Vantage (Becton Dickinson) (Figure 4.3). The Vantage was set up using 6μm beads labelled with FITC and PE fluorochromes. Once the settings were optimised the samples were run and regions drawn denoting the FITC positive and negative populations. There were three populations, one negative which were the $CD8^-$ cells and two positive. The two positive populations divided into the $CD8^{wk+}$, which had intermediate expression of CD8, and the $CD8^+/CD3^+$ cells that strongly

expressed CD8 (Figure 4.4). In this way the $CD3^+$ cells could be excluded from the sort. The $CD8^-$ and the $CD8^{wk+}$ populations were then sorted into two different tubes in the FACSVantage.

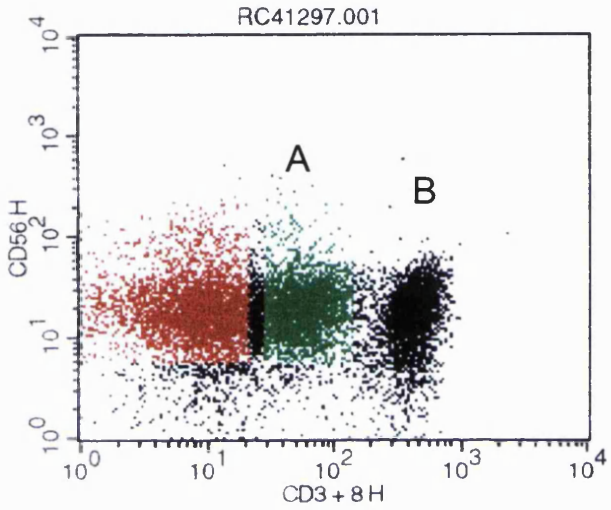
For most of the experiments it was the $CD8^-$ population which was used to set up the assays. The cells were then suspended in serum free medium (X-Vivo 10, Biowhittaker, U.K., with 100units/ml penicillin and 10,000μg/ml streptomycin), and subsequently in DMEM/F12 (Life Technologies, Scotland). Typically $CD56^+/CD8^+/CD3^-$ and $CD56^+/CD8^-/CD3^-$ populations were achieved with >95% purity.

Figure 4.3 Sorting by FACS Vantage



The ultrasonic vibrator creates droplets which contain cells. The cells pass in front of the laser beam and emit light which is focused by the collecting lens onto the barrier filter. The filter only allows light of a certain wavelength to pass through, and this light is recorded by the PMT (photomultiplier tubes). Cell size is also measured by the scattered light reaching the PMTs. The computer registers this information and applies the appropriate electrical charge to the cells which meet the criteria established by the operator.

Figure 4.4 Separation of three populations of CD56 cells by FACS Vantage



The dot plot shown features CD56 on the y axis and CD3 and CD8 on the x axis. A in green highlights the $CD56^+/CD8^{wk+}/CD3^-$ cells. B in black highlights the $CD56^+/CD8^+/CD3^+$ and $CD56^+/CD8^-/CD3^+$ cells. The cells in red are $CD56^+$ and $CD8^-$ and $CD3^-$.

4.2.2 Establishing dilutions of cytokines

Three different cytokines were used in the experiments. IL-2 (Recombinant Human IL-2, Peprotech EC Ltd, London), IL-7 (Recombinant Human IL-7, Genzyme, Kent), and IL-15 (Recombinant Human IL-15, Genzyme, Kent).

4.2.2.1 IL-2 dose response curve

In order to establish the correct amount of IL-2 for these assays a dose response curve was performed. PBMC were used from a healthy volunteer, and the cells were separated by Lymphoprep (section 2.2.1). After monocyte depletion the cells were counted and phenotyped. The cells were sorted for $CD56^+$ expression by CD56 Microbeads (section 4.2.1.1.), and for $CD8^+$ and $CD3^+$ expression by FACSVantage (section 4.2.1.2.). Initially three different concentrations of IL-2 were tested, 0.1ng/ml, 0.5ng/ml and 1.0ng/ml. The cytokine was diluted with serum free medium (X-Vivo 10, Biowhittaker, U.K., with 100units/ml penicillin and 10,000μg/ml streptomycin) and the $CD56^+$ cells were incubated with the different dilutions in the serum free medium. The cells were incubated for 24 hours and then phenotyped again for CD8/CD56/CD3 expression by 3-colour flow cytometry (Figure 4.5).

Figure 4.5 IL-2 dose response curves

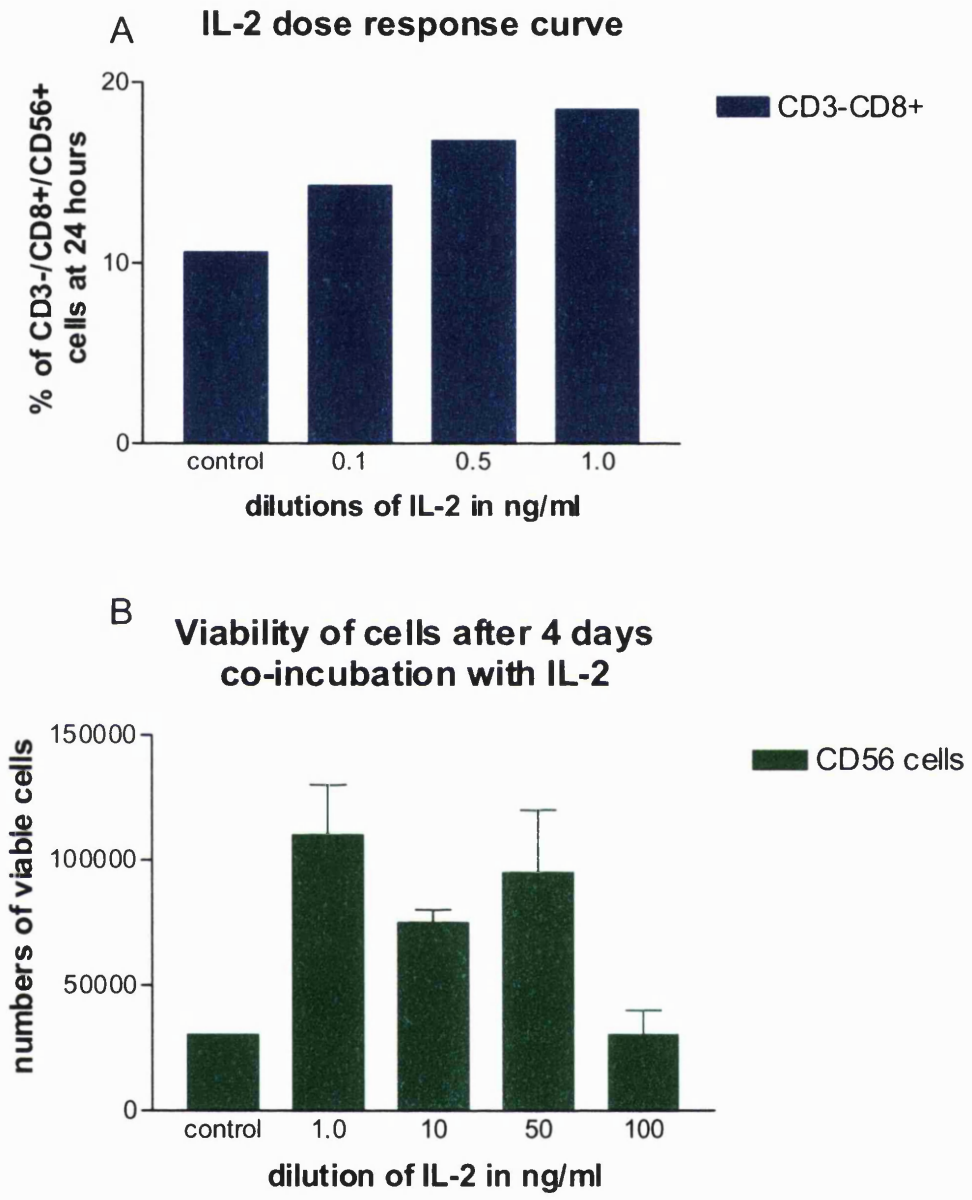


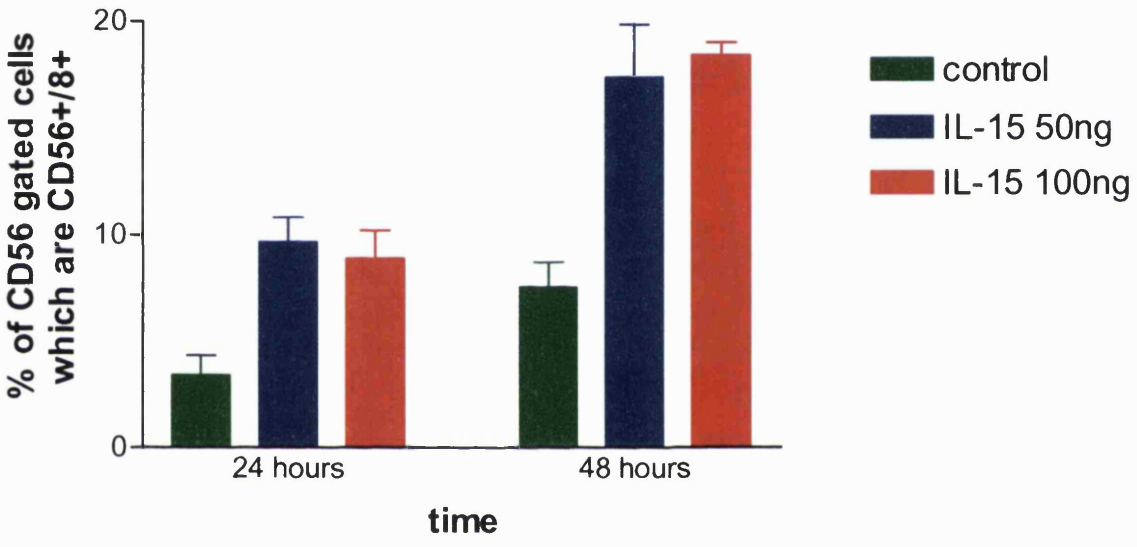
Figure A shows $CD56^+/CD8^+$ cells after 24 hours incubation with escalating doses of IL-2. Figure B shows $CD56^+$ cells after 4 days co-incubation with escalating doses of IL-2. The error bars in B represent the means ± SD of three cultures.

An additional dose response curve was also performed using 1.0ng/ml, 10ng/ml, 50ng/ml and 100ng/ml co-incubated with $CD56^+$ sorted cells (Figure 4.5B). This was done because the aim was to maximise $CD56^+$ and $CD8^+$ expression, whilst maintaining cell viability. The cells were checked under the microscope to confirm viability by trypan blue exclusion after four days in culture. From these experiments 1.0ng/ml of IL-2 proved to be the optimum concentration for the culture conditions.

4.2.2.2 IL-15 and IL-7

IL-15 was used in two different dilutions initially to determine which was the most effective in generating $CD56^+/CD8^+$ cells. Dilutions of 50ng/ml and 100ng/ml were incubated with cells sorted in the same manner as section 4.2.1.1. and section 4.2.1.2. These dilutions were chosen after consultation with the suppliers of IL-15 and a review of the current literature on IL-15. The higher concentration had no significant effect over and above the 50ng/ml dose. Therefore the lower dose was used for all future experiments (Figure 4.6). Similarly IL-7 was used in two dilutions of 50ng/ml and 100ng/ml after consultation with the suppliers and a review of the literature. After dose response curves were performed 50ng/ml was chosen as the optimum dilution for this series of experiments.

Figure 4.6 Expression of $CD56^+/CD8^+$ on CD56 sorted cells after co-incubation with IL-15 in a dose response curve



The graph shows CD56 sorted cells after 24 and 48 hours incubation with escalating doses of IL-15. The cells were gated on CD56 expression because after 48 hours 15% of the cells sorted on CD56 expression no longer expressed CD56. The error bars represent mean ± sd of three cultures.

4.2.3 Characterisation of cytokines

4.2.3.1 Establishing the phenotype of cytokine-treated cells

The cytokines were added to two different serum free medium X-Vivo 10 (BioWhittaker, U.K.) and AimV (Life Technologies) both with 100 units/ml penicillin and 10,000 μg/ml streptomycin. 48-well plates (Nunc, Life Technologies, Scotland) were used in the assay, and 1 ml of the cytokine/cell suspension was placed in each well. The number of cells/well varied inter-assay but not intra-assay and a minimum of $2x10^5$ cells/well was used in each experiment. Each sample was set up in triplicate. The plates were incubated at 37°C/5%CO_2. Initially after 24 hours an aliquot was removed from each well and phenotyped for CD8/CD56/CD3. After 48 hours the contents of each well were recovered and the entire sample was phenotyped as before. The samples were analysed by flow cytometry (FACScan) using Lysis II and the data were analysed using quadrant statistics.

4.2.3.2 The effects of cytokines on NK cell viability in vitro

Purified NK cell subsets were cultured in the presence of a single cytokine or combinations of cytokines for five days. They were cultured in 48-well plates (Nunc, Life Technologies) at 37°C/5%CO_2. On days one, two and five aliquots were removed and counted. Serum free media (section 4.2.2.1.) was compared with DMEM/F12 supplemented with 10% AB serum. The cells were monitored each day by light microscopy for viability and cytospin preparations (as

described in 4.2.3.3.) were used to allow semi-quantitation of blast cell formation.

4.2.3.3 The preparation of cytospins

Glass slides were acid washed (1M HCl) overnight, and rinsed thoroughly in distilled water. The cell density was adjusted to 10^5/ml in HBSS. The slide with a fresh filter was then inserted into the holder, which was placed in the cytospin bucket. 200µl of the cell solution was pipetted into each filter cup and spun for 2 minutes at 40 x 10 rpm. The slides were then left to air dry for 5 – 10 minutes.

4.2.4 Cytotoxicity assays

When sufficient preliminary data on the action of the cytokines and their combined effects was obtained, cytotoxicity assays were performed to see if the cytokines had any effect on the function of the killer cells. These assays entailed co-incubating the cytokine-treated cells with K562 target cells. On day 1 peripheral blood was taken from volunteers and the CD56 cells were positively selected. These cells were split into two aliquots and one was used as control, and a combination of IL-2 (1ng/ml), IL-7 (50ng/ml) and IL-15 (50ng/ml) was added to the other sample. On day two both the control group and the sample group were sorted by flow cytometry into $CD56^+/CD8^+$ and $CD56^+/CD8^-$ fractions.

The K562 cells had been labelled with PKH 26 (for method see 3.2.3) and were co-incubated with all the NK samples at a 1:1 ratio. An additional triplicate culture contained K562 cells alone as a control to monitor spontaneous cell death. Each tube was made up to 1ml with DMEM/F12 supplemented with 10% AB serum and the cells were incubated for four hours at 37°C/5%CO_2. At the end of the incubation the tubes were centrifuged for 10 minutes at 200g and gently resuspended in 500ml PI/PBS. 10,000 target cells were acquired with 1024 channel resolution after electronic gating on the $PKH26^+$ cells, and they were stored in list mode data (Lysis II).

4.2.4.1 Method 1

The cytotoxicity assays were performed with purified CD56 selected cells. They were split into two groups and to one of the groups IL-2 and IL-15 was added and the cells were left overnight at 37°C/5%CO_2. Twenty-four hours later both groups were sorted by FACSVantage into $CD8^+$ and $CD8^-$ fractions. They were then incubated, in triplicate, at a 1:1 ratio with K562 cells (labelled with PKH26). The cells were suspended in 1ml of CM and placed at 37°C/5%CO_2 for four hours.

4.2.4.2 Method 2

As 4.2.4.1. but the $CD56^+/CD8^-$ fraction was sorted by MACS and FACSVantage, and only these cells were used for the experiment. In these experiments IL-2, IL-7 and IL-15 were used.

4.2.4.3. Method 3

As 4.2.4.1, but the $CD56^+/CD8^-$ population was pre-incubated with the three cytokines for 24 hours. By this point the cells were quite overgrown so they were split into two and the media was refreshed without additional cytokines. On day three they were phenotyped for CD8 expression. The $CD56^+/CD8^+$ and $CD56^+/CD8^-$ fractions were sorted by flow cytometry. They were then cultured, in triplicate, in a 1:1 ratio with K562 cells for four hours at 37°C/5%CO_2 (for method see 4.2.4).

Preliminary experiments had shown that cell viability in media without serum was below 10% by day 3, so in these experiments DMEM/F12 plus 10% AB serum replaced the original medium.

4.2.4.4 Method of monitoring cell death

Dead cells can be identified on the flow cytometer by changes in their light scatter properties, in that they usually show a decrease in forward scatter and an increase in side scatter compared to live cells (Schmid *et al.*, 2001). However a more reliable method was required for assay work. To distinguish how the cells died a flow cytometric method was used, essentially that described by Schmid *et al* which makes it possible to measure apoptosis (1994). The cells were stained as in the cytotoxicity assay, using PKH 26 to stain the K562 cells and then PI/PBS to monitor cell viability (section 3.2.3.). Electronic compensation was used between the fluorescence channels to remove spectral overlap. This was done by using single-stained cell aliquots to optimise the photomultiplier tube voltage settings, subsequently dual labelled

aliquots were used to fine tune the settings for the different fluorochrome interactions.

PI fluorescence was collected using a log scale but analysed against forward scatter on linear amplification. Live cells featured in the first log decade of PI fluorescence whereas those cells which had taken up PI, and were taken to be apoptotic, featured in the second log decade. Those cells which had fully absorbed PI were found in the third log decade and were taken to be necrotic (as descibed in Shapiro, 1995) (see Figure 4.16). A minimum of 10,000 events was collected on each sample, and analysis of the data was performed with Lysis II.

4.2.5 Survival, proliferation and viability of cytokine treated cells

To study the growth of the $CD56^+$ populations the cells were obtained from leucocyte preparations from the National Blood Service. They were sorted by magnetic beads and flow cytometry (see section 4.2.1.1. and 4.2.1.2). The cells were cultured in 96-well U-bottomed tissue culture grade plates (Nunc), and grown in 200µl cell suspensions of DMEM/F12 supplemented with 10% AB serum and 100 units/ml penicillin and 10,000 µg/ml streptomycin.

To gain preliminary data the CD56 cells were not sorted by $CD8^-$ and $CD8^+$ expression, and IL-15 was the only cytokine used to supplement the media to look at survival, proliferation and viability. As the experiments progressed the

cells were fractionated into $CD8^+$ and $CD8^-$ subsets, and IL-2 was added to the initial suspension of medium at day 1, after that the medium was refreshed with a suspension of medium containing IL-15 alone. In the preliminary experiments with IL-7, it was found that this cytokine did not greatly enhance the cellular response and it was therefore decided to concentrate on IL-2 and IL-15 in the later experiments.

The cells were split into three groups, one acted as the control, one was supplemented with IL-15 and one with IL-15 and IL-2. The dilution of IL-15 added at each time point after the initial dose was reduced to a maintenance dose of 10ng/ml. Half the medium was replaced every other day when the cells were counted. The controls were fed with the basic medium, all the other cell groups with medium supplemented with IL-15. The medium for all groups was pre-warmed at 37°C/5%CO_2. The numbers of cells cultured in each separate experiment varied according to the success of the sort, and ranged from $9x10^4$ to $5x10^5$. Triplicates were set up in each assay. When the cells were counted, an aliquot of cells was taken and diluted with trypan blue to assess viability.

4.3 Results

4.3.1 The impact of the cytokines

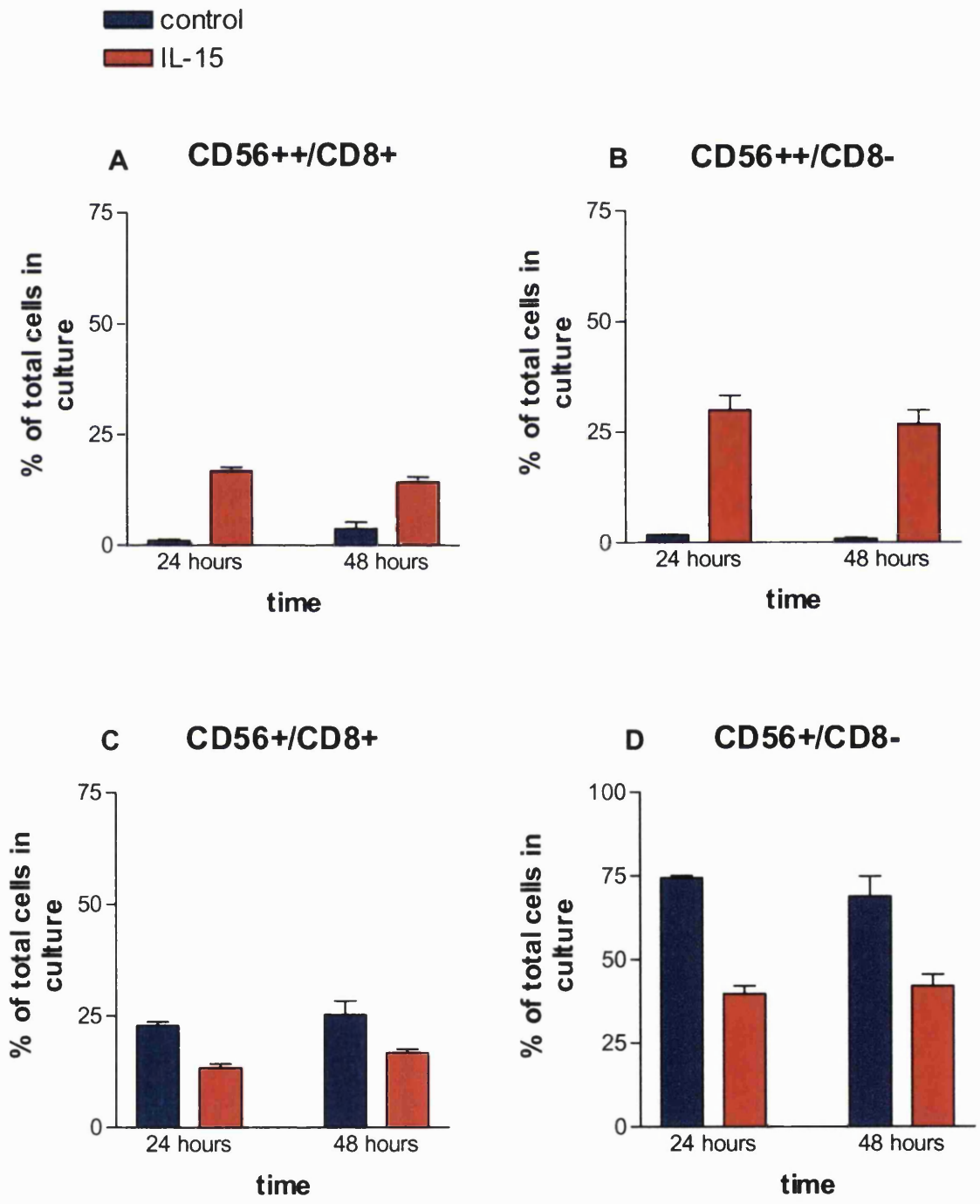
4.3.1.1 The effects of IL-15 on $CD56^+/CD8^-$ cells

Initially IL-15 was used alone and the cells were monitored for up to 48 hours. The cells in the control sample remained small and regularly shaped whilst the cells in the sample treated with IL-15 became enlarged and irregular in shape as would be consistent with activation. This proved to be typical of a pattern that emerged over subsequent experiments, the cytokines driving the cells into blastogenesis. In the absence of cytokines no blastoid cells were seen. The cells were phenotyped to see whether the cytokine had driven the $CD56^+/CD8^-$ cells to express CD8. $CD56^+/CD8^+$ cells appeared in both the control and the cytokine treated group at 24 hours but there was twice the proportion in the cytokine treated group as in the control group (Figure 4.7A and C). At 24 hours 15% of the control group no longer expressed CD56, and none of these cells expressed CD8.

The addition of IL-15 caused a rapid increase in the percentage of $CD56^{bright}$ cells, which peaked at 24 hours and then gradually diminished (Figure 4.7A and B). $CD56^{bright}/CD8^+$ and $CD56^{bright}/CD8^-$ were also generated from an initial pool of $CD56^{dim}/CD8^+$ and $CD56^{dim}/CD8^-$ cells. This was apparent because proliferation had not occurred at this time point, and yet an increase in the proportion of $CD56^{bright}$ expressing cells was seen (Figure 4.7A and B).

Figure 4.7 The effect of IL-15 on $CD56^{+}/CD8^{-}$ cells

50ng/ml of IL-15 was incubated with sorted $CD56^{+}/CD8^{-}$ cells for 48 hours, and the cells were phenotyped for CD56 and CD8 expression at 24 and 48 hours. The experiments were performed in triplicate and the error bars represent standard deviation. Four experiments were performed.



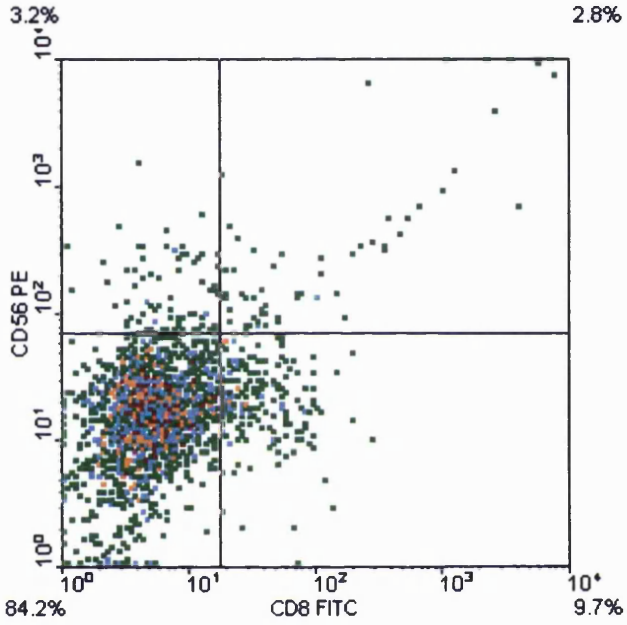
4.3.1.2 The effects of IL-7

The preliminary results with IL-7 alone showed that in the cytokine-treated group there was an increase in the percentage of $CD56^{bright}$ expressing cells at 48 hours over and above the control group of 10.9%. However, the percentage of cells that became $CD56^{+}/CD8^{+}$ cells was 0.6% less in the IL-7 treated cells as against the control group (Figure 4.8). Also those cells treated with IL-7 had a marginally higher survival rate than the cells in the control group, but less than those treated with IL-15. As can be seen from the graphs (Figure 4.9) the effects of co-incubating $CD56^{+}$ cells with IL-7 alone were not as marked as the results with IL-15 alone. Another difference between the effects of the cytokines was that the IL-7 group did not induce the blast morphology seen with co-incubation of CD56 cells either separately or together with IL-2 and IL-15. Also there was no increase in $CD8^{+}$ when $CD56^{+}/CD8^{-}$ cells were co-incubated with IL-7.

Figure 4.8 Dot plots showing the effects on CD56^{+} cells of co-incubation with IL-7

The quadrant statistics are placed so as to show the CD56^{bright} cells as against the CD56^{dim}. The percentages are placed in the corner of the quadrants. Plot A shows the control sample at 48 hours without the addition of cytokines; plot B shows the effect of co-incubation of IL-7 with CD56 cells at 48 hours.

A



B

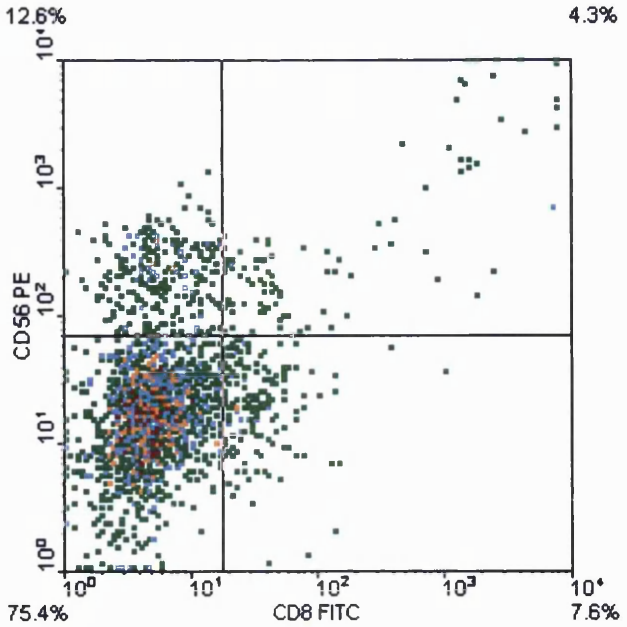
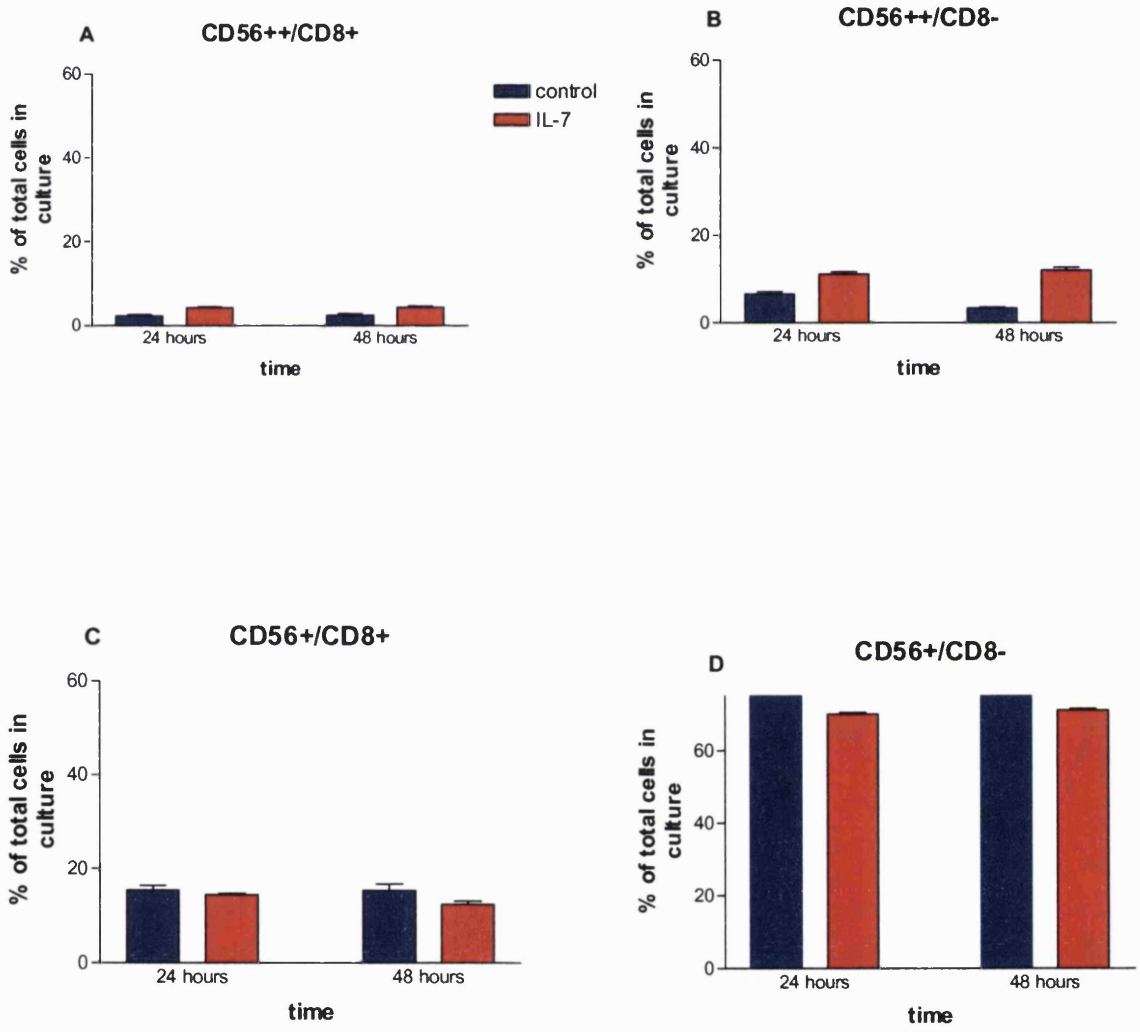


Figure 4.9 The effect of IL-7 on sorted populations of $CD56^{+}/CD8^{-}$ cells

50ng/ml of IL-7 was incubated with sorted $CD56^{+}/CD8^{-}$ cells for 48 hours, and the cells were phenotyped for CD56 and CD8 expression at 24 and 48 hours. The experiments were performed in triplicate and the error bars represent standard deviation. Four experiments were performed.



4.3.1.3 The effects of IL-2 and IL-15

The experiments using IL-15 and IL-2 showed that there was always a substantial increase in the percentages of $CD56^{bright}$ cells with the addition of cytokines. At 24 hours the percentage of $CD56^{bright}$ cells was 5.4% (sd 0.0) in the control group compared with 41.2% (sd 2.8) in the cytokine treated group. By 48 hours the differential in $CD56^{bright}$ expression had lessened and the dot plots (Figure 4.10) showed an increase of 3.8% for the cytokine treated cells as against the control group. The subsets fractionated into $CD8^+$ and $CD8^-$ are shown in Figure 4.11A.

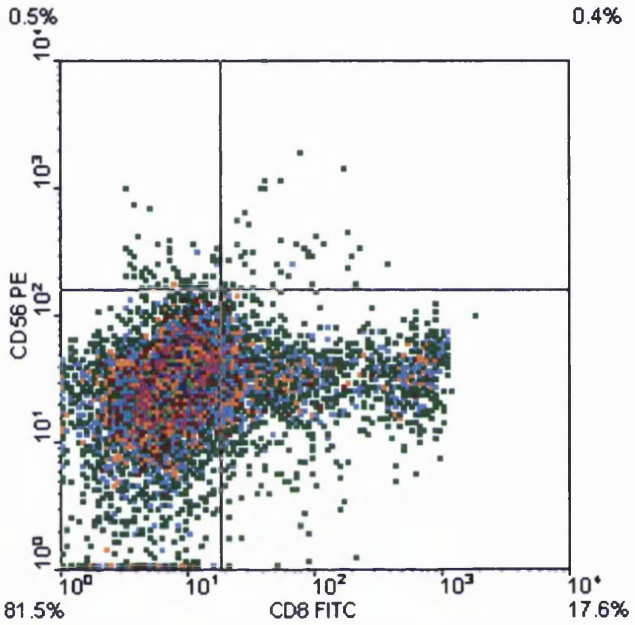
There was no marked increase in the proportion of $CD8^+$ cells, in the control group. However, in the cytokine treated group a slight increase in CD8 expression was apparent at 24 hours (4.5%) which rose to a 12.2% increase over the control at 48 hours. These experiments were run for up to six days by which point 95.7% (sd 1.4) of the cells were $CD56^{bright}$ and of these 93.6% (sd 2.0) were $CD8^+$. By day 6 the cells in the control group had all died (Figure 4.11B).

Figure 4.12 shows the effects of overnight culture of different combinations of the three cytokines on NK cells. The change in CD8 expression of the sorted $CD56^+$ cells is shown as is the lack of CD3 expression.

Figure 4.10 Dot plots showing the effects of IL-2 and IL-15 on $CD56^+$ cells

Plot (a) shows the control sample at 48 hours with no cytokines and plot (b) shows the effects of IL-2 and IL-15 at 48 hours.

A



B

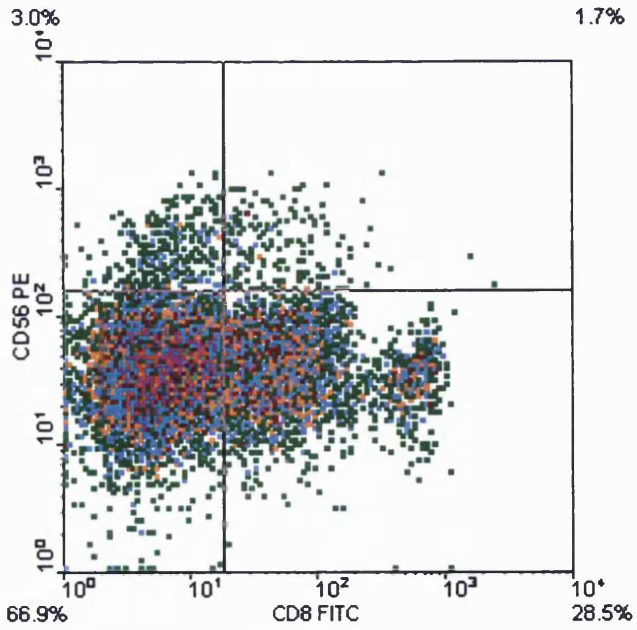
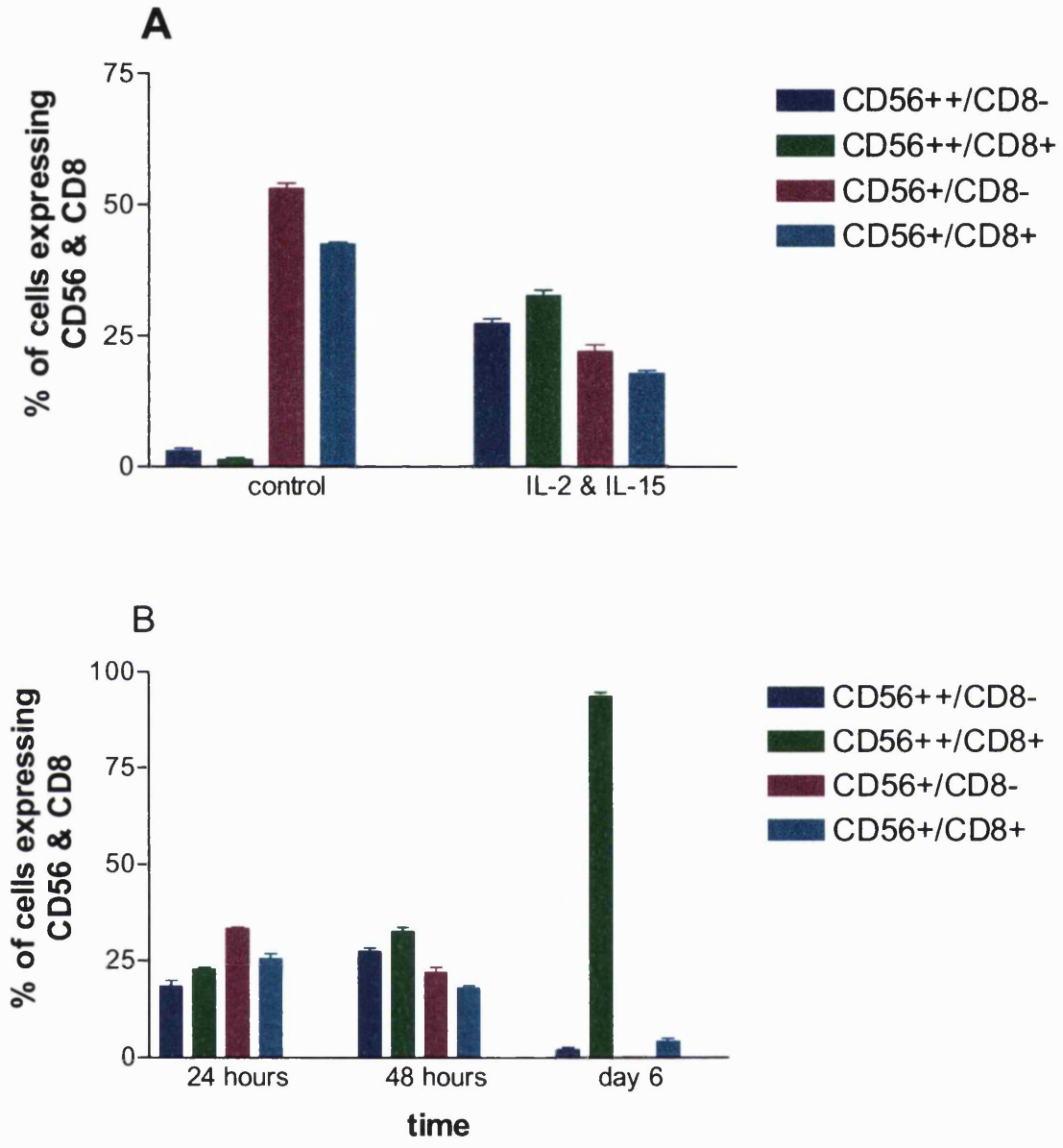
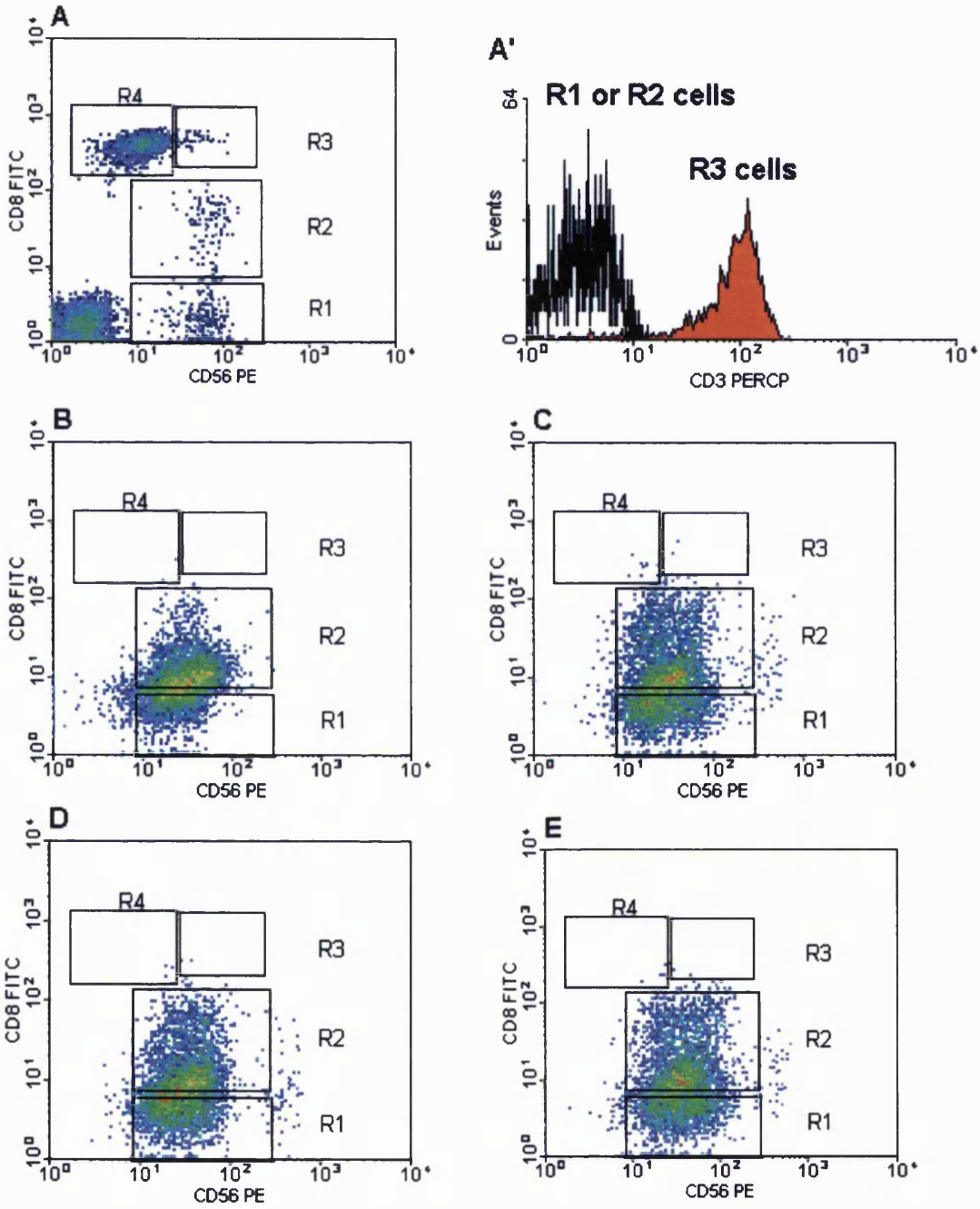


Figure 4.11 The effects of IL-2 and IL-15 on $CD56^{+}/CD8^{+}$ expression



Graph A depicts the comparison between the different subsets of CD56 cells treated with cytokines or alone at 48 hours. Graph B shows the response of the CD56 cells to the cytokines over a 6 day period. In both experiments the cultures were co-incubated in triplicate. These graphs present the means of results from three donors, each analysed in triplicate (error bars are standard deviation).

Figure 4.12 The effect of overnight culture of human NK cells with IL-2, IL-7 and IL-15.



The effect of overnight culture on CD8 expression on human NK cells:
A – 2-D density plot showing the initial PBMC sample containing $CD56^+/CD8^-$ cells (R1), $CD56^+/CD8wk^+$ cells (R2), $CD56^+/CD8^+$ cells (R3) and $CD56^-/CD8^+$ cells (R4). $CD56^+$ cells lacking CD8 or which were $CD8wk^+$ (R1 or R2) were CD3 negative whilst the $CD56^+/CD8^+$ cells (R3) were exclusively $CD3^+$ as shown in histogram A'.
Plots B-E show the change CD8 expression on the sorted $CD56^+$ cells after overnight culture in: B – medium alone, C – medium plus IL2and IL15, D – medium plus IL2 and IL7, E – medium plus IL2 and IL7 and IL15

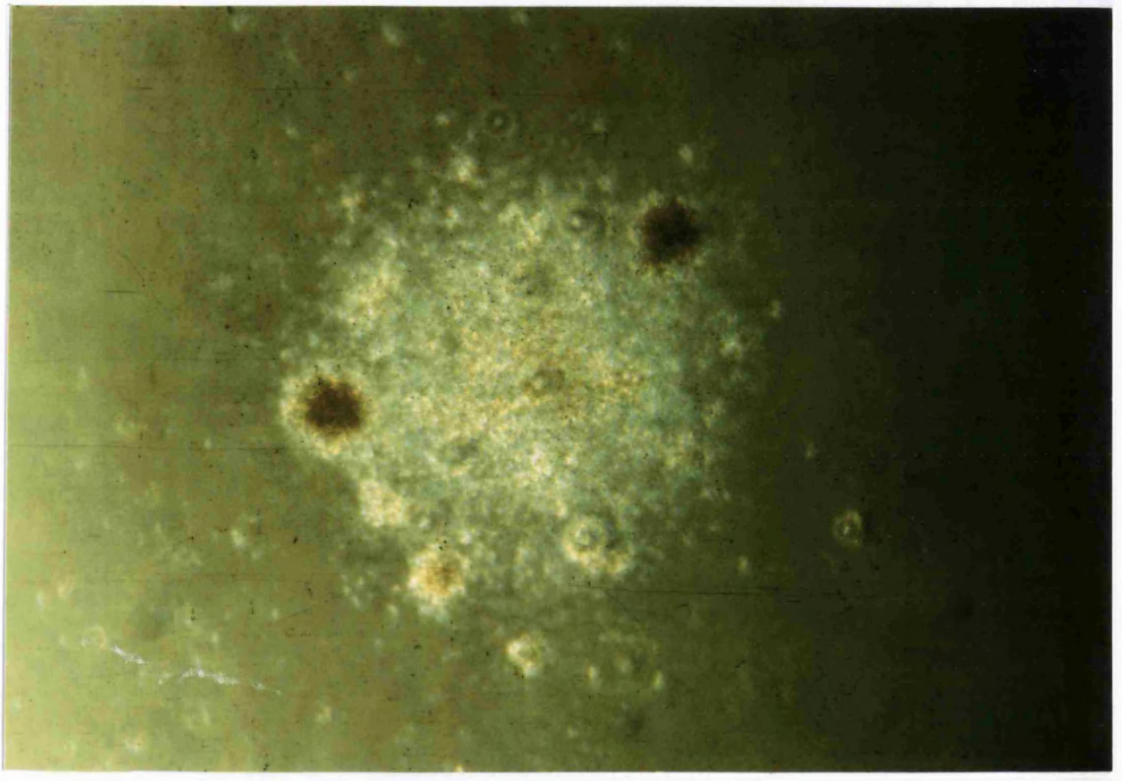
4.3.2..Survival and viability of cytokine treated cells

The cells treated with IL-15 alone looked different to the control group by light microscopy as early as day 1. These cells became elongated and clumped together, but within those clumps discrete cells could be seen. They separated into a number of distinct groups whereas the non-IL-15 treated cells settled at the bottom of the well in no distinct pattern. Over the subsequent days these differences became more pronounced (Figure 4.13). Non-IL-15 treated cells showed signs of poor viability by day 2 and were all dead by day 5, (as determined by Trypan Blue exclusion). Whereas cells treated with IL-15 maintained viability extremely well (Figure 4.14). In preliminary experiments with IL-2 alone there was a 74% loss in survival of the cytokine-treated cells by day 4.

In another series of experiments the cells were maintained for 8 days. The $CD56^{+}/CD8^{wk+}$ and the $CD56^{+}/CD8^{-}$ cells were co-incubated with IL-15, IL-2 and IL-15 or medium alone and monitored for viability. The graphs showed that those cultures receiving cytokines were able to maintain viability for the duration of the experiments, although as expected the controls died. There was double the proliferation in the $CD56^{+}/CD8^{-}$ fraction as against the $CD8^{+}$ fraction (Figure 4.15). The $CD56^{+}/CD8^{-}$ cells following culture with IL-2 and IL-15 for more than 6 days uniformly acquired CD8 expression (section 4.3.1.3.). This lack of proliferative response in the $CD56^{+}/CD8^{wk+}$ fraction suggested that CD8 expression may delineate a chronically activated population that is terminally differentiated into an effector cell.

Figure 4.13 The appearance of cells (A) co-incubated with IL-15 and (B) in medium alone using light microscopy

A



B

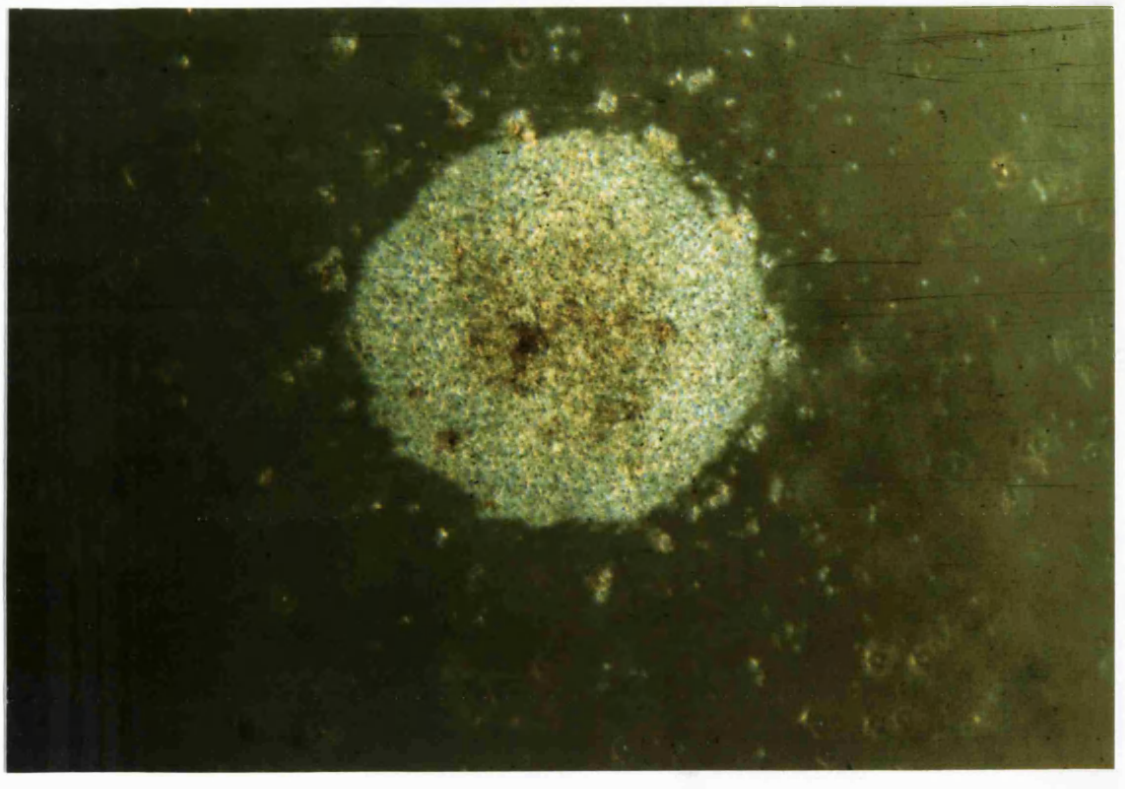
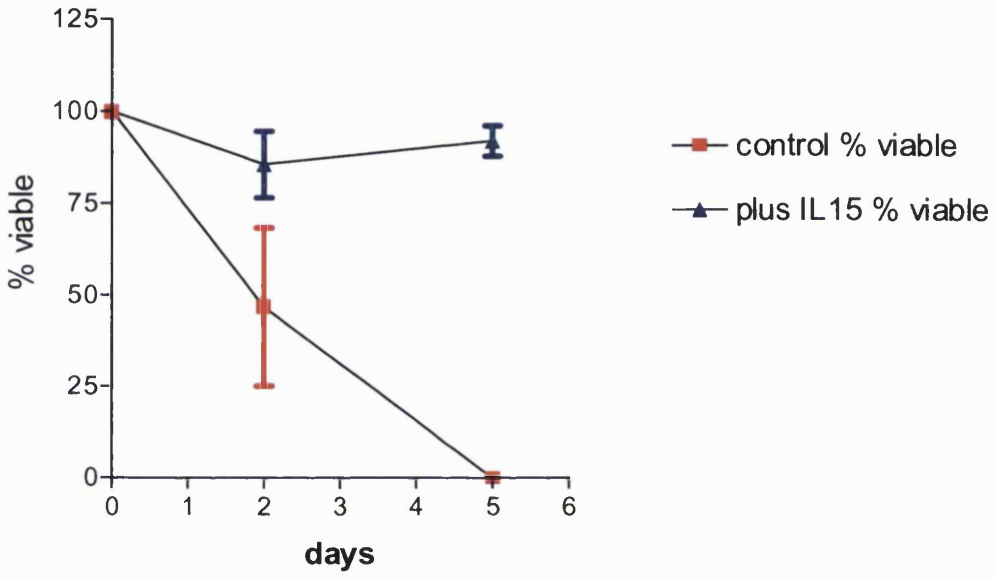
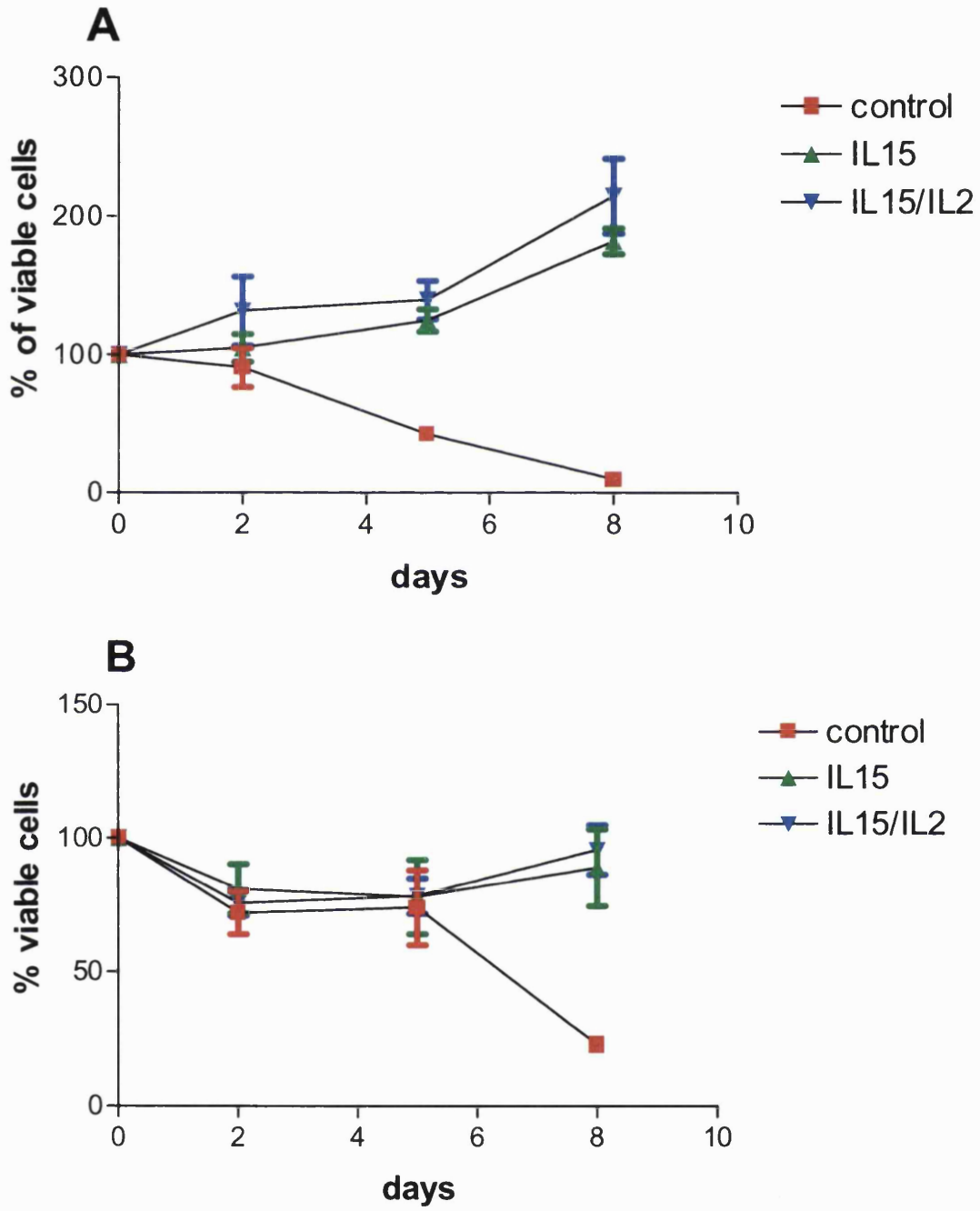


Figure 4.14 Viability of $CD56^{+}/CD3^{-}$ cells co-incubated with IL15



This graph shows the number of live cells counted by Trypan Blue exclusion as a percentage of total cells counted/ml. With this series of experiments the $CD56^{+}$ cells were depleted of $CD3^{+}$ cells but not fractionated into the $CD8^{wk+}$ and $CD8^{-}$ subsets. A maintenance dose of 10ng/ml of IL-15 was used. This graph presents data from three experiments and the error bars depict standard deviation. At day 0 and day 5 the results of the control group were so similar that the error bars do not appear on the graph.

Figure 4.15 Survival and viability of $CD56^{+}/CD8^{-}$ and $CD56^{+}/CD8^{wk+}$ sorted cells with the addition of IL-2 and IL-15.



Graph A shows the survival of $CD56^{+}/CD8^{-}$ cells and graph B the survival of $CD56^{+}/CD8^{wk+}$ cells in culture alone, with IL-15 or a combination of IL-15 and IL-2. The cultures were set up in triplicate and the graphs present mean results from three donors. The error bars depict standard deviation.

4.3.3 Cytotoxicity assays

4.3.3.1 Without cytokines using K562 as target cells

Freshly isolated $CD56^+/CD8^-$ and $CD56^+/CD8^{wk+}$ subsets from three donors were co-incubated with K562 cells at a 1:1 E:T ratio. The background cell death of K562 cells alone was 16.0% (sd 1.8%) and the total death with K562 cells co-incubated with $CD56^+/CD8^-$ was 35.1% (sd 4.9%) making the specific lysis 19.1%. The killing in the $CD56^+/CD8^{wk+}$ fraction co-incubated with K562 cells was 29.4% (sd 4.8), making the specific lysis 13.4%. This meant the difference in killing was 5.7%.

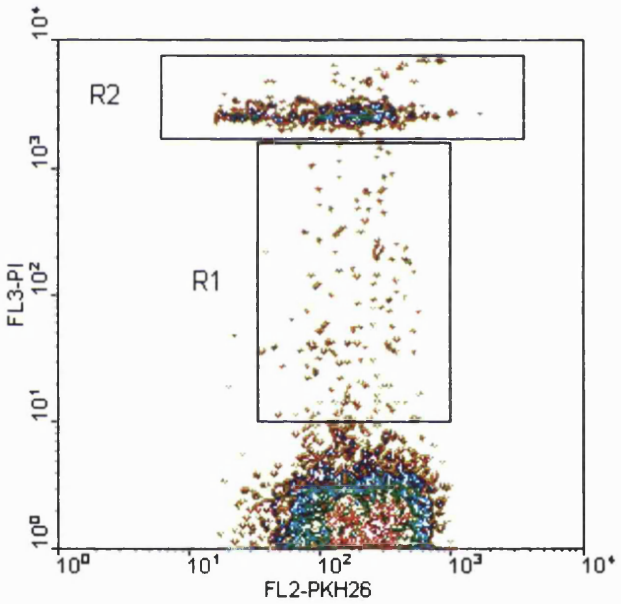
4.3.3.2 With cytokine-enhanced cells and K562 targets

Cytotoxicity assays were performed with cells co-incubated with IL-2 and IL-15 (for method see 4.2.4 and method 1). The cytokine-enhanced group exhibited 17.55% specific lysis over background and 11.49% specific lysis over the non-cytokine-treated group. When studying the fractions separately as $CD56^+/CD8^{wk+}$ and $CD56^+/CD8^-$ cell subsets there was 9.12% specific lysis in the $CD56^+/CD8^-$ cytokine treated group over the non-cytokine treated control group and 2.37% specific lysis in the $CD56^+/CD8^{wk+}$ fraction of cytokine-treated over the non-cytokine treated group. Most of the killing therefore resided in the $CD56^+/CD8^-$ population.

Subsequent cytotoxicity assays concentrated on the $CD56^+/CD8^-$ fraction (see method 2). When the cells were treated with IL-2, IL-7 and IL-15 for the cytotoxicity assay there was 22.7% specific lysis in the cytokine-treated group over the non-cytokine treated group, and 35.5% specific lysis in the cytokine

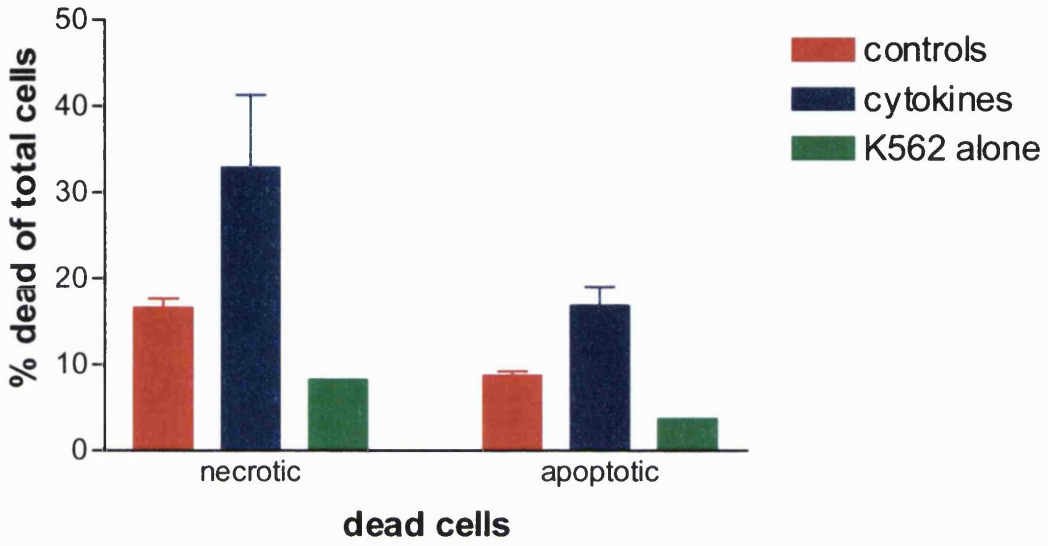
treated group over background cell death. Double the percentage of cells died by necrosis as opposed to apoptosis in the cytokine-treated group (Figure 4.16 and 4.17).

Figure 4.16 The positioning of the live, apoptotic and necrotic populations by dot plot.



R2 shows high PI fluorescence, denoting those cells which are necrotic, and have therefore absorbed PI because the integrity of the membrane has been lost. R1 shows the cells deemed to be apoptotic on the basis that the membrane has absorbed a limited amount of PI.

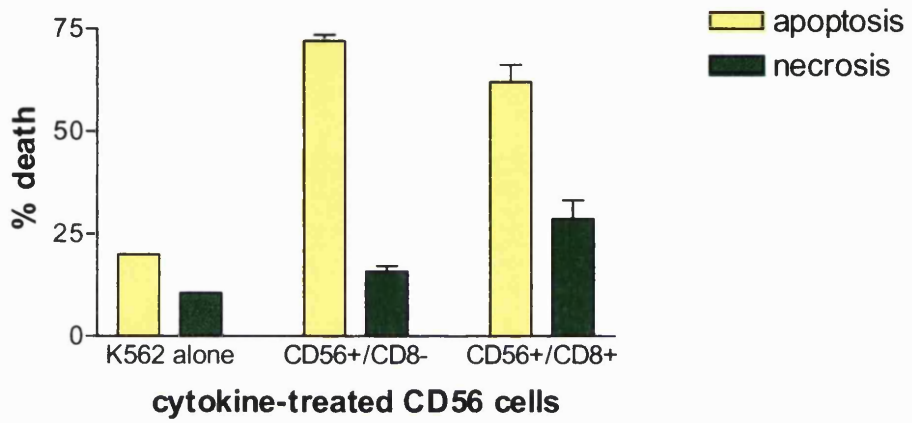
Figure 4.17 Cytotoxicity assay showing killing of K562 by $CD56^+/CD8^-$ cells co-incubated with IL-2, IL-7 and IL-15



The graph shows the total death of target cells by $CD56^+/CD8^-$ cells either alone or after incubation with three cytokines. Data presented are means and standard deviations from three experiments. In the graph the different methods by which the cells died either by necrosis and apoptosis has been shown. The results for K562 cells alone were so similar that error bars do not appear on the graph.

Taking this series of experiments one step further $CD56^+/CD8^-$ cells treated with all three cytokines (see method 3) were sorted into $CD56^+/CD8^{wk+}$ and $CD56^+/CD8^-$ fractions and incubated with K562 (Figure 4.18). When phenotyped on day 3 prior to incubation with the target cells 25% of the $CD56^+/CD8^-$ cells had become $CD8^+$. The results show that in the $CD56^+/CD8^-$ fraction there was 57.3% specific lysis over K562 alone, and in the $CD56^+/CD8^+$ fraction this was 60.1%. Resulting in only a 2.8% difference in killing by $CD8^+$ or $CD8^-$ cells in favour of the $CD8^+$ fraction.

Figure 4.18 Cytotoxicity assays depicting $CD56^{+}/CD8^{-}$ & $CD56^{+}/CD8^{wk+}$ cells co-incubated with IL-2, IL-7 & IL-15 incubated with K562 cells



The graph shows the killing of K562 by $CD56^{+}$ subsets. This data presented are the means from three experiments, and the error bars depict standard deviation. As in previous experiments the results for K562 alone were so similar that no error bars appear on the graph. Although the graph shows the total percentage of death for the controls and the $CD56^{+}$ subsets, the percentage of death has been further divided into death by apoptosis or necrosis determined by the regions in which the dead cells appeared on the flow cytometer screen during analysis.

To clarify by what means the cells were dying they were sorted in one cytotoxicity experiment by the region in which they appeared on the flow cytometer screen. Electron microscopy was then performed on these sorted cells to look for signs of apoptosis or necrosis. Figure 4.19 A, B and C show examples of the cells that were sorted.

Figure 4.19 A Examples of cells from the cytotoxicity assays after electron microscopy

A The E.M. shows cells sorted by FACSVantage. These cells were taken from R1 (Figure 4.16)(a) K562 with the appearance of a necrotic cell (b) an NK cell.

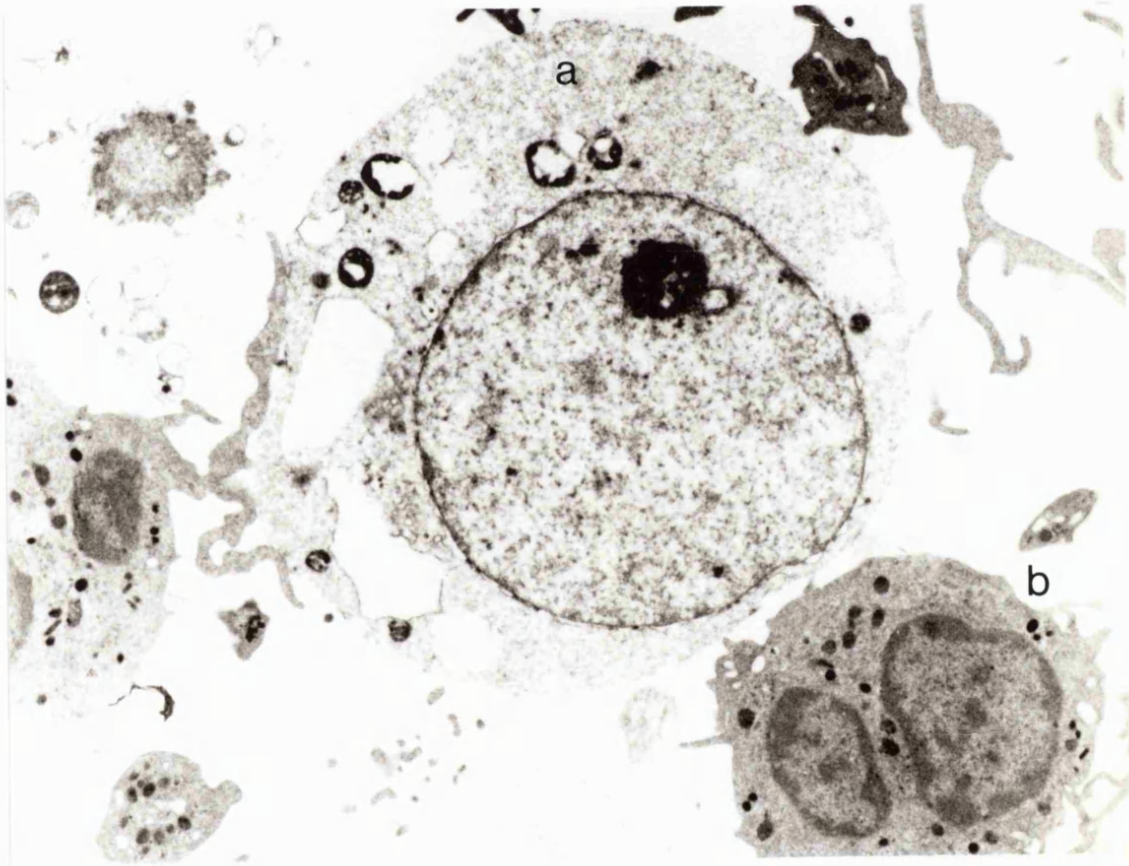


Figure 4.19 B

The E.M. shows cells sorted by FACSVantage. These cells were taken from R1 (Figure 4.16)(a) a K562 cell which appears to be necrotic (b) an NK cell

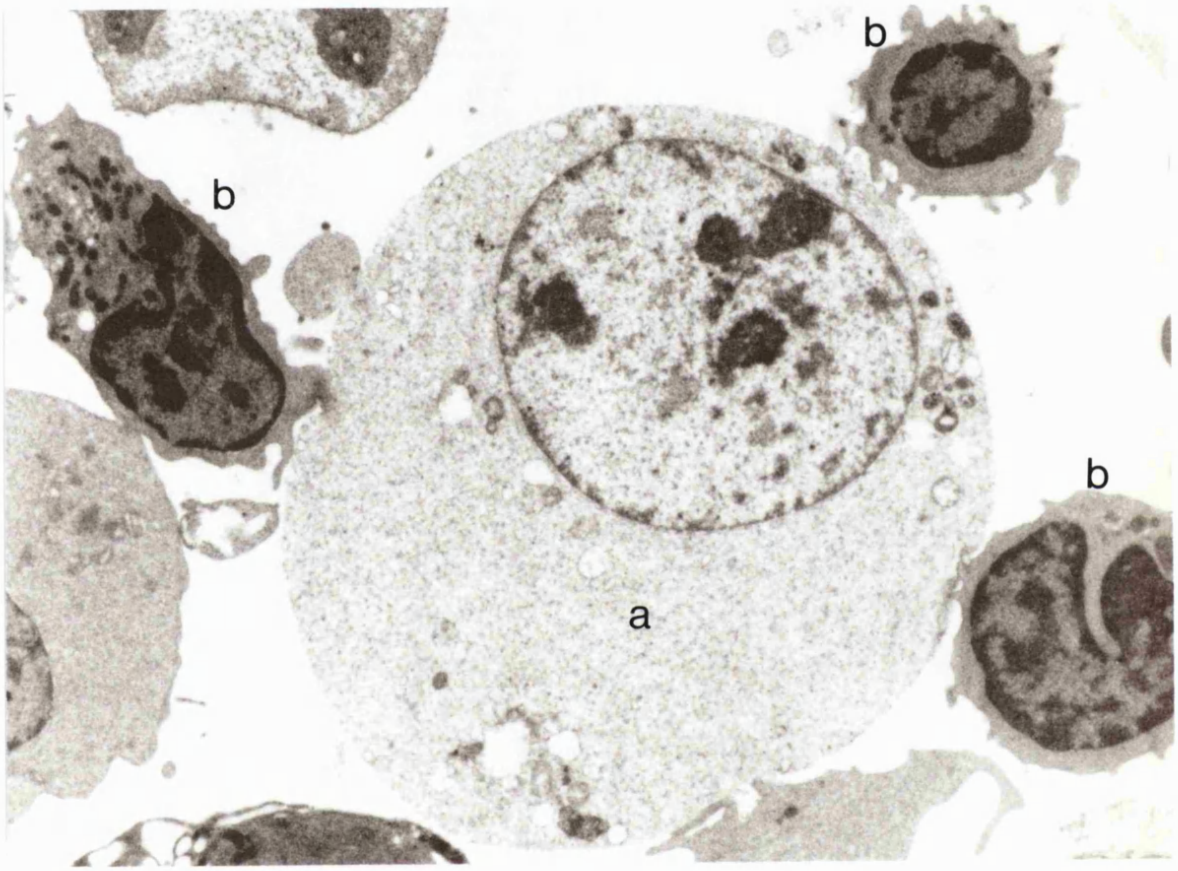
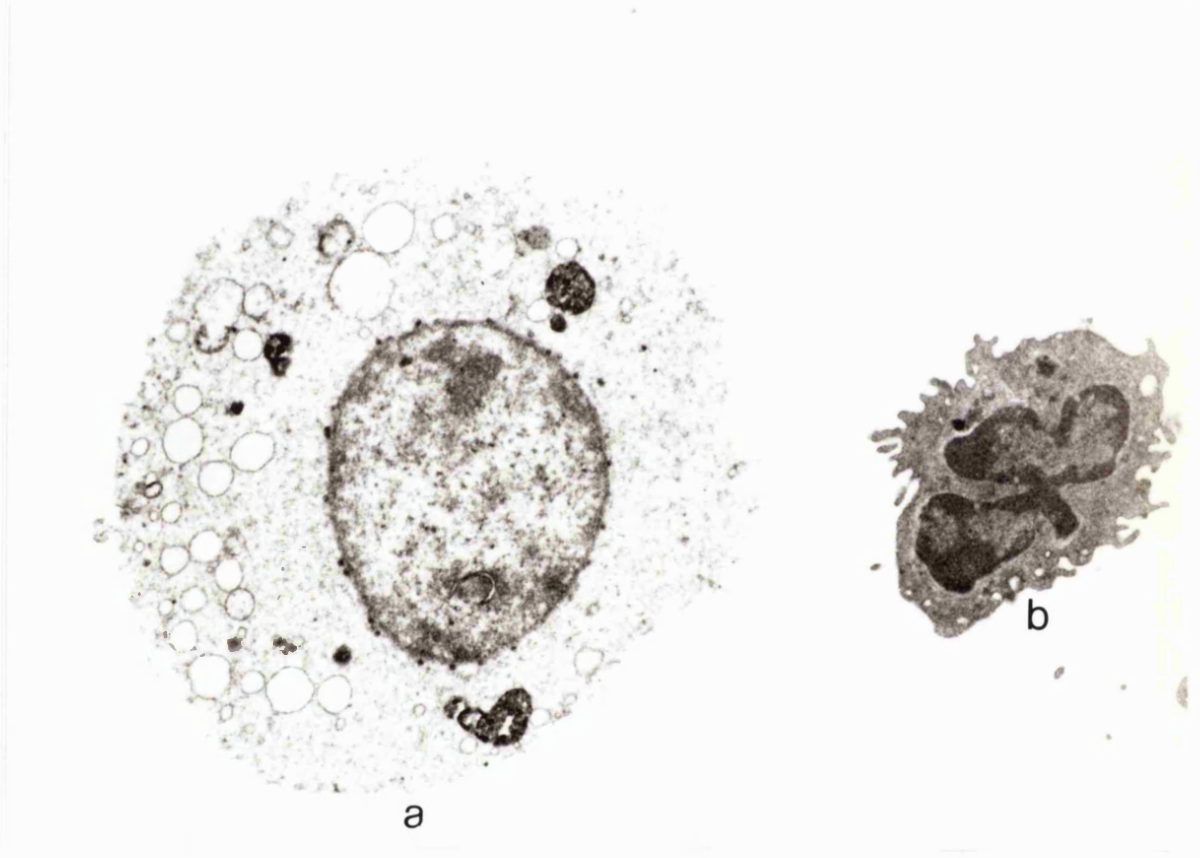


Figure 4.19 C

The EM shows cells sorted by FACSVantage. These cells were taken from R1 (Figure 4.16)(a) a K562 cell which appears to be necrotic and (b) NK cells



4.4 Discussion

The aim of this series of experiments was to see if the cells that showed cytotoxic activity against autologous leukaemic blasts, (detailed in chapter 3) could be generated and maintained *in vitro*. The $CD56^{+}/CD8^{wk+}$ subset does not constitute the majority of $CD56^{+}$ cells usually it makes up about 30% of the CD56 compartment. The experiments were designed to see if these cells could be generated from $CD56^{+}/CD8^{-}$ cells by treating them with designated cytokines, and then to see if these cells also had cytotoxic ability. A further aim was to monitor the ability of these cells to survive and proliferate.

Initially the $CD56^{+}/CD8^{-}$ subset was looked at to see if it was possible to generate the $CD56^{+}/CD8^{wk+}$ subset. There were two reasons for this first in order to sort the $CD8^{+}$ population directly the cells had to be labelled with CD8 monoclonal antibody. Although the role of CD8α on NK cells is unknown there was concern that the monoclonal antibody may block important interactions at the effector: target junction. Second the $CD56^{+}/CD8^{-}$ population, as the majority population, was easier to sort in more substantial numbers, and this gave larger cell numbers on which to conduct the preliminary experiments.

There were two main findings from the series of experiments incubating cytokines with NK cells. The cytokines appeared to expand $CD56^{bright}$ cells whilst leading to a reduction in the proportion of $CD56^{dim}$ cells, and the longer the cells were maintained the greater the proportion of $CD56^{bright}/CD8^{+}$ cells. In both cases the shift in expression could have been due to the fact that the

$CD56^{dim}$ cells and the cells lacking CD8 were preferentially dying. This would increase the proportion of $CD56^{bright}/CD8^{+}$ cells, however this is not supported by the fact that initially there was no significant change in cell numbers. Those cells which down-regulated CD56 (predominately in the non-cytokine treated group) lacked CD8 expression when phenotyped, and it could be that over six days those cells that did not initially express CD8 or shed CD8, lost the protection apparently conferred by cytokines. The ability of IL-2, IL-7 and IL-15 to protect cells from apoptosis by the up-regulation of bcl-2 has been observed previously (Armant *et al.*, 1995, Carson *et al.*, 1997). This does not explain why after only 24 hours in culture, up to 15% of cells in the control group lost their CD56 expression, although it may have been a consequence of the selective procedure itself as it involved ligation of the CD56 antigen with monoclonal antibody.

It has been shown in these experiments that it is possible to generate $CD56^{+}/CD8^{+}$ cells from $CD56^{+}/CD8^{-}$ cells. Also those $CD56^{+}$ cells that became $CD8^{+}$ through incubation with cytokines appeared to be more lytic to the K562 target cells than those which remained $CD8^{-}$ and these data have been confirmed by subsequent work in this group (O'Neill *et al*, manuscript submitted). However the $CD56^{+}/CD8^{-}$ cells were also able to lyse to K562 cells although to a lesser degree. The survival assays showed that given enough time the $CD56^{+}$ cells treated with cytokines that survived all became $CD8^{+}$. It may be that these cytokine-generated $CD56^{+}/CD8^{+}$ cells are terminally differentiated into effector cells.

These experiments show that NK cells treated with cytokines, become $CD56^{bright}$. However the role of $CD56^{bright}$ cells has yet to be fully understood. An earlier study found this population to be more potent LAK (lymphokine activated killer) effectors than the $CD56^{dim}/CD16^{+}$ cells (reviewed in Robertson and Ritz, 1990) but later studies have suggested they are less lytic than their $CD56^{dim}$ counterparts (Jacobs *et al.*, 1992). The evidence to date is that these cells express the high affinity IL-2-receptor and react with greater sensitivity to cytokine stimulation, as one would expect from cells with the high affinity receptor. This might lead one to suspect that they are more lytic because it is known that cells treated with IL-2 become LAK cells and have greater lytic potential than untreated NK cells and can even kill target cells resistant to NK cell lysis.

Some work has been done on characterising $CD56^{dim}$ and $CD56^{bright}$ cells (Sedlmayer *et al.*, 1996, Jacobs *et al.*, 1992). Different theories have emerged to date but one is that these cells represent two distinct types of NK cell, both of which have the potential to proliferate massively if given the right stimulus. This theory suggests that $CD56^{dim}$ cells do not become $CD56^{bright}$, because $CD56^{bright}$ cells are a separate entity deriving from a different precursor cell (Nagler *et al.*, 1989). This is at odds with the idea that these cells represent functional stages, and that $CD56^{dim}$ cells respond to a specific stimulus such as the appropriate cytokine and become $CD56^{bright}$. In the experiments documented here very few cells co-incubated with cytokines were $CD56^{dim}$ by day 8.

In one study, $CD56^{bright}$ cells have been observed for a mean period of 17 weeks post-transplant, increasing their proportion to a maximum of 70% of all NK cells in the culture (Jacobs *et al.*, 1992). In addition to being $CD16^-$ $CD3^-$ these $CD56^{bright}$ cells were characterised as $CD26^+$, $CD2R^+$ (CD2 receptor), $HLA\text{-}DR^+$, $p75^+$ (β chain of the IL-2R). Whereas $CD56^{dim}$ did not express these activation antigens, and it has subsequently been shown that the $CD56^{bright}$ cell subset expressed the intermediate affinity α-chain of the IL-2R (Warren *et al.*, 1996). Despite expressing activation molecules, in cytotoxicity assays freshly isolated $CD56^{bright}$ cells were found to be far less lytic than their $CD56^{dim}$ counterparts against K562 and L1210 (a murine lymphoma cell line). The addition of IL-2 and IFN-γ enhanced their lytic potential to a greater degree than the $CD56^{dim}$, but total killing by the $CD56^{dim}$ subset was still greater (Jacobs *et al.*, 1992).

Another group has shown that cells can survive in serum-free medium with the addition of IL-15 alone (Carson *et al.*, 1994). This was not borne out by this series of experiments, in which the cells in serum-free media only survived marginally longer with IL-15. However when grown in Dulbeccos MEM/F12 medium as detailed by Pierson and colleagues (1995) the addition of cytokines made an immense difference to the ability of the cells to survive. The substrates that make up foetal calf serum, which was used in these experiments, appear to be crucial to the ability of the cells to thrive. The exact components of serum are as yet undefined and despite intensive research synthetic serum for growth of NK cells has not yet been produced. This product would be valuable to ensure that cells that had been co-incubated with

cytokines (in order to be returned to the patient) were not contaminated by animal products. It would also lessen the likelihood of batch variation which can occur with foetal calf serum.

The experiments in this chapter show that in addition to serum the cytokines were important for the survival of the cells. It suggests that there may have been an interaction between the serum factors and the cytokine-enhanced cells. During culture the cells developed small colonies which appeared as clumps of cells in the flasks. The clumping of the cytokine-treated cells could be due to the fact that as the cells became blastoid they expressed adhesion molecules, became stickier and tended to clump together. Unfortunately the clumps could not be reduced to a single-cell suspension to make analysis of the cells by flow cytometer possible. The control sample cells, which had not been activated, remained discrete entities.

In the cytotoxicity assays the mechanism of killing appeared to be split between apoptosis and necrosis. One mechanism for $CD56^+$ NK killing is probably due to the perforin/granzyme system (van den Broek *et al.*, 1998). Killing of the target is preceded by the release of granules containing perforin and the serine proteases granzymes A and B. The perforin allows the granzymes access to the target cell by creating a pore in the plasma membrane (Wickremasinghe and Hoffbrand, 1999). This is known as the secretory (necrotic) mechanism, and is thought to be operative only in the killing of NK-sensitive targets, such as K562, and rare leukaemia cell lines. Non-secretory (apoptotic) mechanisms can destroy a wide variety of NK-resistant solid tissue-derived tumour cells by

the interactions between Fas ligand on effector cells and Fas receptor on targets (Vujanovic *et al.*, 1996, Kashii *et al.*, 1999).

Those cells treated with cytokines had an increased capacity to kill, and the majority of killing appeared to be by necrosis, whether the CD56 cells were CD8 positive or not did not appear to be a key factor. This may be explained by the nature of the target cells which largely showed high levels of resistance to apoptosis, and the type of target cell which in this case was a leukaemia cell line more prone to necrotic cell death than a solid tumour cell line. It may also reflect the relatively short time course of the cytotoxicity assay although high levels of apoptosis have been found after only 3-4 hours of co-incubation of effectors with targets (Rodella *et al.,* 1998). This means that apoptosis could occur within the incubation time frames of experiments in this study.

The work conducted on apoptosis and necrosis in these studies was based on a previous observation that cells differed in the way PI was absorbed depending on whether they were damaged by apoptosis or necrosis (Schmid *et al.*, 1994). After a cytotoxicity assay in which K562 cells were incubated with NK cells, the cells that absorbed PI were subsequently sorted by FACSVantage depending on the degree of fluorescence they emitted and viewed by electron microscopy. However the findings did not confirm Shapiro's earlier observations (1995). In the strongly fluorescent PI region necrotic cells were found. However, in the weakly fluorescent PI region cells were found which appeared to be necrotic and an absence of those with the appearance of apoptotic cells.

This put into question the work on apoptosis and necrosis, and meant further consideration needed to be given to the possible detection of these different mechanisms of killing by FACS analysis. It did not invalidate the data in that the cells that were identified that absorbed PI were definitely dead. It did mean that the different pathways of death were not so easily identified. There are a number of reasons why this may not have been a good predictor of the mechanism of cell death in this assay. One possible reason is that the targets that were used in these assays were prone to die by the necrotic pathway as opposed to the apoptotic (Vujanovic *et al.*, 1996).

It is also possible that untreated NK cells predominantly kill by apoptosis, and cytokine-treated NK cells by necrosis. This is supported by the fact that LAK cells have more cytoplasmic granules than resting NK cells. It may also be that cells kill by different mechanisms in *in vitro* studies as opposed to *in vivo.* There is also some evidence to suggest that the method by which cells kill is dependent on the ratio of effectors to targets and that apoptosis is preferentially induced at low E:T ratios (Rodella *et al.*, 1998).

These experiments show that it was possible by the co-incubation of $CD56^+$ cells with cytokines to generate $CD8^+$ cells, which were cytotoxic. It was shown that even after a number of days in culture these cells were cytotoxic and that it was possible to maintain these cells in culture. The distinction in lytic ability between the $CD56^+/CD8^+$ and $CD56^+/CD8^-$ subsets that was seen in the experiments in Chapter 3 was not so clear in this chapter. This may well be due to the fact that these experiments were conducted with allogeneic targets

whereas the results in the previous chapter showed the difference in the lytic ability of the two subsets with autologous targets.

5 General Discussion

This project set out with the ambitious remit of defining the cellular mediators of allogeneic GvL activity. It started with an extremely broad base monitoring the immune reconstitution of patients post-BMT, with various types of leukaemia, each of whom had undergone one of four different types of BMT. It finished with the study of a small subset of one cell type in one type of transplant and most successfully in one type of disease. Originally it had been hoped that the issue of GvL in allogeneic transplant recipients (given that GvL is predominately associated with such transplants) would be addressed. This invited an initial comparison of TCD allogeneic transplant recipients to see if a high degree of TCD affected GvL activity as has previously been reported. The results of these experiments led to a far more controversial study of GvL in autologous transplant recipients.

As has been documented in the literature it was found that NK cells recovered to normal levels within the first three months post allogeneic and autologous BMT. It is difficult to assess the role of NK cells in GvL activity in the allogeneic setting. $CD8^+$-specific TCD in CML reduced GvHD and yet did not lead to a total loss of GvL as evidenced by the lack of leukaemic relapse in these patients (Champlin *et al.* (1990) and this has been borne out by follow-up studies with $CD8^+$-TCD DLIs (Shimoni *et al.*, 1999). This implies that either $CD4^+$ cells or NK cells are the mediators of GvL in the allogeneic setting. However, it is clear from this study and those of others (Lowdell *et al.*, 1998) that even in T-replete BMT, $CD4^+$ T cell reconstitution is very slow suggesting NK cells may be

possible mediators of GvL activity in allogeneic BMT recipients. The issue is fraught with conflicting observations. It maybe that it is evidence of a 'back-up' device by the immune system in that when the normal balance of lymphocytes is disturbed, for example when T cell subsets are removed, NK cells proliferate and can fulfil the role of other cell types.

Bacigalupo and co-workers (1991) in a study in allogeneic patients, discussed in Chapter 2, suggested that allogeneic GvL is a phenomenon of the first few weeks post-transplant. In Chapter 4 it was found that $CD56^+/CD8^+$ cells were the cells that mediated leukaemia specific cytotoxicity (LSC) in the autologous setting. Previously in Chapter 2 it had been noted that by one month 42% of $CD56^+$ NK cells expressed the CD8α chain in autologous transplant recipients, compared with 18% of NK cells in fully TCD allogeneic transplant recipients, and 24% in non-TCD allogeneic transplant recipients. This high percentage of CD56 cells that express CD8 may therefore be of importance in GvL in autologous transplantation, and may be less important in the allogeneic setting where T cell recognition of minor histocompatibility antigen mismatches is possible.

When T cells are retained within the allograft they have a high frequency of alloreactive cells which may eliminate residual leukaemia cells and are likely to mediate GvHD, increasing morbidity and mortality. However, some methods of T cell depletion eliminate NK cells as well, therefore T-cell specific cell depletion which retains NK cells may improve the relapse risk as compared to non-T-cell specific TCD. The method of TCD may therefore become a crucial factor.

After autologous and allogeneic BMT the pattern of immune reconstitution determines that for some time after transplant the normal balance of lymphocytes will be disrupted. It is possible in this situation that cells may perform a role that is either not usually required of them or is more naturally performed by another cell subset. As with other investigators this study has noted the rise of NK cells and the over-production of $CD8^+$ T cells early on in the post-transplant period, as against the slow return of CD4 T cells.

In the case of allogeneic transplantation this may be due to the influence of the inflammatory cytokine response which is probably crucial to the timing and pattern of the re-population of different lymphoid subsets. Autologous transplant recipients suffer a lesser inflammatory cytokine response because they undergo less intensive conditioning and in this series did not receive radiotherapy; yet they still undergo disruption of the normal lymphocyte balance although the pattern is different to that of allogeneic transplants. It appears that NK cells expand when T cell (particularly CD4 T cell) numbers are depressed. This is supported by observations in HIV^+ individuals where the proportion of NK cells is inversely proportional to CD4 cells, throughout symptomatic disease (Lowdell, 1992).

There were a number of technical problems with this stage of the project, some of which could be overcome and others that had to be accommodated. With the phenotyping studies low lymphocyte numbers were encountered at various stages of recovery which made phenotyping more difficult. Also it was found

that during episodes of GvHD the patients were treated with anti-thymocyte globulin (ATG) which often made phenotyping difficult as the role of ATG is to T cell deplete *in vivo*.

There were also problems with patient recruitment. The Royal Free Hospital acts predominately as a tertiary referral centre for patients with acute leukaemia. As such the majority of patients are referred in remission and so presentation leukaemic blasts are not available. In cases where patients present with active disease it is usual to receive a single sample containing 20 – 40 x 10^6 blasts prior to initial chemotherapy. The studies described above have been conducted on samples taken from patients when presentation leukaemic blasts were available and who achieved a lasting complete remission of >3 months post-completion of therapy. This is by definition a small population but every suitable patient who was treated during the period of the study was included.

The study continued with investigation into functional immune responses as described in Chapter 3 in allogeneic transplant recipients. It then became apparent that broadly expressed alloantigens on recipient leukaemic cells presented immunodominant epitopes and that the donor cells responding to these antigens also lysed non-leukaemic recipient normal myeloid cells, collected in remission pre-BMT. In the autologous setting, however, a cytotoxicity assay on presentation leukaemic blasts in a long-term surviving autologous BMT recipient was performed, and a significant cell lysis in the

absence of autologous killing of normal bone marrow derived myeloid cells from the same patient was seen.

Preliminary experiments identified $CD56^{+}/CD3^{-}$ cells as the mediators of this activity, and subsequent experiments showed CD8α expression to be important. These cells were also identified in other recipients of autologous BMT and in patients in CR after completion of conventional chemotherapy, and the presence of this lytic activity seems to be associated with long-term disease free survival in both groups of patients. Loss of leukaemia specific cytotoxicity (LSC)(Chapter 4) was followed by morphological relapse although it was recovered in two patients by treatment with alpha-interferon.

Once the existence of this subset had been noted and its function established the goal of this work was to see if it was possible to generate these cells *in vitro* and then expand the subset for adoptive transfer back to the patient. These cells were generated as detailed in Chapter 4 but further work is required on expanding the subset. This would probably be achieved by using higher doses of IL-2 as the doses used in this study were purely used for the maintenance of cultures, not designed to provoke proliferation, and a monocyte feeder layer as described by Pierson *et al.* (1995). However, care must be taken not to override the effect of IL-15 which has proved so effective at generating these cells, and it is possible that a compromise would have to be made between stimulating $CD56^{+}/CD8^{-}$ cells to become $CD56^{+}/CD8^{wk+}$ and proliferation.

Obtaining pure NK populations in the numbers required for this series of experiments proved to be very difficult and often meant that the experiments were performed on smaller cell numbers than was originally intended. The experiments were designed to be performed with serum-free medium to ensure that all effects seen could be directly attributed to the cytokines and not to any serum factors. However although the first experiments were done using serum-free medium this eventually limited the scope of the experiments due to an inability to maintain the cells for any length of time without serum. This would mean that clinical cell production is more likely to be complicated by batch variation of serum, and the issues of product safety following concerns about transmissible infectious agents from animal-derived constituents. The only resolution of this may be to use autologous serum.

One problem that was resolved was the elimination of $CD56^+/CD3^+$, by efficient sorting. In a number of experiments performed with the $CD56^+/CD3^+$ these cells became adherent when incubated in the plates with cytokines, whereas the $CD56^+/CD3^-$ cells did not, which appears to confirm that they are another subset with distinct characteristics.

Once an efficient method of proliferation has been established the long term growth potential of these cells *in vitro* also needs to be monitored. In the future it must be demonstrated that NK cells with LSC activity can be generated *ex vivo* from patients with little or no innate LSC activity when in remission after chemotherapy. There maybe difficulties in sustaining these cells long term without incurring a switch in the phenotype. More work is required to establish

how stable the cells are in culture, and how long they would have to be cultured to gain sufficient numbers before being transferred back to the patient.

Crucially a clinical problem that remains unresolved at the moment is how many cells would be required by the patient to effect an anti-leukaemic response, and when and how they could be most effectively administered. The culture conditions for large-scale expansion are currently the subject of a new study under separate funding.

In two of our patients who received alpha-interferon treatment LSC activity has been induced (Lowdell *et al.*, 1997). However an MRC trial in AML using alpha-interferon alone has not been successful and the results remain unpublished. This may be due to the fact that the success of this treatment may depend on more specific selection of suitable patients. The two patients who responded well to this treatment in this study had AML M2 and M4. It may be something pertinent to these types of AML that confirms success, or it may be that the defining constituent of the disease which would allow a response to this treatment or of the individual patients' ability to respond to treatment is yet to be found.

In this study the importance of CD8 expression on $CD56^{+}/CD3^{-}$ NK cells in autologous transplant recipients, and chemotherapy patients not receiving BMT with AML has been observed. However the role of this molecule is not understood. Ten years ago investigators were becoming convinced that NK-cell mediated cytotoxicity was governed by the 'missing self' hypothesis (Ljunggren

and Karre, 1990). This suggested that MHC class I molecules protect cells from NK cell lysis (Gumperz and Parham, 1995). Subsequently numerous inhibitory receptors, which are MHC class I ligands, have been discovered which supports the 'missing self' hypothesis (Yokoyama, 1998).

Inhibitory receptors prevent killing of normal cells and limit the production of inflammatory cytokines by NK cells (this includes IFN-γ, GM-CSF and TNF-α). Co-ligation of activating receptors with inhibitory receptors results in a dominant inhibitory effect, that downregulates the signals initiated by the activating pathways (Moretta *et al.*, 2001). There are two separate families to which inhibitory receptors belong with two different structural forms which are either C-lectin-like receptors or immunoglobulin-like receptors. The inhibitory receptors mediate their effects through cytoplasmic sequences known as immunoreceptor tyrosine-based inhibitory motifs (ITIM). However there are also related isoforms which may activate cells known as immunoreceptor tyrosine-based activatory motifs (ITAM). MHC class I molecules appear to be able to bind a number of different NK cell receptors, and each NK cell may express several different receptors.

Although the ITIMs have been under much scrutiny to discover their ligand specificity and functional activity, the ITAMs on human NK cells have not been fully elucidated. What has been observed is that the interaction between the Natural Killer cell immunoglobulin-like receptor (NKIR) (containing the ITIM in the cytoplasmic tail) and the MHC class I molecule is short lived. The cell:cell interaction will be assisted by integrins and adhesion molecules, but it is the

level of expression of the NKIR and the MHC class I molecule that will be the determining factor in the outcome of the NK target cell interaction. The ligation of the NKIR to the MHC class I molecule must be sufficient to deliver a signal to interrupt a cascade of events. It is a rapid signal transduction pathway which has been shown to be much faster than that following TCR binding to a MHC-peptide complex.

It has yet to be ascertained whether each interaction is sufficient to deliver the inhibitory signal or whether a threshold number of interactions is required (Vales-Gomez *et al.*, 1998). It may be that further study of the $CD56^{+}/CD8^{wk+}$ subset in the context of autologous leukaemic cells will reveal that the number of interactions between NKIRs on the NK cell, and MHC class I on the target cell are insufficient to override the inhibitory signal, and therefore lysis occurs.

CD94 and NKG2 are C-type lectin-like NK receptors. CD94 forms disulphide-linked heterodimers with molecules belonging to the NKG2 (natural killer gene complex) family that require CD94 for surface expression. This heterodimer can be inhibitory or stimulatory depending on the exact cytoplasmic domain of the NKG2 molecule. NKG2A and B contain ITIMs and are inhibitory whereas NKG2C does not contain an ITIM and is stimulatory (currently NKG2A-F have been observed)(Yokoyama, 1998). CD94/NKG2A specificity is promiscuous and interactions have been noted with HLA-A, B, C and G. For some of the inhibitory CD94/NKG2 heterodimers it appears that the non-classical MHC class I molecule, HLA-E is the specific receptor (Yokoyama, 1998, Moretta *et al.*, 2001).

NK cells preferentially kill target cells that lack MHC class I on their surface (Ljunggren and Karre, 1990) and it may be that there are triggering receptors on the NK cell that recognise non-classical MHC ligands on the target cells, such as MICA and MICB. These receptors may not be restricted to transformed cells, but may also appear on normal cells, so that target cells lysis or survival may result from the degree of ligand binding of activatory or inhibitory receptors. There is less known about the activatory receptors but their importance is gradually becoming clear.

The expression of CD94 has been shown to influence the recognition of MHC class I expressed on target cells. In IL-2 activated NK cells CD94 is up-regulated and has the ability to trigger cytolytic effector function (Ida *et al.*, 1997). Therefore at least one of the cytokines used in this study has the ability to up-regulate an NK receptor and result in target cell lysis.

NKG2D does not require CD94 for surface expression and is expressed as a homodimer. It does associate with an adapter protein called DAP10. NKG2D expression is not confined to NK cells, but it has been shown to mediate potent NK cell triggering in redirected killing assays. The ligands for NKG2D are MICA and MICB that are encoded within the human MHC, and it may be that it is an additional triggering receptor involved in target cell recognition and initiation of killing (Moretta *et al.*, 2001).

Three new NK-specific triggering surface molecules have been identified (Moretta *et al.*, 2001). NKp46, NKp30 and NKp44 share molecular heterogeneity and are type1 glycoproteins belonging to the Ig-superfamily. They have been termed natural cytotoxicity receptors (NCR) and are mainly restricted to NK cells and have the ability to mediate NK cell triggering without the engagement of other surface molecules. The ability of NKp46 to act depends on the presence and/or absence of specific ligands on target cells (anti-p46 can inhibit the cytolytic activity of NK cell clones against some target cells but not others (reviewed in Moretta *et al.*, 1998). NKp46 and NKp30 are expressed by all NK cells including $CD56^{bright}/CD16^{-}$ cells and immature NK cells. However, NKp44 was only found on activated NK cells cultured in the presence of IL-2 (Moretta *et al.*, 2001). NKp44 may therefore be the receptor responsible for the increased cytotoxicity of LAK cells.

The surface density expression of the NCRs is also important and may affect their ability to trigger lysis. The relevance of the surface density expression appears to depend on the type of target and the effector. There may be a complementary role played by NCRs and NKG2D. Certain targets susceptibility to lysis depends solely on NCR expression, other targets may require both NCR and NKG2D expression (in the case of NCR^{bright} effector cells) and some may require NKG2D in the case of NCR^{dull} effector NK cells (Moretta *et al.*, 2001).

Also differences in natural cytotoxicity observed in different normal donors may be explained by a differential expression of surface NKp46 molecules. This means that individuals that have a $NKp46^{bright}$ phenotype have higher cytolytic

activity than individuals with a NKp46^{dull} phenotype (Sivori *et al.*, 1999). This may well be true of patients as well as normal donors and may be another reason why some of the patients in this study showed LSC via CD56^{+}/CD8^{wk+} cells. The identification of the NCRs is a major advance in our knowledge of NK cells and the next crucial step will be to uncover their ligands.

Co-stimulatory molecules are preferentially expressed on haematopoietic cells, which makes it tempting to think that ligands for these molecules are present on subsets of NK cells but are as yet undiscovered (Chambers *et al.*, 1998). It is known that the B7.1 – CD28/CTLA4 mediated co-stimulation which is vital for T cells is not the crucial interaction for NK cells (Lang *et al.*, 1998). The next step for this study would be to find what receptors are present on the CD56^{+}/CD8^{wk+} cells that trigger lysis of certain AML cells.

Other subsets are under investigation at present for non-MHC restricted killing including γδ T cells (Lamb *et al.*, 1996) and CD3^{+}/CD56^{+} cells (Zoll *et al.*, 1999). It may be the case that each type of leukaemia is sensitive to different effector cell lineages, and will have to be treated accordingly. Perhaps what should be learnt from this is that the lineages that have traditionally been so clearly delineated are not as discrete as has been thought and that T and NK cells are actually much more closely related. The emergence of particular subsets in force when the normal immune balance is disrupted shows that cells that may normally be dormant, or present in small numbers have a significant part to play.

In retrospect this study would have benefited from adding CD3 monoclonal antibody to the original panel of antibodies. This would have allowed for clarification of the percentages of cells that were $CD56^+/CD3^+$ on the large group of patients in the phenotyping studies.

This work has been the first to demonstrate spontaneous innate immunity to autologous leukaemias in patients in remission after conventional or high-dose chemotherapy which is associated with survival. Others have reported the presence of allogeneic (Reittie *et al.*, 1989, Keever *et al.*, 1993) and even autologous (Reittie *et al.*, 1989) LSC-NK but none have shown them to be clinically relevant. These data promise to provide a predictive test for patients in first CR to determine their risk of early relapse which will assist in selecting those who are most likely to benefit from high-dose therapy with allogeneic BMT or from new immunotherapeutic treatments.

Despite the establishment of international marrow-donor registries which have made HLA phenotypically matched marrow from unrelated donors available to patients who lack a suitable related donor, there are still cases where a donor cannot be found, particularly for those patients who come from a mixed ethnic background. This heightens the need to improve the outcome for autologous transplant recipients. The advantage in autologous transplantation is that there is no wait to establish whether there is a suitable donor and this removes an added stress for the patient and their family. Also the risks of post-transplant complications are reduced and therefore the amount of immunosuppression

required, this also means these transplants can be performed on older patients than allogeneic transplants.

The work described here may in the future make it possible to detect which patients are suitable for expansion of their LSC-NK cells post-chemotherapy. These cells could be expanded in appropriate culture conditions administered at the optimum time, and returned to the patient to boost LSC-NK activity. This may lead to the avoidance of allogeneic transplantation in these cases and possibly even autologous transplantation. It opens up the possibility of such treatment for older patients, and for those with no prospect of finding a donor.

7. References

7. References

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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 μg/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.

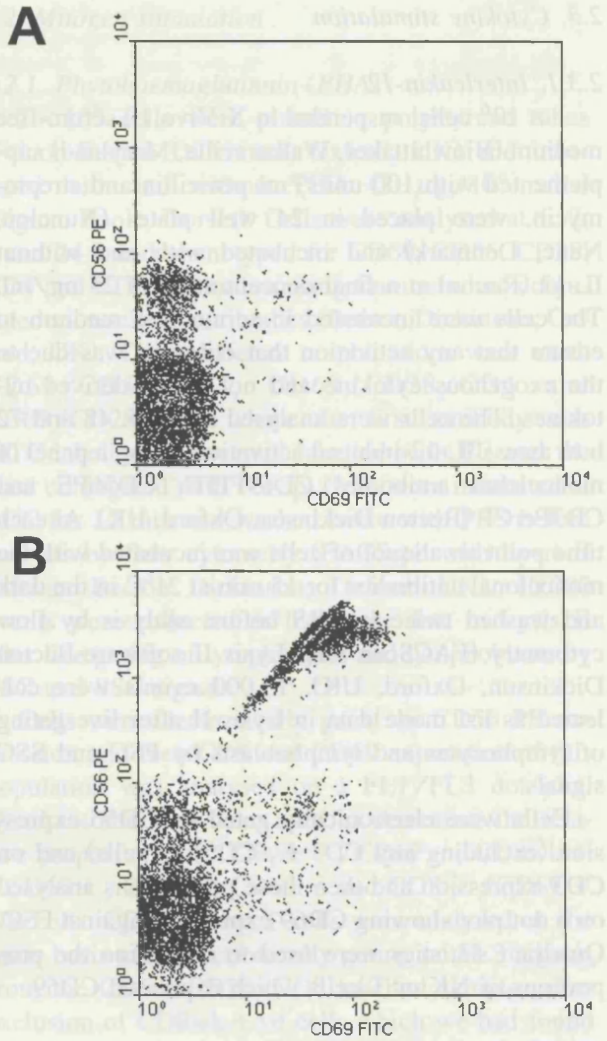

Fig. 1. Resolution of PKH26 labelled mononuclear stimulator cells from monoclonal antibody labelled responder cells. (A) Responder cells alone labelled with CD69 FITC and CD56 PE. (B) Responder and stimulator cells mixed at a 1:1 ratio showing clear resolution of PKH26 labelled stimulator cells in region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

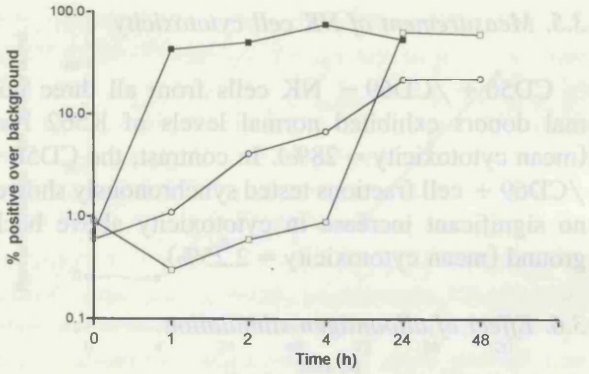

Fig. 2. Temporal expression of CD69 on CD4+ve and CD8+ve lymphocytes after PHA stimulation. CD4+, ■; CD8+/28+, □; CD8+/28−, ○.

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

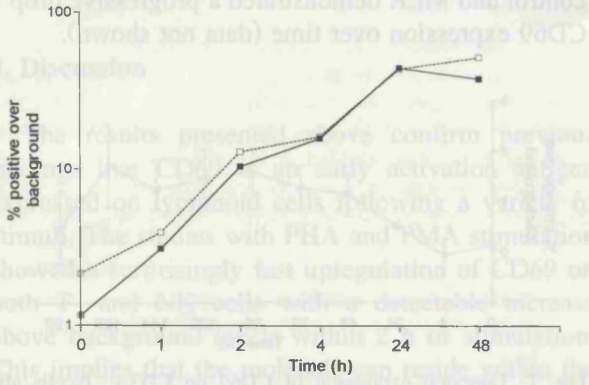

Fig. 3. Temporal expression of CD69 on CD56+ve lymphocytes after PHA stimulation. CD8+ve, ■; CD8−ve, □.

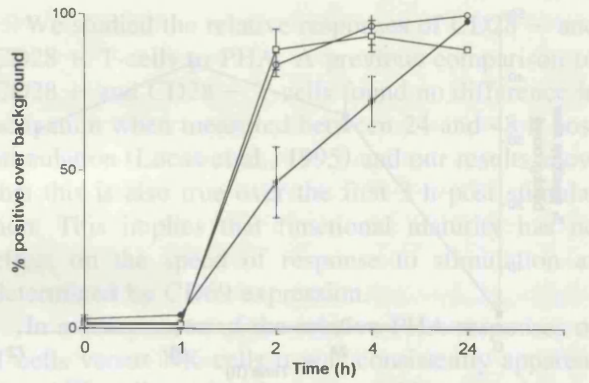

Fig. 4. Temporal expression of CD69 on T-cells and NK cells in response to PMA. CD4+, □; CD8+, ○; CD56+, ●. Data are presented as means and standard deviations.

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.

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-

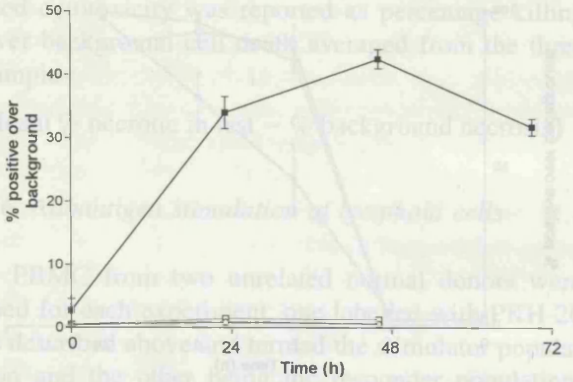

Fig. 5. Temporal expression of CD69 on T-cells and NK cells in response to IL-12. CD4+, □; CD8+, ○; CD56+, ■. Data are presented as means and standard deviations.

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).

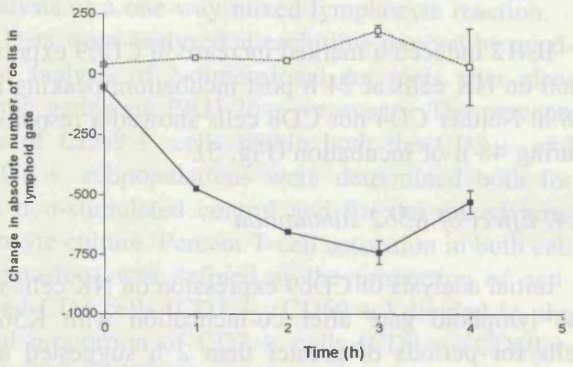

Fig. 6. NK cell subset response to K562. CD69+ve, □; CD69− ve, ■. Data are presented as means and standard deviations.

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).

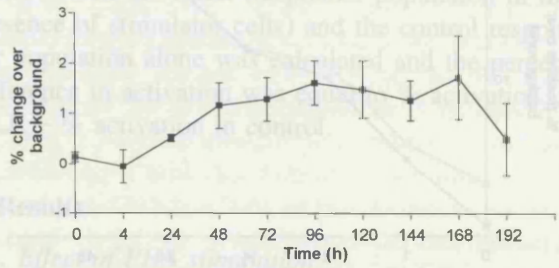

Fig. 7. Temporal expression of CD69 on CD3+ T-cells after alloantigen stimulation. Data are presented as means and standard deviations.

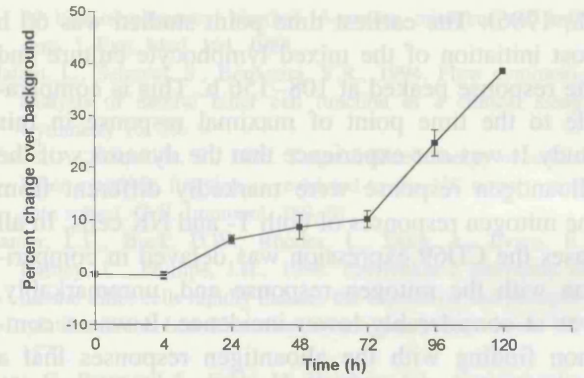

Fig. 8. Temporal expression of CD69 on NK cells after alloantigen stimulation. Data are presented as means and standard deviations.

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).

Acknowledgements

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The *in vitro* detection of anti-leukaemia-specific cytotoxicity after autologous bone marrow transplantation for acute leukaemia

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Summary:

Anti-leukaemia activity after allogeneic bone marrow transplantation has been studied extensively but its antigen specificity and effector cell phenotype remain unknown. Here we report a study in three recipients of autologous bone marrow transplantation done as part of the treatment for acute leukaemia, in whom we were able to detect innate specific anti-leukaemia activity post-transplant. One patient maintained selective cell-mediated cytolytic activity against her autologous leukaemic cells in the absence of cytolysis of her normal bone marrow mononuclear cells (BMMC). She remains in complete remission 3 years after ABMT for acute myeloid leukaemia (M5). A second patient was transplanted for acute lymphoblastic leukaemia and had detectable anti-leukaemia activity up to 20 weeks post-ABMT. At this point anti-leukaemia activity could no longer be demonstrated and the patient suffered a relapse 2 weeks later. A third patient was transplanted for AML (M4 Eo) and lacked detectable leukaemia-specific immune reactivity at 1, 3 and 6 months post-ABMT. She relapsed 6 months after her ABMT and returned to complete remission after further chemotherapy. She commenced treatment with alpha interferon and regained NK function. Furthermore, she developed high level cytolytic activity against her autologous leukaemic cells in the absence of activity against her remission bone marrow samples. She remains in complete remission 17 months after her initial relapse. This is the first report of an apparent association between *in vitro* leukaemia-specific cytolytic activity in individual patients after ABMT and clinical outcome. It encourages the theory that autologous immunomodulation may be useful in the future treatment of leukaemia.

Keywords: NK cells; cytotoxicity; GVL; ABMT; immunotherapy

The role of the immune response in the control and eradication of leukaemia remains controversial but there is increasing evidence that the principal mechanism of cure after intensive chemotherapy and allogeneic BMT is immune mediated. This is evidenced by the observations of a reduced risk of leukaemia relapse after allogeneic BMT when compared with autologous or syngeneic BMT. Furthermore, an increased incidence of leukaemic relapse has been reported after aggressive GVHD prophylaxis with cyclosporin A,[1] or lymphocyte depletion. The association between GVHD and the graft-versus-leukaemia activity of allogeneic BMT is strong and has led to a number of workers concluding that GVL might be inseparable from GVHD. However, the lymphocyte subsets responsible for GVHD and GVL remain to be elucidated in man despite intensive efforts. Certainly $CD3^+$ T cells,[2,3] $CD16^+$ natural killer cells[4] and a population of *in vivo* activated killer cells[5] are involved in GVL.

Studies of GVL in the allogeneic setting are beset with the problem of alloreactivity against major and minor histocompatibility antigens. A number of studies have been performed in the autologous setting but none have reported evidence of innate cytotoxic activity which is specific for autologous leukaemic cells. Lotzova and colleagues[6] demonstrated that patients prior to chemotherapy had low spontaneous NK activity against the erythroblastoid leukaemic cell line K562 and no detectable activity against autologous leukaemic cells. However, both were enhanced following *in vitro* culture with IL-2, although the leukaemia-specificity of the activity remains unknown since cytotoxicity of autologous normal myeloid cells by the IL-2 stimulated cells was not studied. A recent study of CLL patients after fludarabine treatment demonstrated recovery of NK cell killing of K562 targets to normal levels and, in three of five patients, a specific lytic action against their autologous leukaemic blasts.[7] A number of groups have studied NK cell activity after autologous and allogeneic BMT and an increase in NK cell frequency and activity against leukaemic cell lines *in vitro* are a consistent finding but there is no evidence that this is associated with clinical outcome.[5,8–11] In a single study of 146 patients in complete remission of acute leukaemia after allogeneic BMT or chemotherapy alone, the loss of NK activity against the cell line K562 was associated with clinical relapse.[11]

In this study we have followed three patients for up to 3 years after ABMT for acute leukaemia. In all cases we have tested their immune reactivity *in vitro* after ABMT against both their original leukaemic cells and their normal BMMC which were used for the autologous transplant.

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Materials and methods

Three patients were studied after ABMT, the clinical details of whom are presented in Table 1. Peripheral blood and bone marrow samples were obtained prior to ABMT after informed consent. These samples included presentation of leukaemic blasts and autologous remission bone marrow. Mononuclear cells were isolated by density gradient separation (Lymphoprep; Nyegaard, Oslo, Norway) and cryopreserved in RPMI 1640/50% fetal calf serum (FCS)/10% DMSO (Sigma, Poole, UK) in the vapour phase of liquid nitrogen (all tissue culture reagents were purchased from Gibco (Paisley, UK)). After ABMT, heparinised blood samples were collected at regular intervals and tested immediately or cryopreserved as above for future analysis. Before testing for anti-leukaemia activity all patient samples were tested against the erythroleukaemic cell line K562 to confirm NK cell function. No samples were tested against autologous leukaemic cells that had not been shown to lyse K562.

Cell-mediated cytotoxicity was determined by a dye-exclusion assay.[7,12–15] Autologous leukaemic and normal bone marrow were labelled with either the green membrane dye F-18 (a kind gift from Dr Radosevic, University of Twente, The Netherlands) or the red dye PKH-26 (Sigma). The PKH-26 dye was favoured when it became available because of the greater reliability and speed of labelling. Briefly, target cells were thawed rapidly, washed once in RPMI 1640 supplemented with L-glutamine, penicillin and streptomycin (SFM) and resuspended in PKH cell labelling buffer (Sigma). The cells were mixed with an equal volume of 4 mM PKH-26 for 2 min at 21°C. The labelling reaction was stopped by the addition of an equal volume of neat FCS for 1 min and the cells were washed twice in SFM supplemented with 10% FCS (CM). PKH-26-labelled cells showed high levels of fluorescence (Figure 1a) and could easily be resolved from non-labelled effector cells (Figure 1b). Figure 1c and d show the light scatter characteristics of the effector and target cell population, respectively. It is apparent that the two cell populations have overlapping forward (FSC) and side angle (SSC) light scatter properties but can be resolved from each other in a mixed assay system by PKH-26 labelling. The membrane dye was very stable and there was no transfer to non-labelled effector cells during the assay period (data not shown).

Post-transplant peripheral blood mononuclear cells were suspended in CM and were monocyte-depleted on tissue culture plastic for 2 h at 37°C/5% CO_2. The non-adherent fraction was recovered and incubated in triplicate with labelled targets at a ratio of 10:1 for 4 h. After the incubation period the cells were resuspended in a solution of 1 μg/ml propidium iodide (Sigma) in PBS 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 green or 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-mediated cytotoxicity was reported as percentage killing over background cell death averaged from the three samples:

Mean (% necrotic in test – % background necrosis).

This was calculated for both leukaemic and normal autologous target cells which were used as negative controls to confirm leukaemia specificity. Leukaemia-specific killing was determined by subtracting the percent cytolysis of remission BMMC from the percent lysis of leukaemic blasts.

The phenotype of the effector cell population was determined by expression of the activation antigen CD69. Target cells were labelled with PKH-26 as above and incubated with non-labelled effector cells in CM at a ratio of 1:1. Positive control assays were set-up in which PHA (200 ng/ml) was added to the effector cells in the absence of stimulator cells. After 3–4 h incubation the samples were labelled with a combination of CD69 FITC, CD56 PE and CD4 or CD8 PerCP (all from Becton Dickinson). Control samples consisted of effector cells incubated with remission BMMC (in all cases the level of CD69 expression after incubation with remission BMMC was no greater than that of effector cells incubated alone). All samples were set up in triplicate and the mean proportion of CD69 positive cells in each cell subset recorded after analysis by flow cytometry after exclusion of PKH-26 expressing stimulator cells by electronic gating. PKH-26 fluorescence is detected by the second photomultiplier tube on the FACScan cytometer as is the CD56 PE signal. CD56 is expressed at relatively low levels compared to the extremely bright fluorescence of the PKH-26 dye. This allows exclusion of the bright PKH-26-expressing stimulator cells without loss of the CD56 positive effector population.

Table 1 Patient characteristics

UPN	*Diagnosis*	*Disease status at ABMT*	*Age at ABMT (years)*	*Sex*
UPN 633	AML M5	CR2	18	F
UPN 660	AML M4 Eo	CR2	50	F
UPN 664	T-ALL	CR1	27	M

All patients received pre-transplant conditioning with cyclophosphamide 60 mg/kg × 2; single fraction TBI 750 cGy.

Analysis of surface expression of MHC class I molecules

Cryopreserved leukaemic bone marrow samples from each patient and five PBMC samples from normal donors were thawed rapidly, washed in CM and resuspended to 10^6/ml in PBS. 10^5 cells from each specimen were incubated with 10 μl anti-MHC class I (W6/32 FITC; Harlan, Sera Lab, Crawley Down, UK) for 15 min at 21°C, washed once in PBS and analysed by flow cytometry (FACScan, Becton Dickinson).

Fluorescence signals were logarithmically amplified and intensities were analysed after resolution to linear data array channels.[16]

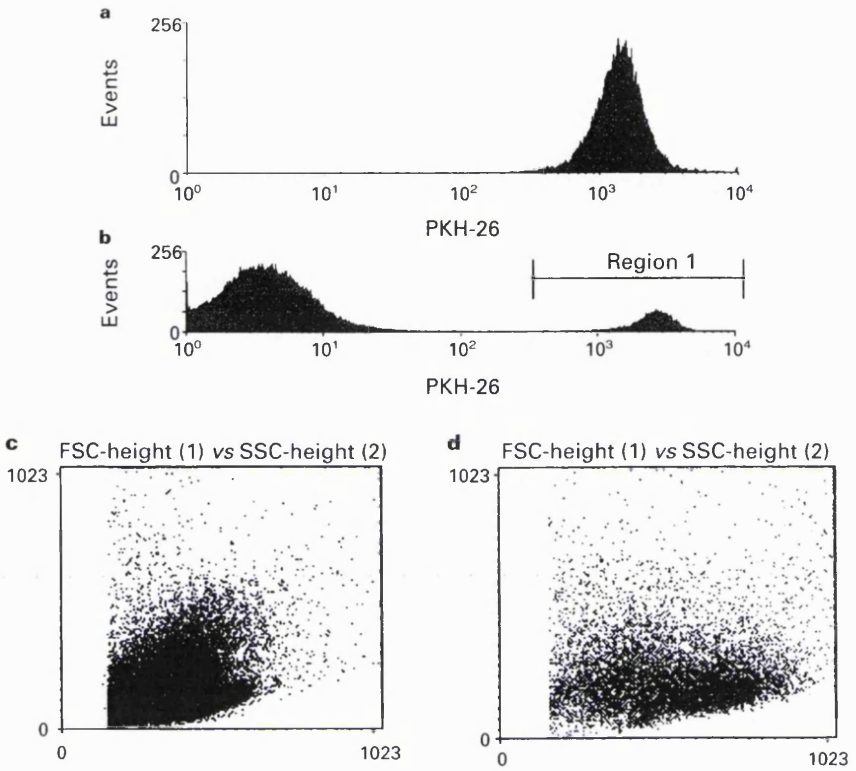

Figure 1 PKH-26 resolution of target cells by flow cytometry: All target cells label strongly with PKH-26 and the dye is retained within the cell membranes after a 4-h incubation at 37°C (**a**) PKH-26 positive target cells are included in region 1 in (**b**) and are readily resolved from the non-labelled effector cells which appear in the first fluorescence decade on a four decade scale after logarithmic amplification. (**c**) and (**d**) show the foward angle (x-axis) and side angle (y-axis) light scatter characteristics of the effector and target cells populations respectively and demonstrate that these populations are overlapping but can be resolved by PKH-26 expression within the target cell population.

Results

Patient UPN 633

The first post-ABMT sample available from this patient was taken at 12 months. Cytotoxicity testing demonstrated specific anti-leukaemia activity of 24%. Subsequent testing at 18 and 33 months showed consistent anti-leukaemia cytotoxicity in the absence of lysis of remission BMMC (Figure 2a). Analysis of the cellular responders in this assay system as determined by CD69 expression upon co-incubation showed them to be CD56+ve natural killer cells. There was no increase in activation of CD4 or CD8 T cell subsets, even when analysis was restricted to CD45RO+ve subsets (Figure 2b). Furthermore, the absence of activation of any cell subset in response to the remission autologous bone marrow supports the leukaemia specificity of this activity. The patient remains in complete remission 38 months post ABMT.

Patient UPN 664

Samples were received from this patient regularly from day 24 post-ABMT. They contained high numbers of activated NK cells at all time points up to 18 weeks post-transplant and he remained in remission. Anti-leukaemia cytotoxic activity was detected at 13 weeks post-ABMT in the absence of lysis of autologous remission BMMC (Figure 3). In addition, no activity could be detected against two allogeneic leukaemic samples from ALL patients. By week 18 post-transplant, the anti-leukaemia activity had fallen but remained detectable. At week 20 post-transplant, no autologous anti-leukaemia activity was detectable although the patient remained in complete remission. The patient relapsed at week 22. Anti-leukaemia activity remained undetectable and he has recently received an allogeneic BMT from an unrelated volunteer donor.

Patient UPN 660

Samples received at 1 and 3 months post-ABMT showed high levels of cytolytic activity against both leukaemic and remission BMMC (Figure 4a). Samples taken at 6 months showed no cytolytic activity against any targets, including the cell line K562. The patient also lacked detectable numbers of NK cells in her peripheral blood. She relapsed 1 week after sampling. After further chemotherapy (FLAG/idarubicin: fludarabine 30 $mg/m^2 \times 5$; Ara-C 2 $g/m^2 \times 5$; idarubicin 8 $mg/m^2 \times 3$) she achieved a second complete remission which was consolidated with a second course of FLAG/idarubicin. Three months after relapse she was commenced on alpha-interferon therapy (IFNα, 1 and 2 megaUnits alternate days, s.c.) (Schering, Burgess Hill, UK). $CD56^+$ cells reappeared in her peripheral blood and she recovered NK cell function. One month after induction of IFNα treatment the patient developed strong leukaemia-specific lytic activity which has been maintained over the

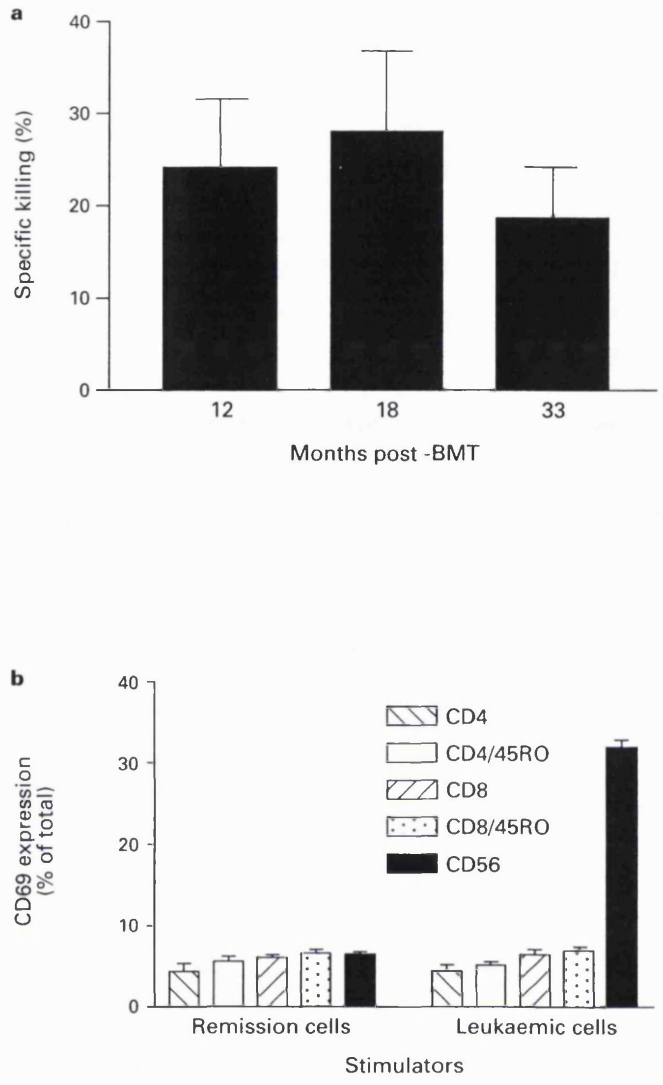

Figure 2 (**a**) Time course of specific anti-leukaemia cytotoxicity after ABMT (UPN 633). (**b**) CD69 expression by PBL subsets at 12 months post-ABMT following stimulation with pre-transplant remission and leukaemic BMMC (UPN 633). The error bars represent standard deviations.

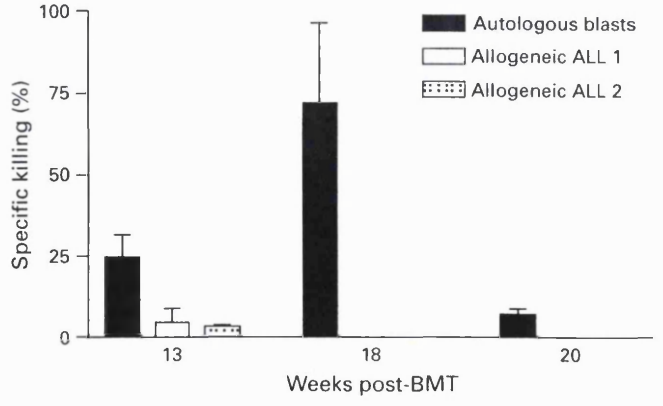

Figure 3 Cytolytic activity post-ABMT against autologous and allogeneic ALL blasts (UPN 664). The error bars represent standard deviations.

subsequent 3 months (Figure 4a). Non-specific lysis of remission BMMC stored prior to ABMT was totally absent in samples taken in second CR (Figure 4a). She remains in second CR 16 months after reinduction chemotherapy.

Analysis of the responding cell population by CD69 expression showed that the principal early reactivity resided in the $CD56^+/CD8wk^+$ cell population (Figure 4b). In contrast, neither CD56+ve nor CD8+ve cells activated in response to autologous remission BMMC over the same time period (Figure 4c). The 24-h response appeared to be within the CD56+ve/CD8−ve population. However, this may simply reflect the loss of CD8α chain following activation as has been reported for CD8+ve T cells.[17]

Stimulation of the PBL with PHA demonstrated that the CD8+ve T cell subset (CD56−ve) was capable of responding, as was the CD4+ve cell subset (Figure 4d). However, neither cell subset showed increased incidence of CD69 expression after incubation with autologous remission or leukaemic BMMC.

Stimulation with the erythroleukaemic cell line K562, which is a commonly used NK cell target cell, led to the activation of $CD56^+/CD8^-$ NK cells and not the $CD56^+/CD8wk$ cells (Figure 4e).

Immunofluorescent labelling of leukaemic blasts in samples from all patients showed all cells expressed MHC class I molecules and at levels comparable to those expressed by normal peripheral blood myeloid cells (data not shown).

Discussion

Although the graft-versus-leukaemic effect (GVL) after bone marrow transplantation was first identified almost 40 years ago[18] the cellular mediators of this activity in patients remain controversial. The use of T cell depletion (TCD) strategies for the prevention of GVHD in allogeneic transplantation and the subsequent increased relapse risk observed in some centres implied that the GVL activity resided within the T cell compartment. However, clinical results from our group[19] demonstrated that TCD with monoclonal antibodies specifically targeted at T cell epitopes combined with effective immunosuppressive pre-transplant conditioning can prevent clinical GVHD without increasing the risk of relapse or failure of engraftment. Furthermore, a comprehensive study of multi-centre unrelated donor BMT has confirmed the low incidence of relapse following T cell-specific depletion in contrast with broad spectrum lymphocyte depletion.[20] Indeed, infusions of CD8-depleted donor leukocyte preparations have been used successfully for the treatment of relapse following allogeneic BMT for chronic myeloid leukaemia.[21] These data support a possible role for natural killer cells in the GVL effect.

The role of immune mechanisms in continued remission after ABMT is highly controversial but we have previously shown that some of the immunological consequences of allogeneic BMT can be detected after ABMT. High numbers of activated NK cells can be detected in the peripheral blood after both autologous and allogeneic BMT.[5,8] Furthermore, high numbers of resting NK cells circulate in the peripheral blood of transplant-recipients for many months

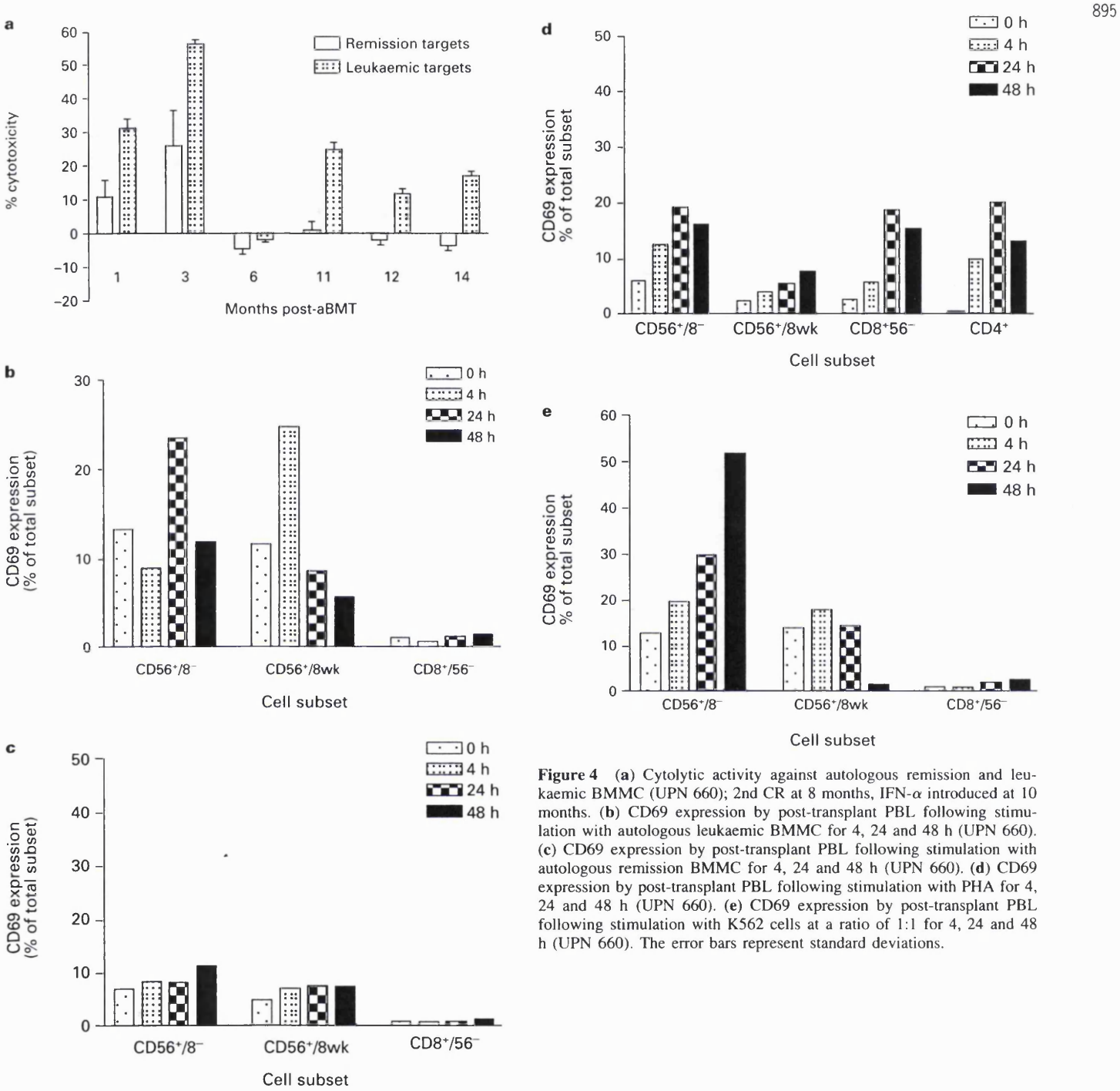

Figure 4 (**a**) Cytolytic activity against autologous remission and leukaemic BMMC (UPN 660); 2nd CR at 8 months, IFN-α introduced at 10 months. (**b**) CD69 expression by post-transplant PBL following stimulation with autologous leukaemic BMMC for 4, 24 and 48 h (UPN 660). (**c**) CD69 expression by post-transplant PBL following stimulation with autologous remission BMMC for 4, 24 and 48 h (UPN 660). (**d**) CD69 expression by post-transplant PBL following stimulation with PHA for 4, 24 and 48 h (UPN 660). (**e**) CD69 expression by post-transplant PBL following stimulation with K562 cells at a ratio of 1:1 for 4, 24 and 48 h (UPN 660). The error bars represent standard deviations.

after the loss of AK cells. That these cells can have anti-leukaemia activity has been reported before,[4] but not in the ABMT setting. In an extensive study of patients with a variety of acute leukaemias it was found that the levels of NK cell activity against the NK-sensitive cell line K562 were significantly lower than in normal blood donors.[11] Furthermore, this activity increased when the patients were in remission and, in all 15 patients studied longitudinally, a reduction in NK activity to pre-remission levels was associated with relapse within 10 weeks. These data suggest that functional NK responses may be important in the control of residual leukaemic cells but there was no direct evidence that NK cells could lyse autologous leukaemic cells. Most recently, a group using a similar dye-exclusion assay for cell-mediated killing as used here has demonstrated specific lysis of autologous CLL cells in three of five patients after chemotherapy without BMT.[7] No data were presented on the relationship between this activity and survival.

The results presented here demonstrate the specificity of the anti-leukaemia response and the activation experiments show this effect is mediated by NK cells. We have chosen to use two novel assay systems in this study. The flow cytometric cytotoxicity assay was preferred over a standard ^{51}chromium-release assay since it did not rely upon the

assumption that all target cells label equally. This is of particular importance when using clinical samples as target cells in contrast to conventional cell lines, since the population is heterogeneous. In a conventional ^{51}Cr-release assay, target cells are loaded with ^{51}Cr and incubated with effector cells. The amount of ^{51}Cr released into the supernatant after a pre-determined period is related to the maximal amount of ^{51}Cr released from the same number of cells by detergent lysis. If the target cell population is heterogeneous regarding cell size and ^{51}Cr-labelling, then there may be no direct correlation between the amount of ^{51}Cr in the supernatant and the degree of lysis. In contrast, the flow cytometric cytotoxicity assays analyse cell death on an individual cell basis and, by combination of dye labelling and cell light scatter characteristics we were able to ensure that the only target cells studied were of blast morphology.

The use of CD69 to identify reacting cell populations is also novel[22] and a more conventional experiment would use selected cell subsets. However, the use of cell selection strategies has severe limitations. Positive selection of specific cells following antibody labelling invariably activates the cells and/or leaves antibody on the cell surface which may interfere in subsequent cell:cell interactions. Negative selection always results in lower purity than positive selection and thus the role of minority cell subsets in the resulting selected population can never be ruled out. This problem was highlighted recently with the discovery of the absolute requirement for NK cells in the initiation of alloreactivity; something which had been previously discounted on the basis of T cell selection experiments in which the minor NK cell contaminant had been ignored.[23] Finally, selection of individual cell subsets from a mixed population may remove cells which control other cells within the mixed population. Thus the use of CD69 to determine which cell subset or subsets within a heterogeneous population respond to a particular stimulus most closely reflects the *in vivo* situation. Furthermore, it has been shown that the induction of CD69 expression on NK cells parallels the acquisition of lytic activity.[24]

The lack of reactivity of the CD56+ve/CD8wk cell fraction with K562 cells was in stark contrast to the reactivity of the CD56+ve/CD8–ve NK cell subset to these cells. This may be evidence of functional subsets of non-MHC restricted killer cells. The data suggest that K562 cytotoxicity assays may not reflect all the NK cell activity within a clinical sample and data derived from assays with this target cell line should be interpreted with caution.

The mechanisms by which NK cells respond to target cells are currently being elucidated. It appears that NK cells have both stimulatory and inhibitory signal receptors.[25,26] Certain MHC class I molecules are the ligands for the inhibitory receptor, principally HLA-C molecules. K562 cells lack surface expression of MHC class I molecules and are unable to provide the inhibitory stimulus, thus making them excellent targets for NK cell assays. In the cases described above, all leukaemic blasts expressed HLA-class I at levels equivalent to normal PBMC. However, HLA-C is expressed at low levels on normal cells and thus reduced expression of these molecules is unlikely to lead to a detectable reduction in total HLA class I expression as determined by antibody labelling. Antibodies specific for HLA-C are poorly defined at present. Thus, while these results are consistent with normal expression of MHC class I on the leukaemic blasts it is possible that the specific inhibitory signal through HLA-C may be absent.

The identification of GVL activity in the absence of GVHD has been a long-term goal of many research groups. We believe that the ABMT setting is ideal for the study of this phenomenon and that the results presented here have important implications for the design of immunotherapeutic treatment strategies.

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Understanding the Graft-Versus-Leukaemia Reaction

Progress Towards The Immunotherapy of Leukaemia

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The role of the immune response in control and erradication of leukaemia remains controversial but there is increasing evidence that the principal mechanism of cure after intensive chemotherapy and allogeneic BMT is immune mediated. This is evidenced by the observations of a reduced risk of leukaemia relapse after allogeneic BMT when compared with autologous or syngeneic BMT. Furthermore, an increased incidence of leukaemic relapse has been reported after aggressive GvHD prophylaxis with cyclosporin or lymphocyte depletion. The association between GvHD and the graft-versus-leukaemia activity of allogeneic BMT is strong and has led to a number of workers concluding that GvL might be inseparable from GvHD although there is increasing evidence that this is not so. The lymphocyte subsets responsible for GvHD and GvL remain to be elucidated in man despite intensive efforts. Certainly T cells, natural killer cells and a population of in vivo activated killer cells are involved in GvL and an effective immune response probably requires a combined approach.

The target antigens of GvL are also controversial. The majority of GvL studies have been conducted in the allogeneic transplant setting in which activity to leukaemia-specific peptides is easily masked by reactivity to undetected MHC mismatches and to minor histocompatibility antigens. Despite this the search for leukaemia-specific peptides has been fruitful in the case of the product of the BCR/ABL translocation in CML.

The ultimate aim of a number of groups in all aspects of oncology is the development of effective and specific immunotherapy. A variety of approaches have been attempted over the last 80 years, including vaccination with irradiated leukaemic blasts and numerous trials of interleukin-2. We now know more about the mechanisms of induction of immunity than ever before and this knowledge, combined with sophisticated molecular biology and virology, promises to revolutionise the immunotherapy of leukaemia over the next ten years.

Keywords: Leukaemia; GvL; GvHD; immunotherapy

The first description of the graft-versus-leukaemia effect (GvL) was published more than 40 years ago[1] but even today the precise mechanisms of the phenomenon are controversial. Barnes and colleagues demonstrated in a murine model that leukaemic animals which received myeloablative radiotherapy followed by syngeneic BMT all relapsed with the original acute leukaemia and died. In contrast, animals receiving the same radiotherapy but allogeneic BMT all developed fatal graft-versus-host disease (GvHD). However, these animals

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survived longer than the recipients of syngeneic marrow and had no evidence of leukeamia relapse at post-mortem. This was evidence both that myeloablative radiotherapy does not eradicate leukaemia and of an anti-leukaemia immune response.

The clinical importance of GvL activity after transplantation only became apparent in the 1980's. During the early years of bone marrow transplantation the principal cause of mortality after transplant was GvHD. It was established in 1968 that acute GvHD is mediated by T-lymphocytes[2] and shown 10 years later that removal of lymphocytes from donor bone marrow prevented GvHD[3]. This led to clinical trials of T cell depletion (TCD) regimens[4,5,6]. In our group we have determined the role of TCD in an homogenous cohort of patients with AML in 1st complete remission (CR) having an HLA-matched sibling transplant. Our studies used ex-vivo depletion strategies with one of two mAb preparations followed by complement-mediated lysis. No post-transplant prophylactic immunosuppressive drugs were given. Initial trials utilised a cocktail of three locally produced mAbs; anti-CD6, anti-CD7 and anti-CD8. In a study of 31 matched sibling transplants for recipients in first complete remission there were four cases of moderate or severe acute GvHD (12.9%). Six patients showed evidence of limited chronic GvHD with a further patient suffering extensive chronic disease. There was only one GvHD-related death (3.2%). Despite this low incidence of GvHD the five year relapse rate was 3.8% and the event-free survival is 70%. Subsequent studies with ricin A-conjugated anti-CD5 mAb in 20 patients transplanted for a variety of haematological malignancies showed no evidence of severe acute or chronic GvHD and only one incidence of mild GvHD (grade I acute). Engraftment was 100% but the relapse rate in 10 patients transplanted in 1st CR of AML from HLA-matched siblings was 30%[7] and event-free survival is 60%.

We have also used Campath IgM (anti-CDw52), a monoclonal mouse IgM reactive with T, B and NK cells[8] as part of a multicentre study, with and without the IgG isotype (Campath 1G) for in vivo conditioning[9]. In a group of patients with AML in 1st CR having HLA-matched sibling donor transplants we have seen no evidence of severe acute GvHD, three cases of grade I acute and one incident of limited chronic GvHD. Use of the Campath IgM ex-vivo TCD alone was associated with a relapse risk of only 14% but the concurrent use of in vivo Campath IgG increased this risk to 50%. Since there was no evidence of GvHD or transplant-related mortality it is hypothesised that the IgG antibody continued to circulate at the time of marrow infusion leading to a greater TCD of the donor marrow. The event-free survival in this group is 49.6% which is little different from the 43% expected in this group of patients treated with chemotherapy alone in this era (Rees - personal communication). These data all suggested that the use of TCD could abrogate GvHD risk and markedly improve long-term survival although our experience with Campath 1G indicates that excessive TCD can lead to markedly increased risk of relapse.

The clinical anti-leukaemic effect of GvHD was first formally reported by Weiden[10] and confirmed later by registry data from IBMTR[11]. This study supported the finding that excessive TCD was associated with significantly higher relapse risk after allogeneic BMT and individual transplant groups reported significantly higher relapse rates after TCD BMT than after non-TCD transplants. Bacigalupo et al[12] showed that inappropriate immune suppression with cyclosporin as GvHD prophylaxis could also remove the anti-leukaemia benefit of allogeneic BMT. These results demonstrate that the increased survival associated with allogeneic BMT over that of autologous BMT is due to the GvL activity of the donor immune system. They are the clinical

correlates of Barnes' & colleagues' original murine studies.

The most widely used prophylaxis for GvHD remains cyclosporin (CsA). Whilst this may be less effective than TCD in GvH prevention it has the advantage that its administration can be stopped if the patient relapses or their is evidence of post-transplant lymphoproliferative disease. The cessation of CsA administration is capable of re-inducing remission but often at the cost of GvHD[13;14;15]. CsA is suppressive of both T and NK cell-mediated cytotoxicity. Collins et al[13] found that, in a single patient, the discontinuation of CsA led to the development of NK-mediated lytic activity against K562 target cells and against autologous CML blasts. In addition, they observed simultaneous peripheral expansion of T cells and concluded that both T and NK cells are likely to be involved in the GvL response.

The effectiveness of cessation of immune suppression in the treatment of relapse after allogeneic BMT was followed by the use of donor T cell therapy in the same setting. Appropriate T cell depletion prevents GvHD; it is therefore logical that donor T cell add-back at time of relapse should induced anti-leukaemia immunity. The bone marrow transplant group at Hadassah University Hospital were the first to add back allogeneic T cells to patients who had received TCD bone marrow[16]. The first report of the use of non-manipulated donor leukocytes for the treatment of haematological relapse in chronic myelogenous leukaemia (CML) after allogeneic BMT was from Germany[17]. This paper reported the use of viable leukocytes from the peripheral blood of the donor in three patients who had relapsed with CML after allogeneic BMT. All three attained remission. Two developed GvHD which responded to treatment with corticosteroids.

The success of donor leukocyte transfusions (DLT) in the treatment of relapsed CML led to its widespread use and the determination of an optimal dosing strategy to maximise GvL benefit while minimising the concomitant risk of GvHD[18]. This study used staged doses of T cells over a protracted period of up to eight months. Haematological remission was achieved in 19 of 22 patients; over half of the responders did so after receiving $1\text{-}5 \times 10^7$ donor T cells / kg. The remaining responders received either 1×10^8 or 5×10^8 T cells per kg. GvHD was limited, with only one fatality. The current status of this treatment in Europe has been reviewed recently for the European group for Blood and Marrow Transplantation (EBMT)[19].

The application of DLT in the treatment of relapsed acute leukaemia after BMT is more restricted[20,21,22]. In a study of 7 patients with acute lymphoid or acute myeloid leukaemia we reported a high incidence of post DLT GvHD (6/7) after administration of between 10^7 and 10^8 T cells/kg. Three patients who had not received cytoreductive chemotherapy prior to DLT did not respond. In contrast, the four patients receiving prior chemotherapy did respond[22]. A UK trial assessing the use of DLT in acute leukaemia is currently underway.

The international status of DLT for relapsed leukaemia after allogeneic BMT has been reviewed and included data from 45 patients with acute leukaemia[23]. Twelve patients (26.7%) achieved "long-term" disease free survival although the definition of long-term was not given. Interestingly, this included none of the patients relapsing with acute lymphocytic leukaemia despite the fact that 5/10 of these patients did show a complete response to the DLT as determined by PCR. These data raise the possibility that lymphoblasts are less immunogenic than myeloblasts.

Only around 30% of patients who would benefit from allogeneic stem cell transplantation have an identifiable HLA-matched donor. This is despite the magnificent efforts of the international bone marrow donor panels. To overcome this limitation a number of groups

have investigated the induction of immunity against leukaemia after autologous BMT (aBMT).

Early in the 1980s, two reports demonstrated that a graft-versus-host disease-like phenomenon could be induced after syngeneic or autologous BMT by the administration of CsA[24,25]. It was shown that the CsA broke tolerance to Class II MHC antigens[25]. A trial was undertaken at the Royal Marsden Hospital to investigate the anti-leukaemia effect of autologous GvHD post-BMT for AML[26]. Nine aBMT recipients received CsA daily (1 mg/kg) for up to 28 days post BMT. Biopsy-proven acute skin GvHD was observed in 3 patients, two of whom subsequently relapsed. A second trial in Genoa confirmed the GvHD-inductive ability of CsA but showed no survival benefit[27].

With the cloning of human interleukin-2 (IL-2) came the perceived ability to manipulate the immune response against tumours. The demonstration that IL-2 treatment of mononuclear cell preparations from leukaemic patients generated NK cells which were lytic to autologous and allogeneic leukaemic blasts[26] led to the establishment of clinical trials in the treatment of leukaemia patients. Many studies were in recipients of autologous BMT[29] whilst others studied patients after chemotherapy alone[30,31,32]. All the trials of IL-2 in aBMT can be divided into one of three basic groups. These are the use of IL-2 in vivo with or without concomitant aBMT; the use of IL-2 in vitro to stimulate NK/LAK responses and the subsequent infusion of activated cells; or the combination of in vivo IL-2 with NK/LAK cell therapy. The timing and route of administration varied as well as the clinical stage of the recipient. In some studies the patients were only treated in remission whilst others treated in a state of minimal residual disease. Basically the results were uniformly poor despite initially encouraging reports[33] and multicentre trials have now ceased. Probably the most interesting and informative trial of IL-2 therapy was a small study in Seattle. This group treated 14 patients with AML, of whom 11 were in first or second relapse, with aBMT and IL-2[34]. These patients with very poor prognosis showed a remarkable response to IL-2 therapy with a four year actuarial disease-free survival of 64%. This is markedly better than the results of a multicentre trial of IL-2 consolidation in leukaemia-free patients after aBMT and suggests that the benefit of IL-2 therapy may be maximised in the presence of minimal disease which acts as a source of leukaemia-specific antigens. Although this is an attractive hypothesis it is partly refuted by the observation that clinical benefit was associated with apparent autologous GvHD[35], suggesting that the response may not have been leukaemia-specific.

The concept of leukaemia-specific antigens remains the most controversial area in GvL research. The data from TCD allogeneic BMT implied that moderate GvHD is essential for manifestation of GvL and the results from DLT studies are similar. Although we and others have shown very low relapse rates after allogeneic BMT in the absence of GvHD it can still be argued that the alloreactivity of the donor lymphocytes induced sub-clinical GvHD.

The target antigens involved in GvL depend upon the effector cells involved and may be miHAgs, leukaemia-specific antigens or undetectable MHC mismatches in the non-related donor setting. Recently miHAg-specific T cell clones been have successfully isolated and characterised[36,37]. Some miHAgs have been cloned and their tissue distribution described[38]. One of these molecules, HA-2, is restricted to haematopoietic cells and represents a potential target for immunotherapy. Although not leukaemia-specific, the immune response to this miHAg would eliminate all residual host haematopoiesis, both normal and malignant, with no overt clinical GvHD. The Leiden group continues to work towards a

clinical application with the generation of stable miHAg-specific cell lines and clones.

The search for leukaemic-specific antigens has been intense. Given the known genetic translocations associated with many acute and chronic leukaemias it is unsurprising that many groups have searched for immunostimulatory peptides within the resultant leukaemia-specific proteins. The most widely studied has been BCR-ABL in chronic myeloid leukaemias (CML). Data have been published describing peptides from BCR-ABL which are MHC Class II-restricted and are in vitro targets for cytolytic activity[39,40]. In contrast, other workers have been unable to identify MHC Class I-restricted peptides from BCR-ABL[40,41]. This leads to the intriguing possibility that class-I restricted CD8 T cells are not required for specific GvL and are only involved in alloreactivity. Indeed, the majority of T cell clones raised against miHAgs have been CD4 cells (Falkenburg - personal communication).

The most convincing evidence of leukaemia-specific immunity must come from patients who have not received an allogeneic BMT. Recently we have been monitoring recipients of autologous BMT as treatment for acute leukaemia. We have demonstrated in vitro cytotoxic activity against presentation leukaemic blasts in three out of three patients in the absence of reactivity against their normal remission myeloid or lymphoid cells[43]. In two patients the loss of this activity was immediately followed by frank relapse. In one patient the response returned after re-induction chemotherapy and has been maintained for 18 months during which time she has remained in remission. The second patient achieved remission after further chemotherapy but his immune response to his leukaemia was not re-established. He relapsed soon after and, after further re-induction chemotherapy, received a transplant from a matched non-related donor. The final patient studied to date received an autologous BMT for AML. She is now 46 months post-transplant and has had a demonstrable anti-leukaemia cytotoxic response when tested at 12, 18, 24 and 33 months. She remains in complete remission. These data are strong evidence for the concept that leukaemic cells express surface molecules which can direct an appropriate immune response and that these molecules are absent or are expressed at a low level on normal myeloid and lymphoid cells. They tell us nothing about the presence or absence of leukaemia-specific antigens.

Much work has been directed to the identification of lymphoid mediators of GvL. Certainly CD3+ T cells[44,45], CD16+ natural killer cells[46] and a population of in vivo activated killer cells[43,47] are involved. It has been established in a murine system that the cells mediating GvL can be dissected from those mediating GvHD[44] although the observations appear to dependent upon the strains of mice used as recipients and donors[48]. In some studies the GvL response is limited to the CD8 cells while in others CD4 cells are exclusively involved. In human BMT, infusions of CD8-depleted donor leukocyte preparations have been used successfully for the treatment of relapse following allogeneic BMT for chronic myeloid leukaemia[49], suggesting that CD4 T cells and natural killer cells may all have important roles in the GvL effect in humans.

High numbers of activated NK cells can be detected in the peripheral blood after both autologous and allogeneic BMT[43,47,50,51]. Furthermore, high numbers of resting NK cells circulate in the peripheral blood of transplant-recipients for many months after the loss of AK cells. That these cells can have anti-leukaemia activity has been reported after allogeneic and aBMT[41,44,45]. In an extensive study of patients with a variety of acute leukaemias it was found that the levels of NK cell activity against the NK-sensitive cell line K562 were significantly lower than in normal blood donors[52]. Furthermore, this activity increased when the patients were in remission and in all 15 patients studied

longitudinally, a reduction in NK activity to pre-remission levels was associated relapse within 10 weeks. These data suggest that functional NK responses may be important in the control of residual leukaemic cells but there was no direct evidence that NK cells could lyse autologous leukaemic cells. Most recently, a group has demonstrated NK-mediated specific lysis of autologous CLL cells in three of five patients after chemotherapy without BMT[53]. No data were presented on the relationship between this activity and survival. Intriguingly, NK cells may also be important in the generation of GvHD and thus non-specific GvL. A recent report has shown that in vitro generation of alloreactive T cells requires the presence of allogeneic NK cells[54].

NK cell are not alone in mediating non-MHC restricted cytotoxicity and there are reports in the literature of $\gamma\delta$ T cell mediated GvL[55,56,57]. $\gamma\delta$ T cells recognise heat shock proteins which are commonly expressed on the surface of tumour cells[58]. In an elegant SCID mouse model of human leukaemia Malkovska et al[59], showed that such $\gamma\delta$ T cells could mediate in vivo antilymphoma activity which significantly improved survival. Furthermore, they confirmed that NK cells were not involved in this activity by depletion studies with monoclonal antibody. In HLA-mismatched transplants a high incidence and frequency of $\gamma\delta$ T cells has been reported in the peripheral blood post-transplant and their presence associated with long-term survival[57].

It is clear from the data described above that the majority of leukaemias respond to immune control but most patients who would benefit from the GvL associated with allogeneic BMT still lack a suitable donor. Immunotherapy trials from the mid-1980s and early 1990s were largely unsuccessful. This was for a variety of reasons but the principal problem was the toxicity of systemic cytokine administration. Future immunotherapy protocols will be more sophisticated and less toxic. More than 20 years ago Bretscher & Cohn proposed their two-signal hypothesis for immune activation of B cells[60].

Increasingly the requirements for T cell activation are becoming defined and it has been shown that T cells require at least two signals for activation. One is provided by the cognate interaction between the T cell receptor and Antigen/MHC complex; a second signal is provided by the interaction between a costimulatory molecule and its receptor on the T cell. T cells receiving signal 1 in the absence of signal 2 become anergic or are deleted. In contrast, CTL receiving both signals expand clonally and are able to respond to target cells which express only signal 1. A proposed reason for the escape from immune surveillance is that tumours do not express appropriate costimulatory molecules and thus render T cells anergic to any tumour associated antigens.

The most studied costimulatory molecules are the B7 family which bind to the T cell antigens CD28 and CTLA 4. We have studied the expression of B7.1 and B7.2 on 25 samples of AML and have found only rare expression of B7.1 and at levels below that seen on normal myeloid cells. In contrast, B7.2 expression was frequently detected, although also at levels below that seen on normal monocytes.

Animal tumour models have demonstrated that vaccination with modified tumour cells expressing B7.1 leads to protective immunity against both the modified cells and the parental tumour. This approach may also be used to treat small established tumours in at least some cases. It has also been shown to stimulate protective immunity to a radiation-induced leukaemia in a murine system[61].

Transduction of B7.1 into some highly immunogenic tumours has led to their rejection in animal models[62;63]. However, spontaneous human malignancies are likely to be of low immunogenicity. To overcome this we and others are combining the gene transfer of B7.1 to AML cells with cytokines such as interleukin-2

(IL-2) or IL-12[64,65]. The advantage of transfer of cytokine genes to leukaemic blasts is that the proteins are secreted locally in vivo. This will reduce the toxicity associated with systemic cytokine therapy.

The proposals outlined above are aimed at engineering tumours cells to mimic antigen presenting cells (APC). An alternative approach which is under investigation is the use of autologous dendritic cells, either as isolated from peripheral blood or cultured from haematopoietic stem cells, to present tumour-specific peptides. This approach has the advantage of using a highly efficient APC which is readily obtainable. Its disadvantage is the requirement for antigenic peptides which have not yet been identified in the case of most leukaemias. The approach has been reviewed recently[66].

The last half century has seen a dramatic advance in our understanding of the pathogenesis and treatment of leukaemia and the role of the immune response in the long-term remission is well established. The future of leukaemia therapy in the next century lies in the combined use of autologous haematopoietic stem cell transplantation with effective autologous immune therapy.

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The effect of T cell depletion with Campath-1M on immune reconstitution after chemotherapy and allogeneic bone marrow transplant as treatment for leukaemia

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Summary:

The prophylactic use of T cell depletion (TCD) strategies for the prevention of graft-versus-host disease (GVHD) following allogeneic stem cell transplantation remains widespread. Initial reports of high incidence of graft rejection after TCD BMT led to a move away from this approach but improved conditioning regimens have reduced this risk substantially. The use of TCD has also been associated with higher relapse risk post-BMT although the success of donor leukocyte infusion (DLI) as treatment for relapse has reduced this problem, especially in chronic myeloid leukaemia (CML). Currently the use of TCD BMT is increasing particularly due to the relative increase in BMT from non-related donors for whom TCD is the optimal GVHD prophylaxis. However, doubts remain over the long-term effect on the reconstituted immune system of recipients of TCD BMT, particularly in adult recipients. In this study we have undertaken a detailed sequential analysis in 23 patients who received allo-grafts from HLA-identical sibling donors after high-dose chemo/radiotherapy for acute or chronic leukaemia. Of these patients, 11 received non-manipulated grafts, five received 'partially TCD' (PTCD) and a further seven received 'fully TCD' (FTCD) bone marrow. T cell depletion was performed *ex vivo* by Campath-1M plus autologous serum as a source of complement. Partial TCD describes grafts with a T cell reduction of 1–2 log. Full TCD refers to grafts with a reduction of >2.5 log. The decision regarding the optimal degree of TCD was clinical and was based upon the perceived relative risk of relapse based upon the disease and remission status. All patients were monitored for up to 12 months post-BMT with regard to reconstitution of T and NK cell subsets. T cell depletion at either level was associated with a slower recovery of CD4 cells. This was most marked in the FTCD recipients and lasted throughout the period of study. CD8 cell recovery was also slower in the TCD recipients but this normalised throughout the 12 months post-BMT. The ratio of CD45RA$^+$:CD45RO$^+$ increased in all recipients after month 3. This suggests that a degree of extra-thymic T cell maturation can occur in recipients of allogeneic BMT. NK cell recovery was more rapid in the TCD recipients and these differences were maintained throughout the first year.

Keywords: allogeneic; Campath; T cells; NK cells; immune reconstitution

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It was established as early as 1968 that acute graft-versus-host disease (GVHD) is mediated by lymphocytes[1] and could be prevented with anti-lymphocyte globulin[2] and a variety of methods have subsequently been employed in both animal models and in the clinical setting to achieve this. In the mid-1970s discontinuous density gradients were used to remove mature lymphocytes from donor mouse bone marrow in allogeneic transplant experiments. These depletions prevented all evidence of GVHD and demonstrated that acute GVHD is mediated by T lymphocytes. This led to clinical trials of T cell depletion (TCD) regimens.[3–6]

That TCD could prevent GVHD was accepted in the early 1980s but it soon became apparent that this beneficial effect was countered by a concomitant loss of suppression of residual host immunity, as well as the anti-leukaemic benefit of the donor T cells, leading to a higher rate of graft rejection and/or leukaemia relapse. Successful use of TCD requires establishment of an appropriate balance between the residual immune response of the recipient, which can be achieved by appropriate conditioning, and the adjustment of the T cell content of the donor bone marrow.

Since the late 1970s we have used a variety of strategies for *in vivo* and *ex vivo* TCD of donor marrow prior to allogeneic transplantation including a cocktail of three locally produced mAbs: anti-CD6, anti-CD7 and anti-CD8;[7] a ricin-conjugated anti-CD5;[6] as well as Campath-1M.

In this study we have used Campath-1M (anti-CDw52) for *ex vivo* TCD. This is a rat monoclonal IgM reactive with T, B and NK cells.[8] In a previous study of a similar group of patients with AML in 1st CR having HLA-matched sibling donor transplants at our centre we saw no evidence of severe acute GVHD, three cases of grade I acute and one incident of limited chronic GVHD. The relapse rate in this group is 14%.[7]

Thus, TCD BMT after appropriate conditioning can prevent GVHD without loss of GVL. However, the question of the effect on immune reconstitution remains. Studies

from our group and others during the 1980s suggested that, although delayed, immune reconstitution was normal after TCD BMT. Helper T cell function as determined by the ability to promote immunoglobulin synthesis has been reported to return early after TCD BMT[9] although plasma cell activity can be delayed for up to a year after transplant.[9,10]

Given that many of the recipients of TCD BMT are adult and consequently are assumed to have little or no thymic activity there has been much speculation about the diversity of the T cell repertoire post transplant. It has been shown that the peripheral blood T cells in long-term survivors of non-TCD allogeneic BMT are oligoclonal in nature and this has been taken as evidence that these cells have derived from mature T cells within the graft.[11] Consequently, it has been suggested that the removal of T cells from allo-grafts will restrict the repertoire further, leading to a degree of immune incompetence post transplant. However, extra-thymic T cell maturation can occur[12] and this may lead to normal T cell reconstitution post-TCD BMT.

In this study we have followed 23 recipients of allogeneic BMT from HLA-identical sibling donors for up to 12 months post transplant. Of these, 11 received non-manipulated grafts, five received grafts after T cell reduction of 1–2 log and the remaining seven received grafts following T cell depletion of >2 logs. Patients were comprehensively monitored for reconstitution of T and natural killer (NK) cell subsets weekly from week +3 for 3 months and then monthly until month 12.

Materials and methods

All patients undergoing allogeneic BMT from HLA-identical sibling donors for haematopoietic reconstitution following high-dose chemotherapy and radiotherapy for haematological malignancy were enrolled in the study after informed consent. Patient demographics are detailed in Table 1. All patients received cyclophosphamide (60 mg/kg/day; 2 days) and total body irradiation (750 Gy, fast rate 15 cGy/min, single fraction). No patients received Campath-1G as part of their conditioning regimen. Donor bone marrow was processed to a mononuclear cell fraction in a closed system (Cobe 2991, Zaventem, Belgium) and either infused immediately or T cell depleted to the planned level of T cell content and infused within 6 h. T cell depletion was by complement-mediated lysis. Briefly, the proportion of T cells in the donor marrow was determined by flow cytometry using anti-CD3-FITC monoclonal antibody (Becton Dickinson, Oxford, UK) and absolute mononuclear cell counts were measured by a particle counter (Coulter Z-1, Luton, UK). Combination of the results allowed the calculation of the absolute T cell content of the donor bone marrow. The volume of bone marrow containing the appropriate T cell dose for re-infusion was removed from each harvest and the residue incubated with Campath-1M. Donor serum was used as the source of complement. The degree of T cell depletion in the Campath-1M-treated bone marrow was assessed in a sample of the treated harvest by flow cytometry using anti-CD3 and the vital dye propidium iodide (5 μg/ml; Sigma, Poole, UK). The proportion of live CD3+ve T cells was determined by propidium iodide exclusion. Samples were simultaneously assessed for residual Campath-1M binding to $CD3^+$ cells using anti-rat IgM PE. T cells which remained viable after *ex vivo* Campath-1M but which continued to bind the rat monoclonal antibody were considered to be part of the depleted fraction since lysis was likely to occur *in vivo* following re-infusion. The final product was a combination of the non-depleted and depleted fractions, adjusted to achieve the desired T cell content.

Table 1 Patient demographics

Sex			
Male	18		
Female	10		
Diagnosis			
AML	14		
ALL	9		
CML	4		
MDS	1		
T cell depletion			
No. of patients analysed at each time-point	*<3 months*	*3–6 months*	*6–12 months*
Non-depleted	11	7	4
Partially depleted	5	5	3
Fully depleted	7	7	7

The degree of T cell depletion was a clinical decision made with respect to the perceived likelihood of disease recurrence. 'Partial' TCD was determined as 1–2 log reduction in total CD3+ve T cells; 'full' TCD represented a reduction of greater than 2 log as determined by the flow cytometric method. In general, 'good'-risk patients received fully TCD grafts while those at high risk of relapse received non- or partially depleted bone marrow. This differential risk led to greater attrition in the non-TCD group, with 12 month survival of 36% compared with 83% in the fully TCD group. However, the larger starting population in the non-TCD group ensured that similar numbers of patients were assessed in each group at each time-point.

Patients were followed at least 3 ×/week after transplant with respect to white blood cell count and leukocyte differential until engraftment. After 3 weeks the EDTA-preserved samples were additionally assayed for lymphocyte phenotypes by a lysed whole blood technique and three-colour flow cytometry. All leukocyte counts and differentials were performed within 6 h of venepuncture whilst immunophenotyping was performed within 24 h. The combinations of monoclonal antibodies (mAbs) are listed in Table 2. Monoclonal antibodies were conjugated with FITC, PE or PerCp. All were supplied by Becton Dickinson Immunocytometry Systems (Oxford, UK). Labelling was by a standard lysed whole blood method with a proprietary red cell lysis buffer (FACSlyse; Becton Dickinson). All samples were analysed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems) within 4 h of preparation. Lymphocytes were isolated by electronic gating on the basis of forward and side angle light scatter signals and at least 5000 events collected as list mode data (Lysis II; Becton Dickinson Immunocytometry Systems).

Table 2 Monoclonal antibody combinations

Tube No.	*FITC reagent*	*PE reagent*	*PerCp reagent*
1	CD45RA	CD45RO	CD4
2	CD45RA	CD45RO	CD8
3	CD57	CD56	CD8
4	CD57	CD28	CD4
5	CD57	CD28	CD8
6	HLA-DR	CD56	CD8

Phenotyping data were analysed in an interrelational three-colour system (Paint-a-Gate Plus; Becton Dickinson Immunocytometry Systems).

Statistical analysis was performed on the means and standard deviations of each comparable distribution by non-paired Student's *t*-test after initial analysis of variance by F test. Homoscedastic distributions were compared by conventional Student's *t*-test while those of unequal variance were compared by Snedecor's modified *t*-test.[13]

Results

Lymphocyte engraftment

The rate of lymphocyte engraftment followed a broad distribution in each of the three groups of patients. The absolute peripheral blood lymphocyte count can be used as a broad indicator of lymphocyte engraftment and the mean value for all patients during the initial 3 months post BMT was 0.52×10^9/l (s.d. 0.49). During the 3–6 month period this rose to 1.21×10^9/l (s.d. 0.67) and was 1.39×10^9/l (s.d. 0.93) in the 6–12 month period.

All patient groups showed similar mean absolute lymphocyte counts at the 3 and 6 month time-points although the fully TCD group showed a trend towards lower absolute counts which was not statistically significant. In contrast, the mean absolute lymphocyte counts in the recipients of non-TCD or partial TCD were significantly higher than that of the fully TCD group (1.33×10^9/l *vs* 2.23×10^9/l *vs* 0.62×10^9/l, respectively) ($P < 0.02$).

T lymphocyte reconstitution

TCD, whether full or partial, was associated with a slower recovery of CD4 cells than non-TCD transplant. In the non-TCD recipients the absolute number of CD4 cells rose steadily after BMT and in the final period of analysis had reached the lower end of the normal adult range, although even by 6 months after transplant it had not exceeded the proportion of CD8 cells. In contrast, although the CD4 cell level rose in the two TCD groups during the period of study, neither reached the lower level of normal at any time during the 12 month period. Analysis between 1–3, 3–6 and beyond 6 months post-transplant showed a consistently lower CD4 cell incidence in the recipients of TCD marrow than in recipients of non-manipulated grafts at each time-point. The CD4 reconstitution in the recipients of fully TCD grafts was significantly slower than either the partial or non-TCD groups at 6 months ($P < 0.001$ for both) (Figure 1a). This was also true of the period between 6 and 12 months post-BMT although at this time the recipients of partial TCD grafts showed lower CD4 levels than the non-TCD recipients ($P < 0.05$).

CD45RA expression is a phenotypic marker of immune naivity since it is lost upon activation. The proportion of CD4 cells expressing CD45RA fell consistently in the fully TCD recipients at each time-point after BMT. In contrast, while there were no significant differences between the relative proportions of $CD45RA^+$ CD4 cells in the three groups in the first 3 months after BMT, both the partial TCD and non-TCD showed a significant increase in CD45RA+ve at the further time-point ($P < 0.01$) (Figure 1b). Although these changes were statistically significant, they represented small increases in the relative proportions since the majority (78–98%) of the CD4 cells in all groups of patients were CD45RA−ve/CD28+ve (Figure 1c).

CD8 T cell reconstitution was more rapid than that of CD4 in all groups and was within the normal adult range within 3 months (Figure 2a). However, the recipients of fully TCD grafts showed significantly lower absolute numbers of CD8 cells in the final period of study due to the low absolute lymphocyte count described above. In addition, despite similar total lymphocyte counts between groups at the 3 month time-point, the absolute number and proportion of CD8 cells was significantly lower at 3 months post BMT in the fully TCD patients as compared with both the partial and non-TCD recipients ($P < 0.001$). At this time, all three groups showed low incidence of $CD45RA^+$/CD8+ve cells and this proportion rose at each subsequent time-point in each group. These two observations suggest that the CD8 cells arising early after BMT are mature cells included in the graft. The subsequent rise in $CD45RA^+$ cells within the CD8 cell subset was accompanied by a concomitant fall in the expression of CD28 (Figure 2c) in all patient groups. The fall was most marked in the two TCD groups and was significantly lower in these than in the non-TCD recipients ($P < 0.001$). The initial incidence of $CD28^+$ cells within the CD8 subset was within the normal adult range in all three groups, further supporting the suggestion that these cells were transplanted with the graft. By the latter 6 months this had fallen below the normal range in the recipients of TCD transplants (Figure 2c).

All three groups showed very high levels of CD8 activation as measured by HLA-DR expression throughout the period of study. There were no significant differences between the three groups in the first 6 months although the non-TCD and partial TCD groups showed a trend towards decreasing CD8 cell activation while the level was maintained in the fully TCD group (Figure 2d). In the latter 6 months the proportion of CD8 cells which were activated in the fully TCD group was significantly higher than in the partial and non-TCD patients ($P < 0.02$ and $P < 0.001$, respectively).

CD57 expression on CD8 cells was consistently in the upper normal range for all groups and showed a trend to increasing with time post BMT (Figure 2e). The distributions were broad in all patient groups and showed no significant differences.

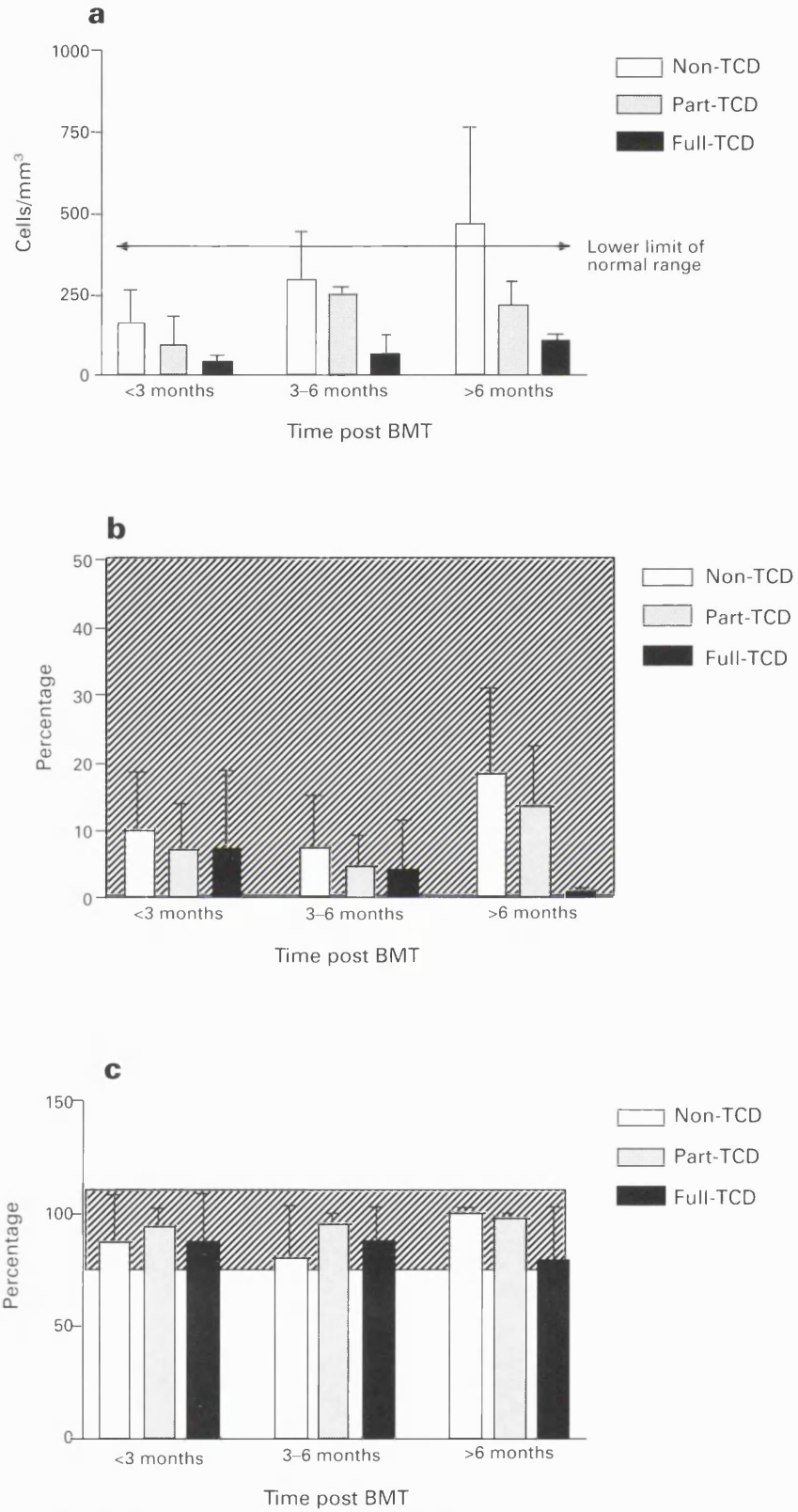

Figure 1 (**a**) $CD4^+$ T cell recovery. The bars depict arithmetic mean values with error bars showing standard deviations. The arrow-headed line represents lower limit of the normal adult range (mean − 2 s.d.) from 20 normal donors. (**b**) CD45RA expression on CD4 cells as a proportion of total CD4 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area represents normal adult range (mean ± 2 s.d.) from 20 normal donors. (**c**) CD28 expression on CD4 cells as a proportion of total CD4 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area represents normal adult range (mean ± 2 s.d.) from 20 normal donors.

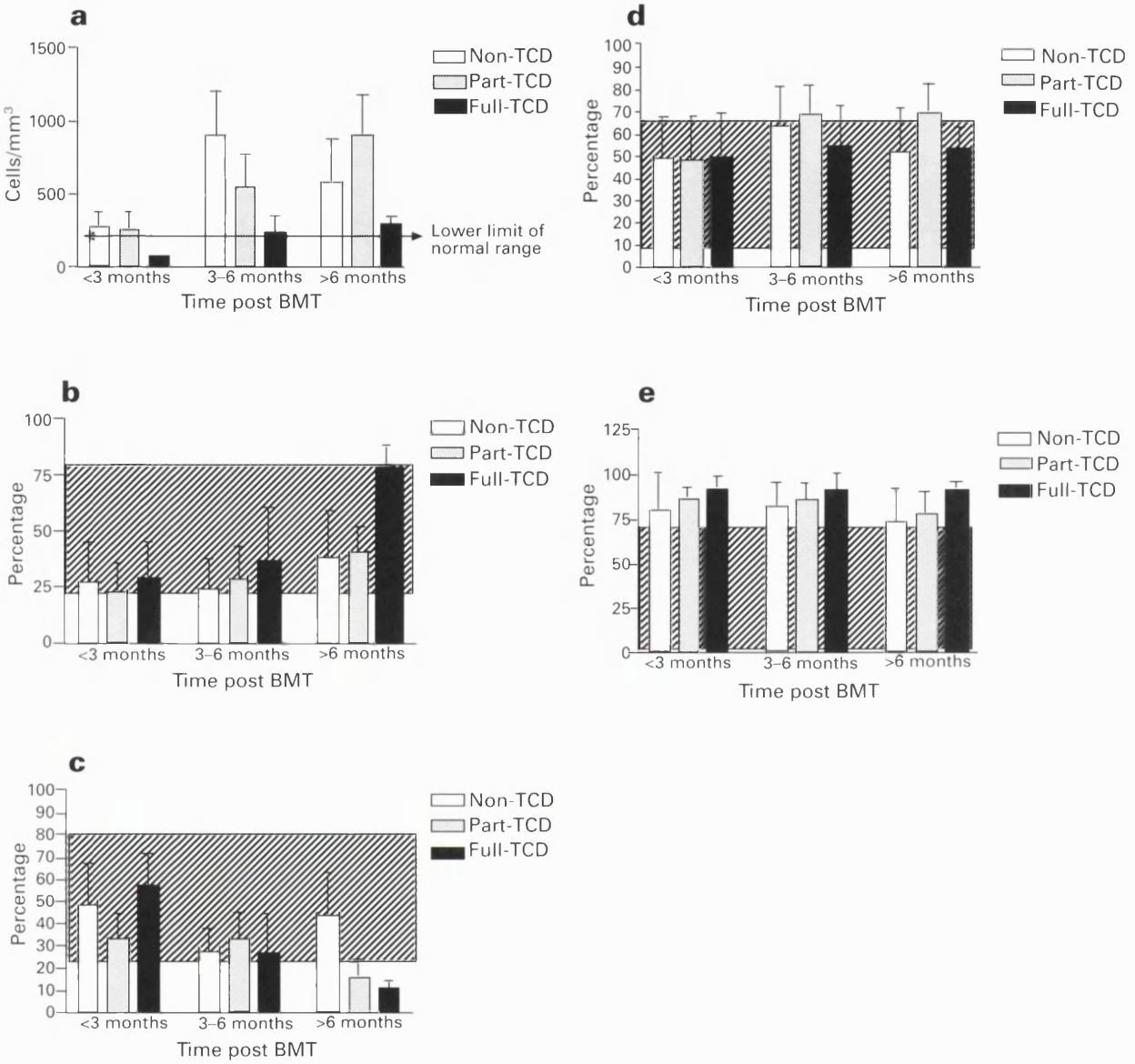

Figure 2 $CD8^+$ T cell recovery. The bars depict arithmetic mean values with error bars showing standard deviations. The arrow-headed line represents lower limit of the normal adult range (mean − 2 s.d.) from 20 normal donors. (**b**) CD45RA expression on CD8 cells as a proportion of total CD8 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area represents normal adult range (mean ± 2 s.d.) from 20 normal donors. (**c**) CD28 expression on CD8 cells as a proportion of total CD8 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area represents normal adult range (mean ± 2 s.d.) from 20 normal donors. (**d**) CD57 expression on CD8 cells as a proportion of total CD8 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area represents normal adult range (mean ± 2 s.d.) from 20 normal donors. (**e**) HLA-DR expression on CD8 cells as a proportion of total CD8 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area repreents normal adult range (mean ± 2 s.d.) from 20 normal donors.

Natural killer cell reconstitution was swift in all groups post-BMT (Figure 3a) and, as a proportion of the total lymphocytes, exceeded the upper limit of normal within 3 months in the fully TCD group (Figure 3c). All three groups behaved identically with respect to NK cell reconstitution and the relative proportion of CD56+ve cells fell consistently with time although the proportion of NK cells in the fully TCD group remained significantly higher in the fully TCD group than in either of the other groups at all time-points ($P < 0.001$). This high relative proportion within the total lymphocyte population in the fully TCD group meant that the absolute number of NK cells per mm^3 in this group was not significantly different from those of either the partial or non-TCD groups The early NK cell expansion seen in the fully TCD group was predominantly within the CD56+ve/CD8−ve subset since the proportion of CD56+/CD8wk+ve cells was lower in the fully TCD group than in the non-TCD group initially (Figure 3d). This NK cell subset declined in the partially and non-TCD groups with time but increased substantially in the fully TCD group such that it was significantly greater by the latter 6 months ($P < 0.001$). The increase of CD8wk+ve cells within the NK cell subset in the fully TCD group was accompanied by a concomitant decline in CD56+ve/CD8−ve cells as shown by the increase in the proportion of CD56+ve/CD8wk+ve cells within the total CD56 cell

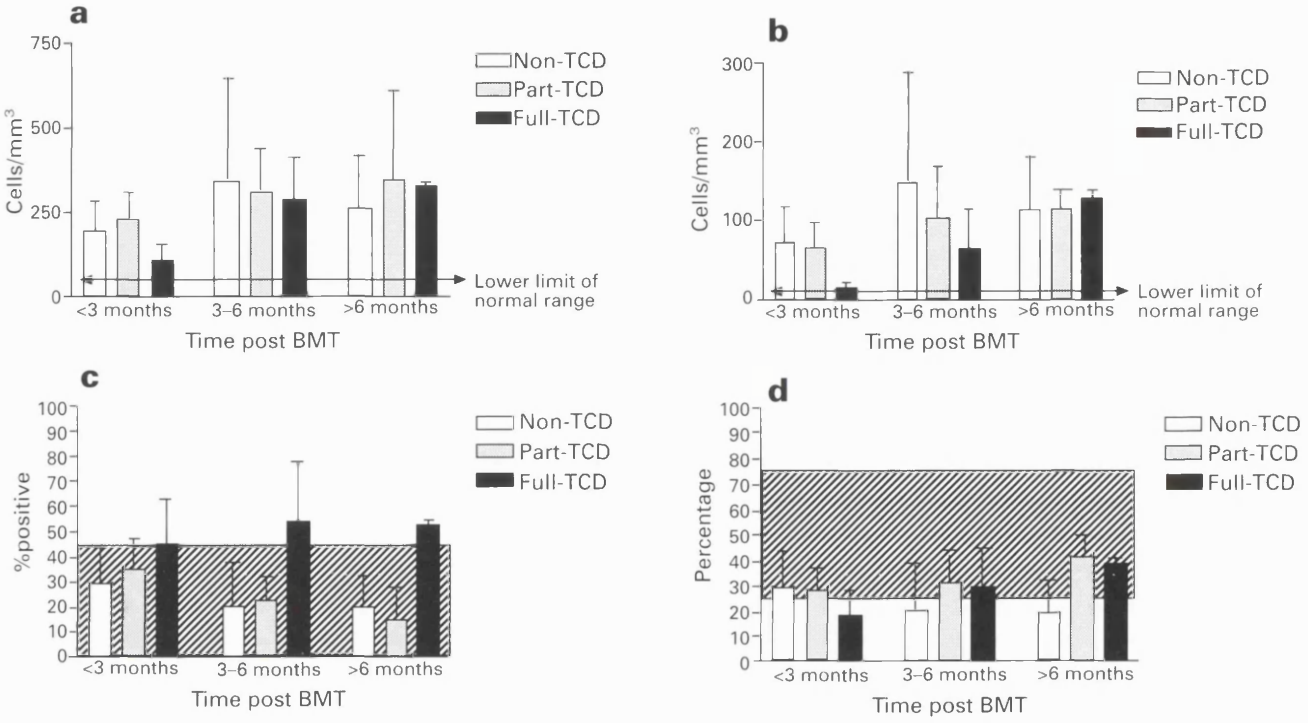

Figure 3 $CD56^+$ NK cell recovery. The bars depict arithmetic mean values with error bars showing standard deviations. The arrow-headed line represents lower limit of the normal adult range (mean − 2 s.d.) from 20 normal donors. (**b**) $CD56^+/CD8wk^+$ cell recovery. The bars depict arithmetic mean values with error bars showing standard deviations. The arrow-headed line represents lower limit of the normal adult range (mean ± 2 s.d.) from 20 normal donors. (**c**) CD56 cell recovery in patients after allogeneic BMT. Results are presented as relative percentage of total lymphocytes and bars represent arithmetic means. Error bars show populations standard deviation. Shaded area represents normal adult range (mean ± 2. s.d.) from 20 normal donors. (**d**) CD8 expression on CD56 cells as a proportion of total CD56 cells. The bars depict arithmetic mean values with error bars showing standard deviations. Shaded area represents normal adult range (mean ± 2 s.d.) from 20 normal donors.

subset (Figure 3d) without a substantial increase in the total proportion of NK cells (Figure 3c).

The percentage of CD56+ve/CD8wk+ve cells as a proportion of the total NK cell subset fell in the non-TCD group with progression from BMT, although their absolute number increased due to the increasing absolute lymphocyte count. In contrast, recipients of either partial or fully TCD BMT showed a constant rise in both absolute number (Figure 3b) and in relative proportion (Figure 3d). In the partial TCD group this was due to a preferential loss of $CD56^+/CD8-$ve cells since the total proportion of NK cells fell during the same period (Figure 3c).

Discussion

We and others have previously described the patterns of cellular and humoral immune reconstitution after TCD BMT[10,11,14,15] including functional studies.[9,16–18] Two published studies have involved recipients of Campath-1M-treated bone marrows[11,15] but this is the first to analyse the effect of returning differing numbers of T cells with the graft. Furthermore, the use of three-colour immunophenotyping has allowed greater dissection of the cell subsets of interest than previously possible.

In common with Foot *et al*[15] and Keever *et al*[18] we found that lymphocyte engraftment was broadly similar after depleted and non-depleted grafts although our group of fully TCD patients did show significantly poorer lymphocyte recovery in the latter 6 months of study. Our observation that CD4 cell recovery was considerably slower after either partial or full TCD and that this was far more prolonged than CD8 cells was also in keeping with the results of Foot and colleagues.

The differential emergence of $CD45RA^+$ CD4 cells in the three groups of patients is of note, particularly in comparison with the dynamics of $CD45RA^+$ CD8 cell repopulation. CD45RA is a marker of naive T cells[19] and the appearance of CD4 cells expressing this antigen in patients after allogeneic BMT implies that donor-derived immature cells are involved in the repopulation of the immune system. However, it is intriguing that recipients of non-TCD grafts showed consistently higher ratios of $CD45RA^+$:$CD45RO^-$ CD4 cells than did recipients of TCD grafts. One might expect T cell repopulation to be faster in recipients of non-TCD grafts since donor T cells will have been transferred with the graft. Indeed, Roux *et al*[11] showed that the T cell repertoire of recipients of TCD transplants was extremely restricted whereas patients who received non-TCD grafts showed broad T cell repopulation. They concluded from this that early T cell reconstitution after allogeneic transplant is due to peripheral expansion of T cells present in the stem cell graft although a late wave of increased diversity was observed in a proportion of patients. Our data show that naive CD4 cells appear early after both TCD and T cell replete transplantation and their relative proportion within the CD4 subset increases substantially in recipients of T cell-replete grafts while declining in recipi-

ents of fully TCD grafts. The Roux data showed that host T cells survived in the recipients of TCD transplants for up to 6 months. If this is the case in our patients, then the $CD45RA^+$ cells in the recipients of fully TCD grafts may have been of host origin and their subsequent loss due to the expanding donor T cell pool.

However, this is difficult to reconcile with the CD8 reconstitution data in which the fully TCD recipients showed a consistent expansion of $CD45RA^+$ cells in common with the non- and partial TCD grafts. Our current interpretation of these results is that naive CD4 and CD8 cells are generated after T cell-depleted or -replete transplants from donor-derived lymphocyte precursors and these cells mature either in the residual host thymus or via extrathymic pathways such as the gut-associated lymphoid tissue.[12] The differences between our results and those of Roux *et al* may be due to the additional GVHD prophylaxis with cyclosporin A and methotrexate which was given to their patients.

With regard to CD8 T cell reconstitution we observed rapid reconstitution to normal proportions in all patient groups although the fully TCD grafts were associated with a slightly slower recovery. In keeping with previous results from studies of TCD BMT recipients, we saw no evidence of a CD8 'overshoot' beyond normal levels.[14,15,18] However, neither did we observe this in the recipients of non-TCD transplants although it has been reported previously.[20,21]

We did observe very high levels of activation in the CD8 subset irrespective of T cell depletion and the majority of these cells expressed CD57. High levels of activated CD8 cells and the expansion of the $CD57^+$ subset after BMT have been reported before[14] and it was suggested that this may be associated with cytomegalovirus infection. We found that increased incidence of HLA-DR and CD57 expression on CD8 cells was universal among our patients and thus we could not associate it with CMV. It may reflect the interplay between donor immune system and the allogeneic host environment in the establishment of peripheral tolerance.

One of the most consistent findings in studies of TCD and non-TCD stem cell transplantation is the early and rapid expansion of natural killer cell subsets. We and others have shown previously that these cells are functional[16,18] and that they can mediate anti-leukaemia activity.[22] In this study we show that this NK cell recovery is equivalent in both TCD and non-TCD groups and that relative expansion is faster in recipients of fully TCD grafts. Furthermore, it is sustained for a longer period in recipients of fully TCD grafts than in either non- or partially TCD transplant groups. In a similar group of patients receiving autologous BMT for acute leukaemia we have shown specific anti-leukaemia cytotoxicity mediated by NK cells post transplant.[23] This activity was greatest within the $CD56^+/CD8wk^+$ cells. In this study we have shown that $CD56^+/CD8wk^+$ cells are present in all three groups of patients although their incidence is highest in the recipients of fully TCD grafts. This may be significant in the low relapse rate observed in our unit despite the long-term use of TCD as GVHD prophylaxis without additional immunosuppression.[7]

T cell depletion in allogeneic stem cell transplantation is currently seeing a revival in HLA-matched unrelated and HLA-mismatched related donor transplants where it appears to be the optimal GVHD prophylaxis. Indeed, in many groups such as ours, it is used in early disease (AML/ALL, CR1; CML, CP) where prevention of acute and chronic GVHD is almost complete even in the absence of post-transplant immune suppression. We have previously demonstrated that it can be used safely with low incidence of graft rejection and of leukaemia relapse but the issue of post transplant immune responsiveness has remained a source of concern. Here we have shown that CD8 T cell and NK cell reconstitution is rapid after Campath-1M-depleted allografting although CD4 repopulation is severely delayed even after partial TCD of the graft. Despite this, naive CD4 cells are present in the peripheral blood of patients after TCD grafts and these may ultimately contribute to a normal T cell repertoire.

The advantage of Campath-1M plus autologous complement for *ex vivo* TCD is that the degree of TCD can be regulated, which we believe is important in maintaining the balance between graft rejection, GVHD and GVL. It must be remembered that laboratory tests of lymphocyte subset recovery are simply markers of an *in vivo* phenomenon and the real test of immune reconstitution is the clinical outcome of the transplant. We have not observed a higher incidence of fatalities due to infectious disease in our fully or partial TCD graft recipients as compared with our non-TCD patients and are confident that the use of Campath-1M for *ex vivo* TCD at the levels used here is a safe alternative to post transplant immunosuppression. The high survival rates in the recipients of partial and fully TCD grafts (Table 1) are testaments to the success of this strategy in prevention of GVHD while maintaining the GVL effect.

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Generation of autologous immunity to acute myeloid leukaemia and maintenance of complete remission following interferon-α treatment

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Abstract

Interferon-α (IFN-α) is established as part of the treatment for chronic myeloid leukaemia, although its precise mode of action remains largely unknown. Its use in acute myeloid leukaemia (AML) has been limited. We have previously documented autologous cytolytic activity against AML blasts in patients after autologous bone marrow transplantation. Here we present a patient with poor-risk AML who relapsed from first complete remission (CR) and was unwilling to undergo high-dose chemotherapy with stem cell rescue. In second chemotherapy-induced CR, the patient had no evidence of antileukaemia cytolytic activity in an in vitro assay, and she commenced IFN-α (Roferon). She subsequently developed high levels of leukaemia-specific cytotoxicity, and has remained in second CR for two years. These findings support the use of IFN-α in patients with poor-risk AML, and suggest that one mechanism of action may be immunological.

Keywords:
interferon-α; acute leukaemia; NK cells; cell-mediated cytotoxicity; immunotherapy

Case report

A 26-year-old Sudanese lady presented at another hospital in November 1994 with acute myeloid leukaemia (AML, FAB type M2) and commenced chemotherapy with doxorubicin (Adriamycin) and cytarabine. At completion of the first course of therapy, a bone marrow aspirate was taken, and 69% blasts were detected. She received a second course of the same regimen in January 1995, and was referred in first complete remission (1st CR) to our hospital for consolidation chemotherapy in February 1995. She began her third course of chemotherapy in March with cytarabine (1.33 g/m^2/day, days 1–3); daunorubicin (45 mg/m^2/day; days 1–3) and etoposide (400 mg/m^2/day; days 8–10). The patient received granulocyte colony-stimulating factor (G-CSF, filgrastim) (300 μg/day, days 1–3) post chemotherapy for mobilization of haematopoietic stem cells, which were harvested and cryopreserved at the end of April 1995 with the intention of future use in autologous transplantation, should it be required.

A further course of consolidation chemotherapy was undertaken in May 1995 to the same schedule as above, and, following bone marrow recovery, the patient was discharged in complete morphological remission in mid-June. No cytogenetic or molecular markers of the leukaemia were available for disease monitoring.

The patient remained in 1st CR until August 1996, when she relapsed in the 14th week of pregnancy. After termination of the pregnancy, she commenced re-induction chemotherapy in September with the FLAG/IDA regimen as described previously.[1] Briefly, she received fludarabine (30 mg/m^2/day, days 1–5), cytarabine (2 g/m^2/day, days 1–5), idarubicin (5 mg/m^2/day, days 1–5) and G-CSF (600 μg/day, days 1–5). Two weeks after completion of this course, the patient's bone marrow was in morphological CR, and she then received a repeat course of FLAG/IDA for consolidation. In November 1996 the patient was in 2nd CR. A blood sample taken in outpatient clinic 10 days later was tested for cytolytic activity against her presentation AML M2 blasts and her remission bone marrow as previously described.[2] The patient was unwilling to undergo autologous stem cell transplantation, and, following previous success with interferon-α (IFN-α) in a patient with AML M4Eo who had relapsed rapidly after autologous bone marrow transplantation (BMT),[2] it was proposed that she commence self-administered subcutaneous IFN-α at 2 MU/day for five days each week as of the beginning of December 1996. This dose was poorly tolerated, with reports of high temperatures and headaches that failed to respond to conventional analgesics. The patient also reported lethargy, and refused to continue treatment after the fourth dose.

In February 1997 a bone marrow aspirate confirmed maintenance of morphological CR, and in April the

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patient recommenced IFN-α treatment at the same initial dose as before. This second attempt was better tolerated, and after three weeks the dose was increased to 3 MU five times per week. Three weeks later, the IFN-α dose was increased further to 5 MU four times per week, and, finally, two months later, this was increased to the full dose of 5 MU five times per week. This dose is well tolerated, and the patient has been maintained on this treatment to date.

Methods

In vitro detection of leukaemia-specific cytotoxicity

Cell-mediated cytotoxicity was determined by flow cytometry as previously described[2] on peripheral blood samples taken in CR pre and post commencement of IFN-α therapy. Autologous leukaemia and normal bone marrow mononuclear cells (BMMC) were labelled with the red membrane dye PKH-26 (Sigma, Poole, Dorset, UK).

Patient-derived peripheral blood mononuclear cells were monocyte-depleted and incubated in triplicate with labelled targets at a ratio of 10:1 for four hours. After the incubation period, the cells were resuspended in a solution of propidium iodide (Sigma, Poole) in phosphate-buffered saline) (1 μg/ml) and analysed by flow cytometry (FACScan or FACS Vantage, Becton Dickinson UK Ltd, Oxford). At least 10 000 target cells were acquired, and the mean proportion of propidium-iodide-positive cells from the triplicate samples was determined. Background target cell death was determined from cells incubated in the absence of effector cells. Cell-mediated cytotoxicity was reported as percentage killing over and above background cell death: this was calculated for both leukaemic and normal autologous target cells that were used as negative controls to confirm leukaemia-specificity. Leukaemia-specific killing was determined by subtracting the percentage cytolysis of remission BMMC (never >2%) from the percentage lysis of leukaemic blasts.

Results

With regard to the development of cytolytic antileukaemia activity, the results have been remarkable (Figure 1). Having shown no detectable lysis of autologous leukaemic blasts in samples prior to commencement of IFN-α, the patient developed specific antileukaemia cytotoxicity within six days of commencing IFN-α treatment. The level of this activity increased over the next 50 days of treatment, and has been maintained since then. The patient remains in morphological CR, and is currently well. Furthermore, she has successfully taken a second pregnancy to term while continuing IFN-α therapy, and has been delivered of a healthy child.

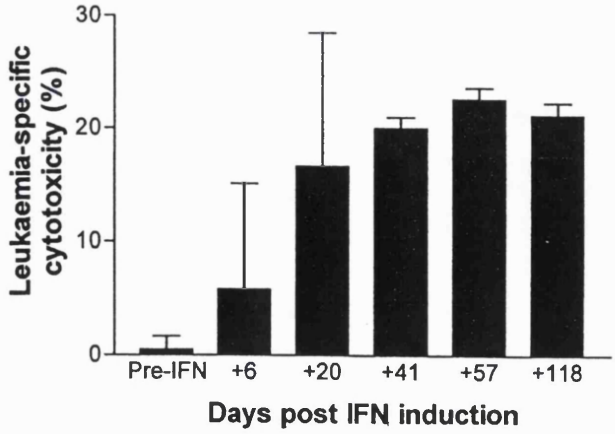

Figure 1
Effect of IFN-α on immune response to leukaemia: leukaemia-specific cytotoxicity after four hours incubation in vitro. Results are presented as arithmetic means of triplicate assays (error bars represent standard deviation).

Discussion

The use of IFN-α in the management of chronic myeloid leukaemia (CML) is well established, although the mechanism of action remains unclear. Natural killer (NK) cells derived from patients with CML in chronic phase show impaired proliferative and cytotoxic potential as compared with normal donor NK cells. This impairment can be corrected in vitro by exogenous interleukin-2 (IL-2) during the early stages of the disease, but progression to blast crisis is associated with a loss of responsiveness to IL-2.[3] This would seem to be of clinical relevance, given that patient-derived NK cells have been shown to suppress growth of autologous CML precursors in vitro,[4] and may thus be responsible for controlling the disease in some patients. Although it remains to be seen whether IFN-α can achieve the same degree of restoration of NK-cell lytic function in this group of patients, it is known that IFN-α does activate the cytolytic function of NK cells.[5]

Experience with IFN-α therapy in AML is far more restricted. However, we have previously reported its use in a patient who relapsed six months after autologous BMT for AML M4Eo.[2] Prior to her relapse, she had lost all evidence of autologous leukaemia-specific cytotoxic NK activity (LSC). Having achieved 3rd CR after FLAG/IDA chemotherapy, she commenced IFN-α, and, within one month, regained high levels of LSC, which have now been maintained for over four years of follow-up. It is noteworthy that, concomitant with the commencement of IFN-α, the proportion of $CD56^+/CD3^-$ cells in her peripheral blood increased eightfold to more than 40%. This fell back to around 30% after six weeks of therapy, and has subsequently remained at this level. She remains in molecular remission more than four years after re-induction chemotherapy.

IFN-α has been used to used to induce graft-versus-host disease (GvHD) to treat relapsed disease after allogeneic BMT for AML,[6] but none of the patients receiving IFN-α alone survived. One of the 13 surviving patients in this study had received IFN-α, but this had been supplemented with subsequent IL-2. None of the survivors were studied to investigate the mechanism of action of the therapy, but the high incidence of acute and chronic GvHD implies that the response was unlikely to be leukaemia-specific. The same group has reported a single long-term survivor after relapse following allogeneic BMT who was treated with IFN-α alone.[7] This patient suffered chronic GvHD with scleroderma-like changes that were resistant to treatment, but remained in remission 41 months after the haematological relapse. The mechanism and specificity of this response was not studied.

A mixed group of patients receiving IFN-α with IL-2 for treatment of relapse after allogeneic BMT have been reported from the MD Anderson Cancer Center.[8] This group included both AML and CML patients, and 6 of the 10 developed acute GvHD symptoms and 2 were described as having responded to treatment. The combination therapy was well tolerated.

Granulocyte–macrophage colony-stimulating factor (GM-CSF) has also been used in combination with IFN-α without increasing the toxicity of either cytokine used alone.[9] In this study, CML patients who had shown only partial responses to 12 months of IFN-α alone were given GM-CSF 30–60 μg/m^2 subcutaneously. The results were encouraging, with three patients showing sustained complete haematological responses. The mechanism of action of the combined cytokines was not studied. Both IFN-α and GM-CSF are known immunomodulatory cytokines, and in vitro studies have shown that monocyte-mediated lysis of leukaemic cell lines is enhanced following GM-CSF treatment of the donors.[10]

We believe that this is the first report of the induction with cytokine therapy of autologous leukaemia-specific immunity in a patient after chemotherapy. We have recently found a strong relationship between the presence of antileukaemia cytotoxicity in AML patients in CR after chemotherapy and their long-term survival (manuscript in preparation), and regard IFN-α therapy as a potential alternative to autologous transplantation in this setting. The low toxicity of the treatment should also be considered, given that the patient described above was able to conceive and sustain a pregnancy to full term while on therapy.

Finally, given the ease of application of this assay for antileukaemia cytotoxicity, we would encourage other groups to monitor patients undergoing treatment with other immunomodulatory cytokines.

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