DEVELOPMENT OF A VACCINE FOR THE IMMUNOTHERAPY OF ACUTE MYELOID LEUKAEMIA BY THE MODIFICATION OF AUTOLOGOUS LEUKAEMIA CELLS

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Contents

Declaration

I declare that this thesis is my own work and effort, planned, performed and written by myself, unless otherwise specifically stated in the acknowledgements.

Dedication

To R.A.R.

Acknowledgments

My thanks go to Marc Turner who, as my supervisor, allowed me room to explore and develop my own ideas whilst at the same time being always available as a source of support, focus and direction.

I also wish to express my gratitude to the following for providing invaluable assistance:

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Abstract

Although leukaemia specific antigens have been identified that are recognisable by cytotoxic T lymphocytes (CTLs), there is little evidence to suggest that clinically relevant immunity, directed against these antigens, develops in patients with acute myeloid leukaemia (AML). A major reason for this is believed to be a consequence of the lack of expression of costimulatory molecules by leukaemia cells. Studies examining mechanisms of T cell activation have demonstrated the crucial role that costimulatory molecules have in providing the signals necessary for the induction of T lymphocyte activation and proliferation following initial T cell receptor engagement of the MHC-peptide complex. A further reason for this failure to develop leukaemia specific immunity is believed to be that the leukaemia microenvironment is one in which the local production of immunosuppressive cytokines and other soluble factors favours immune escape rather than control.

The hypothesis represented within this study is that modification of leukaemia cells in order to allow expression of costimulatory molecules and/or production of immunostimulatory cytokines would render these cells capable of generating autologous leukaemia reactive CTLs. Two main approaches were employed in an attempt to test this hypothesis. The first was based on the transfer of genes encoding costimulatory molecules and immunostimulatory cytokines to leukaemia cells. The second approach was the attempted induction of differentiation of leukaemia cells to dendritic-like cells through the use of specific cytokine combinations.

A critical requirement for the success of any gene transfer strategy, in a clinical setting, is a highly efficient and robust system of gene transfer to primary leukaemia cells. Using Green Fluorescence Protein as a reporter gene a variety of gene transfer methodologies were evaluated in myeloid leukaemia cell lines and primary cells. All the physical and chemical methods tested generally had a low efficiency of gene transfer. Two viral vector systems were evaluated. Although a retroviral vector yielded high gene transfer efficiencies in 3 of 4 leukaemia cell lines efficiency was very low in primary cells. An adenoviral vector similarly was efficient at gene transfer in the myeloid leukaemia cell lines but gave a very variable level of gene transfer in primary leukaemia cells. None of the methods tested was felt to fulfil the requirements of a clinically useful gene transfer system.

The second approach involved the attempted differentiation of leukaemia cells to dendritic-like cells (DLLC) by the use of specific cytokine combinations. Leukaemia cells from 42 patients with AML were tested for evidence of differentiation following their culture with cytokines GM-CSF, IL-4 and TNFα/CD40 ligand. In 22 cases the leukaemia cells were deemed to have undergone DLLC differentiation based on upregulation of expression of costimulatory molecules and dendritic cell associated markers, as measured by flow cytometry, and the acquisition of a dendritic-cell like morphology. Antigen presenting function was measured in mixed leukaemia lymphocyte reactions where DLLC were found to be potent stimulators of allogeneic T lymphocyte proliferation. In 2 cases co-culture of DLLC with autologous T lymphocytes led to the generation of CTLs that were capable of

recognising and destroying unmodified cells in cytotoxicity assays. In 4 cases the leukaemic cells had poor viability over the culture period and were therefore not evaluable. In the remaining 16 cases the leukaemia cells were deemed to be resistant to cytokine induced differentiation. Examination of the clinical features of these cases showed a preponderance of poor risk karyotypic features. A variety of differentiating agents were used in combination with cytokines in an attempt to overcome differentiation resistance. Bryostatin-1 was found to be successful in permitting DLLC differentiation to proceed but only in a proportion of the resistant cases.

In summary DLLC derived from leukaemia cells by cytokine induced differentiation might be suitable for use as vaccines for the immunotherapy of AML.

Chapter 1

INTRODUCTION

1.1 Limitations of current treatment approaches in acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a disease characterised by a clonal neoplastic proliferation of cells of haematopoietic origin. Without treatment patients will usually succumb to the complications of leukostasis or bone marrow failure within days to weeks of the diagnosis being made. Conventional therapy aims at inducing remission (defined as <5% blasts in the bone marrow) through intensive multi-agent chemotherapy, followed by further blocks of consolidation chemotherapy. With this type of approach it is possible to induce a morphological remission in >60% of patients (Bennett *et al*, 1997). However, despite this, only a small number of patients enjoy long-term survival, with the majority relapsing and eventually succumbing to the disease or complications arising from its treatment. A review of the data from the Eastern Cooperative Oncology Group trial serves to illustrate this, with of the 62% of patient's who achieved complete remission, 70% subsequently relapsing and dying from their leukaemia (Bennett *et al*, 1997). The major cause for relapse is due to the presence of small amounts of residual morphologically undetectable leukaemic cells, so-called minimal residual disease (MRD). In MRD the leukaemic blasts are often resistant to the majority of the chemotherapeutic agents used to treat AML, thus enabling them to survive through further courses of treatment. In an attempt to eradicate MRD dose escalation has been employed, with autologous or allogeneic

haematopoietic stem cells being given to 'rescue' patients following myeloablative chemotherapy. Autologous transplantation in AML confers some degree of benefit in respect of improved disease-free survival but is associated with high rates of treatment-associated mortality (Burnett *et al*, 1998; Ravindranath *et al*, 1996). Allogeneic haematopoietic stem cell transplantation (SCT) has a more established role in the treatment of AML (Casper *et al*, 1995). The improved disease-free survival rates observed with allogeneic compared with autologous transplantation is due, in large part, to a 'graft versus leukaemia' (GVL) effect (Korngold *et al*, 1994). In GVL donor T lymphocytes are able to recognise and destroy host allogeneic leukaemia cells (Falkenburg *et al*, 1997). In an attempt to harness this GVL effect, donor lymphocyte infusion (DLI) has been used to treat relapse following allogeneic haematopoietic SCT (Helg *et al*, 1998; Johnson & Truitt, 1995; Kolb *et al*, 1995; Kolb, 1998; Mackinnon *et al*, 1995; Pati *et al*, 1995; Porter & Antin, 1995; Slavin *et al*, 1993). DLI has had most success in patients with relapsed chronic myeloid leukaemia (CML) but complete remission of relapsed AML has also been reported with DLI alone (Helg *et al*, 1998; Kolb *et al*, 1995; Pati *et al*, 1995; Porter & Antin, 1995). Unfortunately GVL is almost invariably associated with the related but distinct phenomenon of graft versus host disease (GVHD). In this condition normal host tissues are attacked by donor T lymphocytes leading to a severe and potentially life threatening multi-system disorder. It has not as yet proven possible to separate the beneficial GVL effects from those of GVHD. However the presence of GVL provides evidence that immunological control of AML is achievable, albeit within the context of an allogeneic stem cell transplantation setting. Further laboratory evidence supporting the importance of host cell-mediated immunological responses

in the defence against leukaemia derives from a study examining the frequency of cytotoxic T lymphocytes (CTL) specific for a leukaemia-specific antigen, PR1, in patients with CML (Molldrem *et al*, 2000). PR1 specific CTL were only found in patients who had received treatment that potentially could have stimulated host immunity against leukaemia i.e. only in patients who had received immunomodulatory therapies such as allogeneic haematopoietic SCT or IFNα, but not in patients receiving chemotherapy alone. In addition the presence of these PR1 specific CTL correlated with clinical responses and survival. Patients with higher numbers of circulating PR1 specific CTLs enjoyed more favourable clinical outcomes (Molldrem *et al*, 2000).

Given the limitations of current conventional AML therapies and based on the evidence suggesting that in certain settings immunological control of leukaemia is possible, there is a need to develop novel immunotherapeutic strategies. Any new therapy would need to fulfil three basic criteria:

- Specifically target the leukaemia cells
- Little or no toxicity against normal tissues
- Be able to eradicate MRD

Creating a tumour vaccine by modification of autologous leukaemic cells might potentially offer a novel treatment approach that would fulfil these criteria (Arceci, 1998).

1.2 Early studies of immunotherapy in acute leukaemia

Early human studies that attempted to harness the immune system against cancer gained inspiration from observations such as those of Coley-Nauts and colleagues, which showed that exposure to bacterial toxins would sometimes induce a regression in cancer patients (Coley-Nauts *et al*, 1946). These human studies, in addition to results obtained from animal studies, led investigators to reason that it might be possible to enhance the immune system of patients to eradicate tumour cells especially in the MRD state. The first clinical trials in leukaemia were performed using bacilli Calmette-Guérin (BCG) adjuvants which were believed to enhance overall immune activity and possibly generate anti-tumour responses in the process. In 1969 Mathe *et al* reported that immunisation with BCG following remission-induction chemotherapy prolonged first remission in children with Acute Lymphoblastic Leukaemia (ALL) (Mathe *et al*, 1969). Although subsequent studies failed to confirm these findings other investigators tried immunotherapeutic based treatment approaches in patients with AML (Gutterman *et al*, 1974; Powles, 1974; Powles *et al*, 1975). Cellular vaccination using autologous and allogeneic leukaemia blasts, either intact or as subcellular extracts, resulted in some studies in a minimal prolongation of remission and survival (Foon *et al*, 1983; Zuhrie *et al*, 1980). Laboratory support for the use of vaccine immunotherapy in patients with AML derived from studies that showed that autoimmunisation of patients in remission with irradiated leukaemia cells resulted in an increased *in vitro* response to leukaemic blasts (Powles *et al*, 1971). However a review based on 1491 patients treated in various vaccination studies concluded that overall this form of immunotherapy conferred no definite clinical benefit (Foon *et al*, 1983).

In all these studies the assumption was made that immunostimulatory antigens existed in AML, although the nature of these antigens was unknown. Improved understanding into the molecular and cell biology of tumour cell antigens and into the mechanisms of T lymphocyte activation paved the way for the next wave of cell based anti-tumour vaccines.

1.3 Identification of Immunostimulatory Antigens

Using techniques of cytotoxic T cell detection of immunostimulatory antigens in combination with recombinant DNA procedures for expression cloning of genes it has been possible to identify a range of different tumour antigens (Boel *et al*, 1995; Boon, 1993; Boon *et al*, 1994; Boon *et al*, 1995; Cheever *et al*, 1995; Coulie *et al*, 1995; Coulie, 1995; Ostrand-Rosenberg, 1994; Pardoll, 1994; Slingluff *et al*, 1994; Van Pel *et al*, 1995). Most of the immunostimulatory tumour antigens recognised by CTL are short peptides (8-12 amino acids) held in the groove of Major Histocompatibility Complex (MHC) class I molecule on the tumour cells. The majority of tumour antigens have been MHC class I restricted and provide stimulation for CTL responses. However class II MHC restricted T helper cell responses have been shown to be important in anti-tumour responses observed in a murine sarcoma model (Baskar *et al*, 1993). Immunostimulatory tumour antigens thus far identified fall into several major classes of genes including:

1.4 Mechanisms of T cell activation

In order for T cell activation to take place there are a number of essential requirements:

- Cell-cell adhesion
- Antigen presentation usually by MHC class I or II molecules
- T lymphocyte recognition of antigen and signaling through the T cell receptor TCR (termed signal 1)
- A costimulation signal involving specific cell surface receptors on antigen presenting cells (APC) and T cells (termed signal 2)
- Induction of interleukin-2 (IL-2) and its receptor by activated T cells resulting in their proliferation and terminal effector function.

If adhesion or antigen presentation is blocked then T cell activation does not develop. However subsequent re-challenge of T cells with antigen results in a normal immune response. In contrast if antigen presentation (signal 1) occurs in the absence of the second costimulatory signal, then antigen-specific hypo-responsiveness or anergy results. This means that a subsequent antigen challenge does not lead to specific T cell proliferation and function. Importantly this condition of anergy can in part be overcome by continuous exposure of T cells to IL-2 in the presence of antigen (Beverly *et al*, 1992).

One especially well-studied costimulation pathway involves the B7 family of antigen-presenting cell surface molecules and their T cell receptors CD28 and CTLA-4. Recent studies have demonstrated the pivotal importance of the B7 pathway in tumour-mediated costimulation. B7-1 (CD80) was first described as a 30kDa membrane protein appearing on B cells following activation (Freedman *et al*,

- Normal differentiation antigens.
- Genes expressed during early embryonic development that are re-expressed following malignant transformation (onco-foetal antigens).
- Mutated proto-oncogenes or fusion proteins that are created secondary to chromosomal translocations.
- Viral derived proteins.

Although the majority of studies on tumour antigens have focused on solid tumours a number of putative leukaemia-specific antigens have also been identified, based on their ability to generate specific CTL responses. These include the AML-associated DEK-CAN fusion peptide (Ohminami *et al*, 1999), the peptide BCR1/25, which encompasses the fusion region of the hybrid molecule PML/RAR alpha and is selectively expressed by blast cells in acute promyelocytic leukaemia (APL) (Gambacorti-Passerini *et al*, 1993), the Wilm's tumour gene encoded transcription factor, WT-1 (Gao *et al*, 2000; Gaiger *et al*, 2000; Inoue *et al*, 1997; Menssen *et al*, 1995; Ohminami *et al*, 2000) and PR1, a peptide derived from proteinase-3 found in the primary granules of myeloid leukaemia cells (Molldrem *et al*, 1996; Molldrem *et al*, 2000).

The identification of immunostimulatory antigens has been an important advance in understanding anti-tumour immunity. However the mechanisms through which malignant cells evade immune recognition and eradication, despite their expression of immunostimulatory antigens, have only recently become evident. Critical observations on antigen recognition and T cell activation have now emerged that have allowed insight into the process through which T cells become activated.

1987; Yokochi *et al*, 1982) as well as on other antigen-presenting cells (APC) such as monocytes, macrophages, Langerhans cells and dendritic cells (Hathcock *et al*, 1993). CD80 can be induced by a variety of stimuli; for B cells this includes anti-immunoglobulin cross-linking (Hathcock *et al*, 1994) and stimulation with IL-4 (Valle *et al*, 1991) or IL-7 (Yssel *et al*, 1993) and for macrophages stimulation with γ interferon (Hirano *et al*, 1996). A second B7 family gene was cloned and termed B7-2 (CD86) (Freeman *et al*, 1993; Freeman *et al*, 1993; Hathcock *et al*, 1993). CD86 is constitutively expressed on monocytes and upregulated within 24 hours on B cells following activation, in comparison to CD80, which is expressed on APC only following activation (Azuma *et al*, 1993; Boussiotis *et al*, 1993; Freeman *et al*, 1993; Hathcock *et al*, 1993). Thus CD86 may provide a very early costimulation signal and CD80 contributes a subsequent signal. Further studies have shown that CD80 and CD86 may provide signals leading to both qualitative and quantitative differences in the immune repertoire (Kuchroo *et al*, 1995; Thompson, 1995). In animal models costimulatory vaccines utilising CD80 and CD86 have led to quite different degrees of anti-tumour responses (Gajewski, 1996; Yang *et al*, 1995). The primary receptor for the B7 costimulatory molecule is a 44kda homodimeric cell surface glycoprotein named CD28, which is found on T cells (Aruffo & Seed, 1987). Increased surface expression of CD28 occurs following TCR binding to antigen (Hara *et al*, 1985; June *et al*, 1987; Ledbetter *et al*, 1985; Linsley & Ledbetter, 1993; Turka *et al*, 1992). Following TCR activation alone T cells are able to undergo minimal amounts of proliferation and cytokine secretion. However following the additional engagement of the CD28 receptor, T cell proliferation, function and cytokine secretion become maximally stimulated (Schwartz, 1992).

It has been hypothesized that tumour cells may escape immune recognition and subsequent eradication by failing to satisfy one or more of the above requirements for T cell antigen recognition and activation (Bluestone, 1995; Bluestone, 1996; Chen *et al*, 1992; Chen *et al*, 1993; Guinan *et al*, 1994; June *et al*, 1994). Thus if antigen presentation does not occur because of low or absent expression of MHC or lack of a recognisable tumour antigen, then tumour cells would not be recognised. On the other hand, if antigen recognition occurs by T cells but tumour cells do not express a costimulatory molecule, then T cells become anergic. Studies examining costimulatory molecule expression in AML have demonstrated that in a minority of AML cases the leukaemia cells do express CD86 but in no cases was significant expression of CD80 seen (Brouwer *et al*, 2000a; Costello *et al*, 1998; Hirano *et al*, 1996; Maeda *et al*, 1998; Zheng *et al*, 1998). Therefore, based on the evidence from these studies, the leukaemia cells in AML would be incapable of stimulating T cell mediated anti-leukaemia immunity because of their lack of expression of costimulatory molecules. A potential strategy for eliciting a specific anti-leukaemia T cell response would be to modify autologous leukaemia cells in such a way that they express the costimulatory molecules that are critical in providing the second signal necessary for T cell activation and proliferation (Figure 1.1).

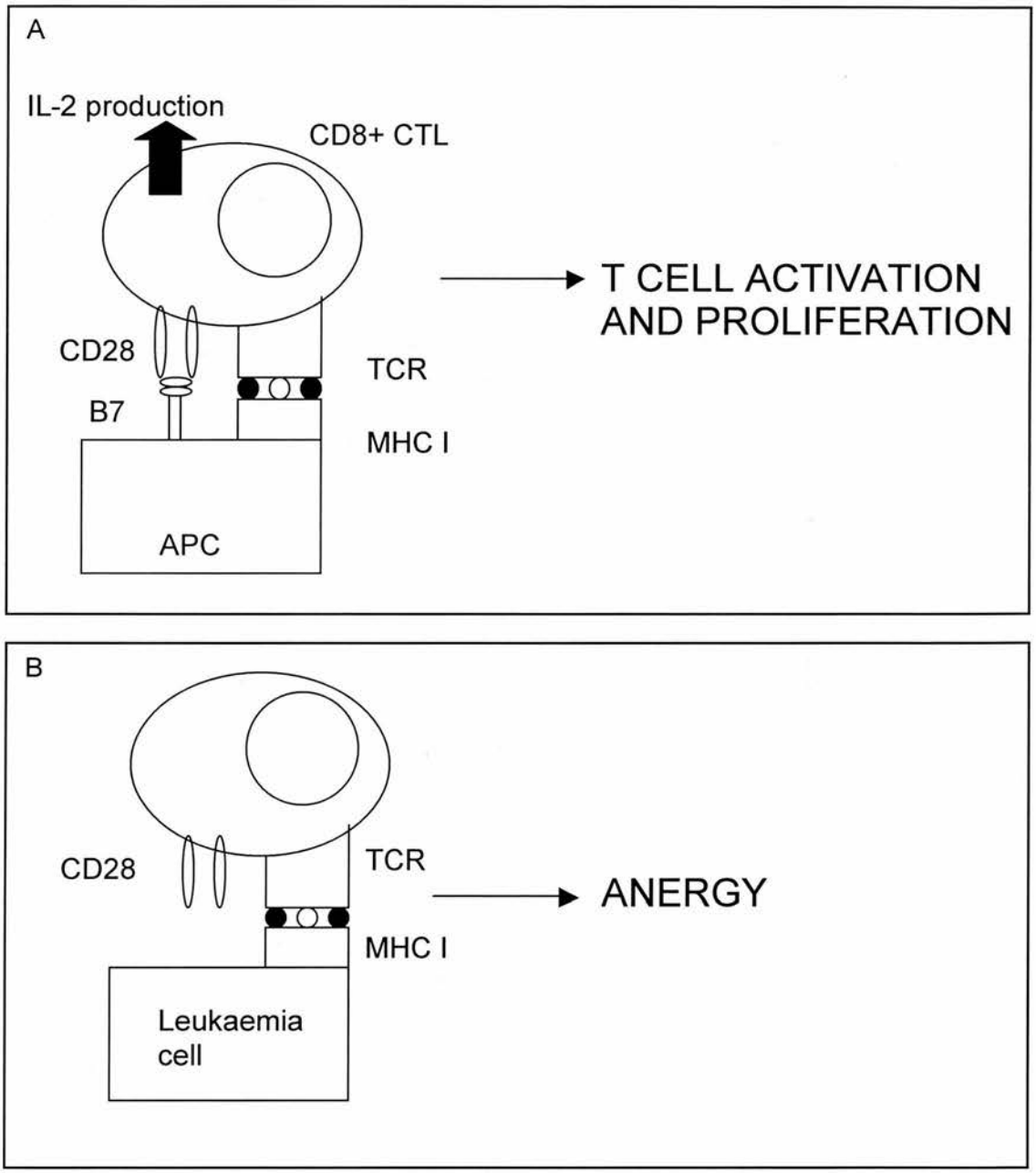

Figure 1.1 Mechanisms of T cell Activation
A. Recognition of MHC/peptide complexes on the surface of an antigen presenting cell by TCR in the presence of a second costimulatory signal mediated by B7 leads to T cell activation and proliferation.
B. Absence of B7 expression by leukaemia cells means that there is no costimulatory signal following TCR binding of MHC/peptide complex with consequent T cell anergy

1.5 The Influence of the cytokine microenvironment

Although the decision to initiate an immune response is dependent upon antigen recognition by the TCR in conjunction with costimulatory molecule signalling, the cytokine microenvironment plays an important role in determining the nature of any response generated (Buggins *et al*, 1999). Leukaemia cells have been shown to produce a number of soluble factors that can prevent or undermine T cell activation (Buggins *et al*, 1999; Chouaib *et al*, 1997). Immunosuppressive factors produced by tumour cells, including leukaemia cells, include vascular endothelial growth factor (Aguayo *et al*, 1999; Gabrilovich *et al*, 1996), TGFβ (de Visser & Kast, 1999; Espevik *et al*, 1987; Espinoza-Delgado *et al*, 1994), IL-10 (Akdis *et al*, 1998; de Waal *et al*, 1992; Groux *et al*, 1996) and soluble Fas ligand (Buzyn *et al*, 1998; Buzyn *et al*, 1999). Therefore if a tumour vaccine is to be effective it must be capable of altering the cytokine microenvironment so as to favour an appropriate T cell response. Dendritic cells (DC) are professional APC that play a crucial role in the initiation of immunity (Avigan, 1999; Banchereau & Steinman, 1998; Colaco, 1999; Hart, 1997; Reid, 1998; van Schooten *et al*, 1997). The cytokine profile produced by DC at the time of antigen presentation dictates the nature of T cell activation through influencing the Th1/Th2 decision during T cell differentiation (Kalinski *et al*, 1999a; Vieira *et al*, 2000). One of the most important cytokines in the development of T cell responses is IL-12, a heterodimeric disulphide-linked glycoprotein consisting of a 35 and a 40kD subunit (Kobayashi *et al*, 1989). It has been shown to be a potent immunostimulatory cytokine that mediates a number of biological important properties. These include stimulation of cytotoxicity and proliferation of NK cells and CTL, induction of cytokine and chemokine secretion,

especially IFN-γ, and generation of T helper type 1 (Th1) cells (Brunda, 1994; Trinchieri, 1994; Trinchieri, 1998). DC that produce high levels of IL-12 cause polarization of T cells towards a Th1 phenotype whereas DC that produce low levels of IL-12 generate T cells with a Th2 phenotype (Brunda *et al*, 1995; Kalinski *et al*, 1999a; Macatonia *et al*, 1995; Palm *et al*, 1996; Vieira *et al*, 2000). Effective anti-leukaemia immunity is generally considered to require a cell-mediated Th1 response. In order for DC to produce high levels of IL-12 they must first have undergone maturation. One of the most important pathways for DC maturation involves the binding of the cell surface receptor CD40 by its ligand CD40 ligand (CD40L) present on activated T cells (Van Kooten & Banchereau, 1997; Van Kooten & Banchereau, 2000). CD40, is a 50kD molecule expressed on B cells, DC and other professional APC (Van Kooten & Banchereau, 1997; Van Kooten & Banchereau, 2000). Binding of CD40L to CD40 on DC leads to the up-regulation of costimulatory molecule expression and to the production of IL-12 (Caux *et al*, 1994; Cella *et al*, 1996; Mackey *et al*, 1998; Mosca *et al*, 2000; Van Kooten & Banchereau, 1997; Van Kooten & Banchereau, 2000) and IL-15 (Kuniyoshi *et al*, 1999). Other maturation stimuli that promote IL-12 production by DC include IFN-γ (Kalinski *et al*, 1999a; Kalinski *et al*, 1999b; Vieira *et al*, 2000 and TNF-α (Sallusto & Lanzavecchia, 1994). Following maturation induction DC become potent immunostimulators capable of initiating Th1 responses (Mackey *et al*, 1998; Terheyden *et al*, 2000). It is this ability to alter the cytokine microenvironment at the time of antigen presentation and so determine the nature of subsequent T cell immune responses that makes DC such effective APC.

1.6 Development of vaccines by modification of autologous leukaemia cells

The principle of vaccination with autologous leukaemia cells is based on the assumption that leukaemia cells possess antigens recognisable by autologous T cells and that it is solely due to a lack of costimulatory molecule expression that immune activation fails to take place. Therefore if the leukaemia cells could be modified in such a way that they gain expression of the required costimulatory molecules and/or produce immunostimulatory cytokines then they might become capable of stimulating autologous T cells to recognise and destroy unmodified leukaemia cells (Allison *et al*, 1995; Bruserud & Gjertsen, 2000). This could be achieved by either:

- Gene transfer of costimulatory molecules and/or cytokines
- Induction of differentiation thereby leading to upregulation of costimulatory molecule expression

1.6.1 Gene transfer of costimulatory molecules or cytokines

Immune gene transfer strategies are all reliant on a robust and efficient gene transfer method for success. The gene transfer technique needs to fulfil a number of requirements in order to be suitable for use in an immunotherapeutic strategy. It must give a high efficiency of gene transfer to the target cell, with low toxicity, and transgene expression must be sufficiently high and of long enough duration to allow the modified cell vaccine to stimulate an immune response.

Gene transfer technologies can be broadly divided into three types:

- Physical methods e.g. electroporation
- Chemical methods e.g. polycationic liposomes, activated dendrimers
- Viral methods e.g. retroviral vectors, adenoviral vectors

1.6.1.1 Gene transfer methods

i/ Electroporation

Electroporation involves using a short duration high voltage pulse that causes the membrane potential of the cells to break down. As a result pores are formed through which macromolecules such as DNA can enter. The main advantage of electroporation is its applicability for transient and stable transfection in all cell types. A major drawback however is the high cell mortality that can result, typically in the region of 50-70% cell death.

ii/ Cationic liposomes

Felgner and colleagues first introduced liposomes in 1987 (Felgner *et al*, 1987). Liposomes typically contain a mixture of cationic and neutral lipid, the latter often being L-dioleoyl phosphatidyl ethanolamine (DOPE). The cationic portion of the lipid molecule associates with the negatively charged nucleic acid, resulting in a liposome/DNA complex. The overall net positive charge of the liposome/DNA complex generally results in high transfer efficiencies because it allows closer association of the complex with the negatively charged cell membrane. The uptake of the liposome/DNA complex is then thought to take place by endocytosis.

iii/ Activated dendrimers

Dendrimers are highly branched spherical molecules with branches radiating from a central core molecule and terminating at amino groups (Tang & Szoka, 1997). Activation of newly synthesised dendrimers involves complete removal of some of

the tertiary amines, resulting in a molecule with a higher degree of flexibility (Tang *et al*, 1996). Activated dendrimers assemble DNA into compact structures through interaction of the negatively charged phosphate groups of nucleic acids with the positively charged amino groups of the dendrimers. The resulting DNA-activated dendrimer complexes possess a net positive charge on the surface molecules of the cell membrane. The transfection complexes are taken up by non-specific endocytosis.

iv/ Retroviral vectors

Retroviruses are a class of enveloped viruses containing a single stranded RNA molecule as a genome. Following infection the viral genome is reverse transcribed into double stranded DNA, which is integrated into the host genome and expressed as proteins (Figure 1.2). The viral genome is approximately 10Kb, containing at least three genes, gag (coding for core proteins), pol (coding for reverse transcriptase) and env (coding for envelope protein). At each end of the genome long terminal repeats (LTR), which include promoter/enhancer regions and sequences involved in integration (Figure 1.3). In addition there are sequences required for packaging the viral DNA (ψ) and RNA splice sites in the envelope.

Vector systems based on retroviruses can be divided into two components, the retroviral vector and the packaging cells. The retroviral vector is manipulated in its DNA form as part of a bacterial plasmid. The vector does not encode viral proteins but serves as a vehicle for the genes to be transferred. The retroviral packaging cells provide all the viral proteins necessary for encapsidation of the vector RNA into

virions and for subsequent infection, reverse transcription and integration of the vector into the genomic DNA of cells (Figure 1.2).

The design of retrovirus packaging cell line has evolved to address the problem of spontaneous helper production such that gag-pol and env viral proteins are separated on different expression plasmids that are independently transfected into the packaging cells, meaning that three recombination events are necessary for wild type virus production (Figure 1.3).

Retroviral vectors are most frequently based on the Moloney-murine leukaemia virus (Mo-MLV), which is an amphotrophic virus capable of infecting mouse and human cells. Attachment of the retrovirus to a target cell is dependent upon the binding of retroviral envelope protein with specific target cell receptors. Different viruses differ with respect to their tropisms therefore by replacing the env gene with that of another virus the host range can be extended in a technique known as pseudo-typing (Karavanas *et al*, 1998). An example of this is the vesicular stomatitis virus-G (VSV-G) protein which has been incorporated into a Mo-MLV derived vector thereby markedly broadening the vector's host range (Yee *et al*, 1994). Transgene expression can be driven by the promoter/enhancer region in the 5' LTR or by an alternative viral (e.g. Cytomegalovirus) promoter. The available carrying capacity for retroviral vectors is approximately 7.5kb, which is too small for some genes even if cDNA is used (Verma & Somia, 1997). A requirement for retroviral integration and expression of viral genes is that the target cells must undergo at least one round of cell division. This limits the use of these vectors to proliferating cells.

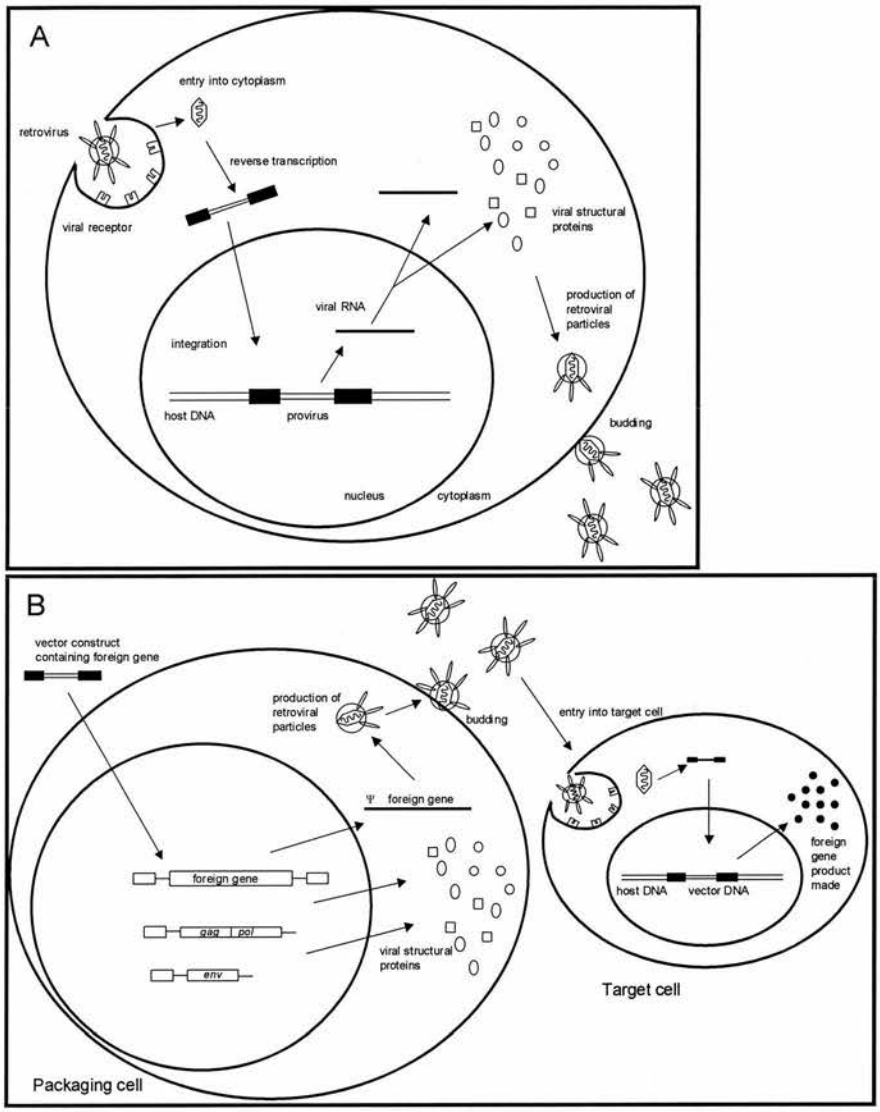

Figure 1.2 Retroviral life-cycle and production of recombination retroviral vectors by packaging cell lines

A. Retroviral replication cycle. Binding of the virus to the host cell takes place by the interaction between viral envelope proteins and specific receptors on the host cell surface. This is followed by viral internalisation and release of the viral core into the cytoplasm. Viral RNA is uncoated, reverse transcribed and transported to the nucleus. The viral DNA is then stably integrated into the host genome. During DNA synthesis viral RNA is produced and translated to produce viral proteins and enzymes. Mature virons are formed following encapsidation of the viral core proteins. The virions are released from the cell where they are able to infect other cells.

B. Retroviral packaging cell line and viral vector production. Viral genes encoding retroviral structural and enzymatic proteins are stably introduced into cells. When a retroviral vector is introduced into the cell, the viral vector RNA can be packaged resulting in the production of a infectious virus particle. These viral particles can be harvested and used to infect a target cell in order to introduce a foreign gene on the vector into the cells.

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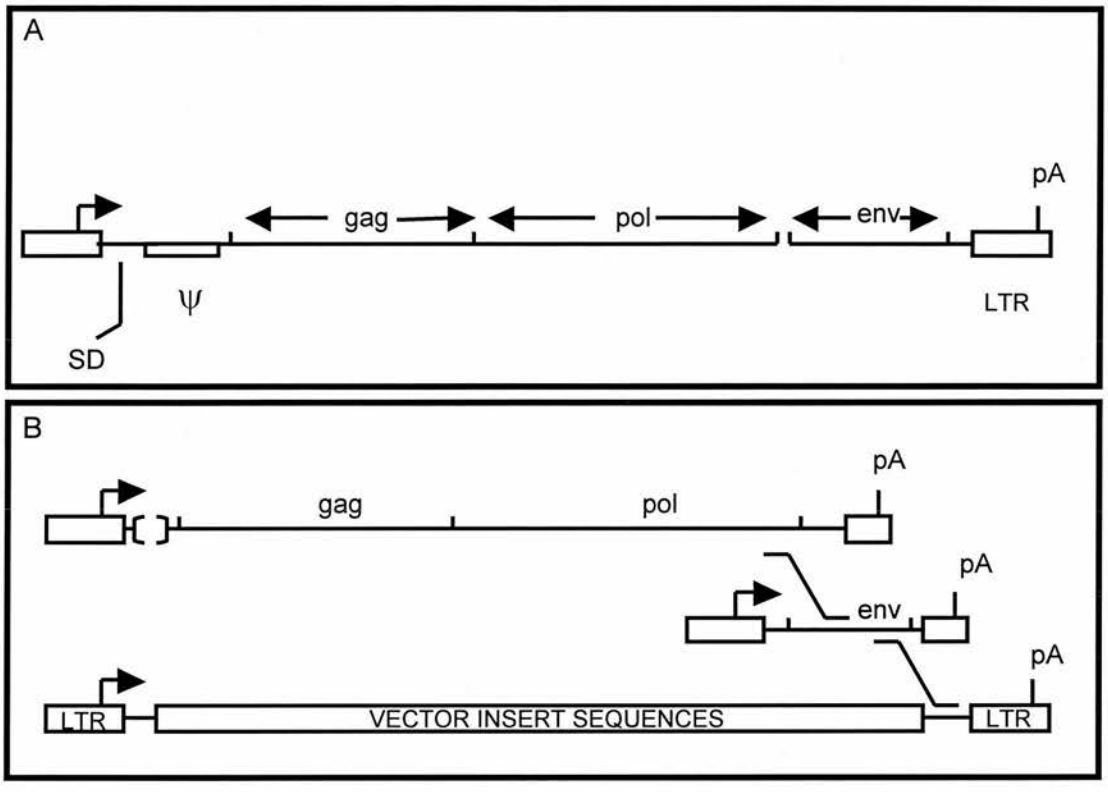


Figure 1.3 Comparison of wild type retrovirus and replication-incompetent retroviral vector
A. Generic helper virus-Mo-MLV is shown
B. Generic retroviral vector with non-viral sequences inserted into the vector
ψ indicates the retroviral packaging signal
Gag, pol and env represent the viral genes
SD and SA are the splice donor and acceptor used for expression of the env gene
Promoters are indicated by boxes with arrows pointing in the direction of transcription
PA indicates the polyadenylation site
LTR indicates the retroviral long terminal repeat

v/ Lentiviral vectors

Lentiviruses are a subclass of retroviruses, which are able to infect both proliferating and non-proliferating cells (Buchschacher, Jr. & Wong-Staal, 2000; Naldini *et al*, 1996; Uchida *et al*, 1998). Their genome is considerably more complex than simple retroviruses containing an additional six proteins; tat, rev, vpr, nef and vif. Because of this ability to infect non-dividing cells there has been great interest in the development of lentivirus vectors for use in clinical gene transfer studies (Buchschacher, Jr. & Wong-Staal, 2000). However the complex genome and replication cycle of lentiviruses, best exemplified by HIV-1, has meant that development of vectors and packaging cell lines has been more difficult in comparison with retroviruses. With recent advances in the understanding of HIV replication it has been possible to generate relatively high-titre lentivirus vectors (Stripecke *et al*, 2000). Lentivirus vectors based on other primate viruses, such as HIV-2 and simian immunodeficiency virus, and non-primate lentiviruses such as feline immunodeficiency virus have also been designed.

vi/ Adenoviral vectors

Adenovirus is a non-enveloped icosahedral structure with 20 triangular surfaces, 12 vertices and a diameter of around 70nm (Morsy & Caskey, 1999). The capsid proteins are composed of 20 hexons, 12 pentons and a fibre protein. The fibre protein is a rod like structure that protrudes from each penton base. The viral genome comprises linear, double stranded DNA (around 36kb) which encodes early- and late- expressed genes; their replication is early or late in relation to the onset of viral replication (Morsy & Caskey, 1999). All viral proteins can be supplemented in trans,

thus coding sequences can be eliminated. The only essential cis elements required for viral propagation and packaging are the packaging signal sequences and the inverted terminal repeats (Yeh & Perricaudet, 1997). The most important advance in the development of adenoviral vectors was the finding that replication defective adenoviruses lacking portions of the E1 region of the viral genome could be propagated in cells engineered to express the E1 genes (Jones & Shenk, 1979). Most of the adenovirus vectors currently in use carry deletions in the E1A-E1B and E3 regions of the viral genome. The elimination of adenovirus coding sequences allows generation of vectors that lack expressed viral proteins thus giving a greater safety profile, reduced immunogenicity and a large insert capacity (up to 37kb) (Yeh & Perricaudet, 1997). Expression of the inserted sequence is usually promoted either by the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter or exogenously added promoter sequences. Adenoviruses attach to the cell surface and are endocytosed via two receptors, the coxsackie and adenovirus receptor (CAR) and the integrin cell surface receptor (Bergelson *et al*, 1997). Vectors based on adenovirus are able to infect non-dividing cells. In contrast to retrovirus vectors, transgene expression is not dependent upon integration with the host genome. In fact integration of adenoviral DNA sequences into the chromosomal DNA of a target cell is an uncommon event and as a consequence transgene expression is not permanent.

1.6.1.2 Comparative Studies of Gene Transfer Methodologies

As stated above the success of any immune gene transfer strategy is critically dependent upon a highly efficient gene transfer technique. There have been no comparative studies examining different gene transfer methods to leukaemia cells from patients with AML, but there has been a single such study in ALL (Mascarenhas *et al*, 1998). In this study a variety of gene transfer approaches were tested in the precursor B ALL cell lines, Nalm-6 and Reh, and in human primary ALL blasts. The gene transfer methods used were electroporation, polycationic liposomes, activated dendrimers, retrovirus and lentivirus vectors.

In order to determine gene transfer efficiency (GTE) a reporter gene coding for the enhanced form of green fluorescent protein (GFP) was used. GFP is derived from the jellyfish Aequora victoria. It absorbs blue light (absorption wavelength peak 395nm) and emits green light (emission wavelength peak 508nm), which is detectable using a fluorescence microscope or by flow cytometric analysis. Enhanced GFP is a red shifted variant of GFP that contains two mutations, S65T and P64L (Zhang *et al*, 1996). In addition enhanced GFP has been re-engineered with more than 190 silent base changes corresponding to human codon usage preferences (Zhang *et al*, 1996). The GFP variant exhibits higher expression and fluorescence intensity than wild type GFP (Yang *et al*, 1996). The combined effect of these changes results in an increased sensitivity of up to 350 times over wild type GFP when excited at 488nm thus making detection more sensitive (Cormack *et al*, 1996).

By flow cytometric measurement of enhanced GFP expression the GTE for each of the gene transfer methods was determined in ALL leukaemia cells. Gene transfer to leukaemia cells derived from the ALL cells lines, Nalm-6 and Reh cells, by electroporation was minimal and associated with significant cell mortality. No measurable gene transfer was achieved with the activated dendrimer (Superfect). Of the polycationic liposomes used only one, Cellfectin, produced reproducible low-level gene transfer. Retroviral vectors based on the Mo-MLV, pseudotyped with VSV-G envelope, transduced Nalm-6 cells more efficiently than Reh. High levels of stable gene transduction could be achieved in Nalm-6 cells by co-cultivation with murine packaging cells and using cell free virus supernatant on either bone marrow stroma or with the recombinant fibronectin fragment, CH-296 (Mascarenhas *et al*, 1998). The highest level of gene transfer in both Nalm-6 and Reh cells was achieved using lentivirus vectors pseudotyped with the VSV-G envelope. Primary B-precursor ALL cells from 5 patients were found to be efficiently transduced by both Mo-MLV based (range 4.1-74%, mean 35.1%) and lentivirus based vectors (range 29.1-43.3%, mean 31.1%) (Mascarenhas *et al*, 1998).

Although no comparable study exists for primary AML leukaemia cells studies have been performed in which the GTE for individual gene transfer methods has been assessed. Leukaemia cells from a single patient with AML underwent electroporation in the presence of plasmid DNA containing the cDNA for CD80 (Mutis *et al*, 1998). Expression of CD80 by the leukaemia cells, measured by flow cytometry, was of a low level and transient. All other studies examining gene transfer to AML cells have utilised viral-based vector systems (Summarised in Table 1.1).

Gene transfer to primary human AML blasts derived from 10 patients, using a Mo-MLV-based retroviral vector containing the cDNA for CD80, gave a GTE ranging from 0.3-8.2% (mean 1.5%) (Hirst *et al*, 1997). Because of the requirement for the target cell to be in cycle to allow transgene expression to take place, the cytokines IL3, GM-CSF and SCF were used to stimulate cell division. This led to an increase in mean GTE to 3.9%. However there was no apparent correlation between the GTE and the degree of cellular proliferation as measured by bromodeoxyuridine uptake (Hirst *et al*, 1997).

There have been two studies in which lentiviral vectors were used for gene transfer to primary AML blasts. In the first of these a lentiviral vector containing the cDNA for CD80 was used to infect leukaemic cells from 5 patients with AML (Stripecke *et al*, 2000). GTE ranged from 61.7-87.6% (mean 77.6%) (Stripecke *et al*, 2000). In the second study, using a lentiviral vector containing cDNA coding for GFP, GTE in leukaemia cells from 5 AML cases ranged from 12.6-32% (mean 19.5) (Biagi *et al*, 2001). In both these studies the lentiviral vectors were pseudotyped with VSV-G envelope protein. Although cell division is not a requirement for lentiviral vector expression leukaemia cells were cultured in growth-stimulating cytokines prior to infection in both studies (Table 1.1).

GTE to primary AML blasts using adenoviral vectors has been examined in one study. Using an adenoviral vector containing the cDNA for B-galactosidase as a reporter gene, GTE in leukaemia cells from 25 AML cases was highly variable, ranging from 0-74.7% (mean 10.26%) (Gonzalez *et al*, 1999). Previous studies examining adenoviral infection of haematopoietic cells provide insight into why adenoviral vectors should show such variability in GTE to leukaemia cells.

Haematopoietic cells do not express the main adenovirus receptor CAR and viral attachment is therefore dependent upon binding to integrins (Huang *et al*, 1996). In general haematopoietic cells have low levels of expression of the αvβ3 and αvβ5 integrins, which mediate adenovirus internalisation by binding to the adenoviral penton base via a RGD motif (Huang *et al*, 1995).

In an attempt to improve adenovirus vector attachment, and therefore GTE, in haematopoietic cells various approaches have been tried. Combining adenoviral vectors with polycationic lipids has been shown to improve GTE in haematopoietic cells (Clark *et al*, 1999; Dietz & Vuk-Pavlovic, 1998; Fasbender *et al*, 1997). An alternative approach is to modify the adenoviral vector by retargeting it to bind to different cell surface receptors (Douglas *et al*, 1996; Douglas *et al*, 1999; Smith *et al*, 1999; Wickham *et al*, 1996). Wickham et al described an adenoviral vector AdZ.F(pk7) containing a modified fibre protein where polylysine had been added to the c-terminal end of the fibre (Wickham *et al*, 1996). This vector, through binding of polylysines to heparan-sulphate containing receptors, could increase virus adsorption and GTE in cell types expressing low levels of adenovirus receptors. As it is known that haematopoietic stem cells express heparan-sulphate receptors the AdZ.F(pk7) vector was evaluated in primary AML cells. Leukaemic cells from 25 patients with AML were infected at multiplicity of infection (MOI) of 100 with the AdZ.F9(pk7) vector containing the cDNA for B-galactosidase (Gonzalez *et al*, 1999). GTE ranged from 70-100% (mean 94.3%) (Gonzalez *et al*, 1999). The addition of cytokines SCF, IL-3 and GM-CSF was essential as their omission led to a significant fall in GTE and a decrease in cell viability.

	Hurst et al 1997	Stripecke et al 2000	Biagi et al 2001	Gonzalez et al 1999	Gonzalez et al 1999
Viral Vector	Retroviral vector	Lentivirus vector (pseudotyped with VSV-G)	Lentivirus vector (pseudotyped with VSV-G)	Adenoviral vector	Adenoviral vector with modified fibre protein
No. of AML samples	10	5	5	25	25
Reporter gene	CD80	CD80	GFP	B-galactosidase	B-galactosidase
Method of analysis	FACS	FACS	FACS	FACS	FACS
Range of GTE (mean GTE)	0.3-8.2% (mean 1.5%)	61.7-87.6% (mean 77.6%)	12.6-32% (mean 19.52%)	0-74.7% (mean 10.26%)	70-100% (mean 94.3%)
Growth factors used	Nil (addition of SCF, GM-CSF and IL-3 increased TE to 3.9%)	SCF and IL-3	SCF, Flt3-L and IL-3	SCF, GM-CSF and IL-3	SCF, GM-CSF and IL-3

Table 1.1 Studies comparing gene transfer efficiency of viral vectors in AML

1.6.1.3 Immunostimulatory properties following gene transfer

In vitro studies, based on the gene transfer of cDNA coding for CD80 to primary human AML blasts, have shown that leukaemia cells expressing CD80 are capable of stimulating the proliferation of allogeneic T lymphocytes (Hirst *et al*, 1997).

A number of *in vivo* studies have been performed with murine leukaemia models. Using the M1 murine leukaemia model, leukaemia cells were transduced with CD80 and then tested for their ability to induce anti-leukaemia immunity (Hirano *et al*, 1997). When syngeneic, immunocompetent SL mice were injected with two independent CD80 transduced monoclonal sublines, M1-B7-1/F/clone F20 and M1-B7-1/F/clone F7, 57% and 43% of mice, respectively, were able to reject the modified leukaemia cells. *In vivo* depletion of T cell subsets showed that both CD4+ and CD8 T cells were indispensable for the development of anti-leukemic immunity. Although a single exposure of irradiated monoclonal M1-B7/1/F cells was not fully effective, multiple exposures induced protective immunity against subsequent challenge with parental M1 cells (Hirano *et al*, 1997). Furthermore, multiple vaccinations with irradiated monoclonal M1-B7-1/F/clone F7 cells could cure 67% of mice previously injected with a lethal number of M1 cells.

Other investigators have utilised a radiation/dexamethasone-induced murine leukaemia model that closely mimics AML in humans (Resnitzky *et al*, 1985). Initial studies using this model involved the transduction of leukaemic cells with a retroviral vector containing the cDNA coding for CD80. Infusion of CD80 transduced mAML cells into a syngeneic host led to complete eradication of the leukaemia if less than 10^6 cells were infused but if greater than 10^6 cells were infused although time till death was prolonged animals were not cured (Dunussi-

Joannopoulos *et al*, 1996). CD80 transduced mAML cells were also capable of protecting animals from the development of leukaemia when later challenged with unmodified mAML leukaemia cells. In animals immunised intravenously with 10^5 irradiated CD80 transduced mAML cells subsequent infusion of 10^5 unmodified mAML cells did not result in the development of leukaemia (Dunussi-Joannopoulos *et al*, 1996). This anti-leukaemic immune response was dependent on the interaction of CD80 and the T cell CD28 receptor as blocking of this with CTLA4/Ig eliminated the efficacy of the vaccine (Dunussi-Joannopoulos *et al*, 1996). To test whether this approach would be successful in treating established disease animals were first inoculated with unmodified mAML cells and allowed to develop leukaemia. Mice were then infused with CD80 transduced mAML cells. If given early in the course of the disease the tumour vaccine led to cure of the animals, however, in late-stage disease although time to death was prolonged animals were not cured (Dunussi-Joannopoulos *et al*, 1996; Dunussi-Joannopoulos *et al*, 1997). When vaccination with CD80 transduced mAML cells was given to treat animals with established leukaemia following prior cytoreductive therapy, it proved more effective in eliciting cure than chemotherapy or vaccination alone (Dunussi-Joannopoulos *et al*, 1997). This suggests that the setting for such a strategy would be following remission-induction therapy when there is a relatively low tumour burden.

Using this model the same group of investigators evaluated the efficacy of vaccines based on leukaemia cells transduced with the cDNA for a variety of immunostimulatory cytokines. Vaccination with mAML cells transduced with cDNA for GM-CSF led to improved cure of established disease in comparison with CD80 transduced mAML cells (Dunussi-Joannopoulos *et al*, 1998). The effectiveness of

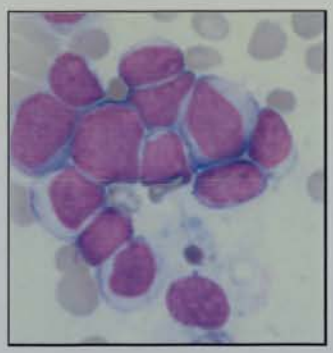
the GM-CSF transduced mAML based vaccine did not appear to be dependent upon the presence of T cells and it was hypothesized that the production of GM-CSF by the leukaemic blasts might have promoted direct leukaemia killing through the recruitment of immune effectors such as activated natural killer cells, macrophages, neutrophils and eosinophils (Dunussi-Joannopoulos *et al*, 1998). More recently IL-12 transduced mAML cells have been evaluated using this model (Dunussi-Joannopoulos *et al*, 1999). Vaccination with IL-12 transduced mAML cells was able to cure established leukaemia and generate long-lasting leukaemia-specific immunity (Dunussi-Joannopoulos *et al*, 1999). This effect was shown to be dependent upon the presence of IL-12 as use of blocking anti-IL-12 monoclonal antibodies (mAb) abrogated the therapeutic effect. It was shown that IL-12 mediated its effect through stimulating the local production of IFN-γ, as depletion of IFN-γ also abolished the efficacy of the vaccine (Dunussi-Joannopoulos *et al*, 1999).

These *in vivo* studies with cytokine gene transduced leukaemia vaccines provide evidence in support of the hypothesis that the generation of therapeutic immunity is dependent upon the creation of a suitable immunostimulatory cytokine microenviroment. Based on the studies that suggest that the leukaemia microenvironment is immunosuppressive, tumour vaccines based solely on the gene transfer of costimulatory molecules might prove to be ineffective at promoting anti-leukaemia immunity, as they would be incapable of favourably altering the cytokine microenvironment. In an attempt to address this problem vaccines have been designed in which the tumour cells are transduced with vectors that contain cDNA for both a costimulatory molecule and an immunostimulatory cytokine, for example CD80 combined with IL-12 (Zitvogel *et al*, 1996) or GM-CSF (Stripecke *et al*,

1999). In a murine ALL model higher leukaemia rejection rates were achieved using leukaemia cells transduced with the cDNA for both CD80 and GM-CSF in comparison with leukaemia cells transduced with CD80 alone (Stripecke *et al*, 1999).

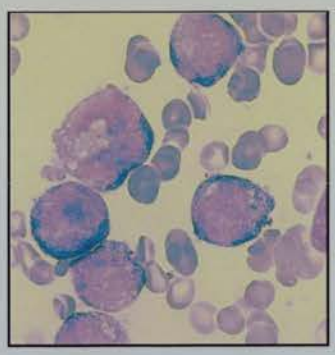
1.6.2 Differentiation induction in human myeloid leukaemia cells

An alternative way of modifying leukaemia cells in such a way as to increase their immunostimulatory properties is by inducing leukaemia cell differentiation. AML is characterised by a block in normal haematopoietic differentiation with the consequence that there is an accumulation of leukaemia cells showing differentiation up to but not beyond the stage of the arrest. In the FAB classification of AML there is a subdivision into seven categories depending on morphological and phenotypic evidence for myeloid, monocytic, erthyroid or megakaryocytic differentiation and on the degree of subsequent cell maturation (Fig 1.4). Recent studies have advanced our understanding of the molecular mechanisms underlying this differentiation arrest. Several lines of evidence point towards a common theme in the molecular pathogenesis of AML namely that the end result of many of the common chromosomal rearrangements found in AML is the transcriptional repression of genes involved in haematopoietic differentiation (Redner *et al*, 1999). In the case of three of these chromosomal rearrangement; t(15;17), t(8;21) and inv(16), the fusion protein products exert their repressive effect by altering histone acetylation status thereby leading to transcriptional inactivation. Despite the differentiation block it is still possible to induce leukaemia cell differentiation by using differentiating agents or by the use of certain cytokines.



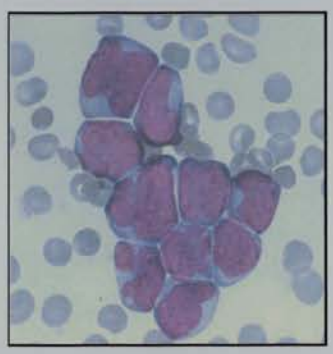
M0

AML with minimal evidence of myeloid differentiation



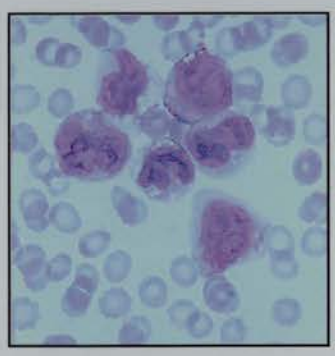
M4

Acute myelomonocytic leukaemia



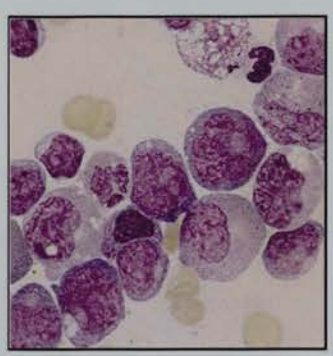
M1

AML without maturation



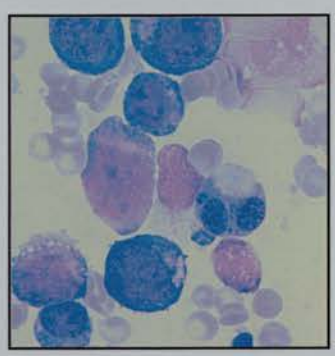
M5

Acute monocytic/ monoblastic leukaemia



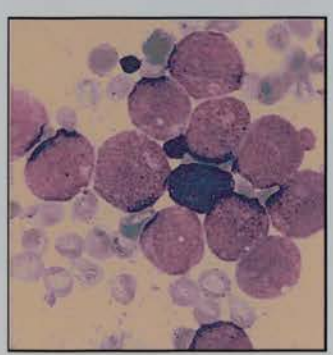
M2

AML with granulocytic maturation



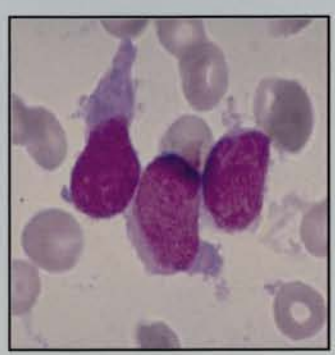
M6

Erythroleukaemia



M3

Acute promyelocytic leukaemia



M7

Acute megakaryoblastic leukaemia

Figure 1.4 FAB classification of Acute Myeloid Leukaemia

1.6.2.1 Differentiating agents

Studies have shown that a variety of agents are capable of inducing differentiation in leukaemia cells. Examples of differentiating agents include vitamin D3 (Miyaura *et al*, 1981; Olsson *et al*, 1983), retinoic acid (Breitman *et al*, 1980), azacytidine (AZA) (Attadia, 1993; Lubbert, 2000; Pinto *et al*, 1984), trichostatin (TSA) (Kosugi *et al*, 1999; Wang *et al*, 1999; Yoshida *et al*, 1987; Yoshida *et al*, 1990), bryostatin (BRYO) (Kaneki *et al*, 1999; Steube & Drexler, 1993) and calcium ionomycin (CI) (Engels *et al*, 1998; Engels *et al*, 1999; Koski *et al*, 1997; Koski *et al*, 1999a; Koski *et al*, 1999b) (Summarised in Table 1.2).

Only the retinoic acid derivative, ATRA, has thus far proven to be clinically useful (Fenaux *et al*, 1993; Grimwade *et al*, 1998). At pharmacological doses ATRA is able to relieve the transcriptional repression mediated by the PML-RARA fusion protein, present in t(15;17) APML, thereby causing the differentiation of leukaemia cells into mature granulocytes (Zhang *et al*, 2000). In combination with chemotherapy the treatment of patients with t(15;17) APML with ATRA has lead to leukaemia-free 5 year survival rates of greater than 70% (Grimwade *et al*, 1998).

The rationale for using TSA and AZA to induce leukaemia cell differentiation is based on studies that have demonstrated the important role that histone acetylation and DNA methylation status have in controlling transcriptional activity and on evidence that many of the chromosomal and molecular rearrangements characterising acute leukaemia act by repressing transcription of genes involved in haematopoietic differentiation through the recruitment of histone deacetylases and DNA methylaters. AZA is able to induce differentiation in myeloid leukaemic cells by relieving transcriptional repression caused by increased DNA methylation (Attadia, 1993;

Lubbert, 2000; Pinto *et al*, 1984). TSA, a specific histone deacetylase inhibitor (HDAI), has been shown to relieve transcriptional repression caused by histone deacetylation thereby allowing differentiation to proceed (Kosugi *et al*, 1999; Wang *et al*, 1999; Yoshida *et al*, 1987; Yoshida *et al*, 1990). In a study examining the effects of TSA on differentiation in AML, treatment of leukaemia cells with TSA, and other HDAIs, was shown to lead to induction in the expression of the costimulatory molecule CD86 (Maeda *et al*, 2000).

A different class of differentiating agent, BRYO, exerts its action by interfering with protein kinase C activity. BRYO has been shown to be able to induce monocytic differentiation in myeloid leukaemia cells (Kaneki *et al*, 1999; Steube & Drexler, 1993).

CI, a calcium ionophore has been shown to induce the differentiation of haematopoietic cells into dendritic-like cells (Engels *et al*, 1998; Koski *et al*, 1999a; Koski *et al*, 1999b). The mechanism of action of CI is thought to be through increased calcium mobilisation thereby leading to activation of calcium-dependent signaling pathways, which trigger DC differentiation. CI was found to induce the differentiation of leukaemia cells derived both from patients with CML and the myeloid leukaemia cell line, HL60, into dendritic-like cells based on costimulatory molecule and CD83 expression and on the acquisition of a DC-like morphology (Engels *et al*, 1999; Koski *et al*, 1999b).

Name	Type of agent	Mechanism of action
Vitamin D3	Hormone	Activates transcription of genes involved in haematopoietic differentiation
All-Trans Retinoic acid	Retinoic acid derivative	Relieves transcriptional repression mediated by PML-RARA fusion protein
Trichostatin	Histone deacetylase inhibitor	Relieves transcriptional repression by restoring histone acetylation status
Azacytidine	DNA demethylase	Relieves transcriptional repression by restoring DNA methylation status
Bryostatin	Protein Kinase C activator	Interferes with protein kinase C dependent signal transduction pathways
Calcium ionomycin	Calcium ionophore	Activates calcium dependent cytoplasmic signaling pathways

Table 1.2 Differentiating agents in AML. A selection of differentiating agents that have been used to induce differentiation in leukaemia cells with a brief description of their mechanism of action.

1.6.2.2 Cytokine-induced differentiation

One of the earliest studies in AML differentiation induction showed that in the murine myeloid leukaemia cell line, M1, differentiation into mature granulocytes and macrophages was possible using the cytokine IL-6, but only in certain subclones (Fibach *et al*, 1972; Sachs, 1978). More recent investigations into cytokine-induced differentiation in AML have been stimulated by studies showing that it is possible to induce the differentiation of peripheral blood monocytes and CD34+ haematopoietic progenitor cells into DC by culture with certain cytokine combinations (Brossart *et al*, 1998; Caux *et al*, 1994; Hart, 1997; Reid *et al*, 1992; Reid, 1998; van Schooten *et al*, 1997). Culture of $CD34^+$ bone marrow haematopoietic progenitor cells or $CD14^+$ PB monocytes with cytokines GM-CSF and IL-4 induces their differentiation into functional DC (Romani *et al*, 1994; Sallusto & Lanzavecchia, 1994). DC generated in this way are considered 'immature' and are efficient at antigen uptake and processing but are relatively inefficient at antigen presentation (Sallusto & Lanzavecchia, 1994; Zhou & Tedder, 1996). Immunophenotypically 'immature' DC express the DC-associated cell marker CD1a, but have relatively low levels of expression of costimulatory molecules CD40, CD80 and CD86 and HLA class II molecules. CD1a is a type I transmembrane protein that associates non-covalently with β_2 microglobulin (Bauer *et al*, 1997). Expression of CD1a has been used as a marker for DC populations although its expression is not restricted to this cell type. Its function is believed to be in the presentation of foreign lipids, such as those derived from mycobacteria, to T cells. Following culture of 'immature' DC in TNFα or CD40L there is down-regulation in the DC ability to take up and process antigen (Figure 1.4). In contrast matured DC show upregulation of CD54, HLA class II

molecules, CD80 and CD86 and gain expression of CD83. CD83 is a type I membrane glycoprotein and is a member of the immunoglobulin superfamily (Zhou 1992). Its expression is highly restricted to mature DC (Kozlow *et al*, 1993). The function of CD83 has been unknown but a recent study presents evidence to suggest that it is an adhesion receptor that binds to a ligand present on circulating monocytes and a fraction of activated and/or stressed $CD8^+$ T cells. Based on the evidence in this study it has been suggested that it may have a role in intracellular communications involving DC, circulating monocytes and certain populations of activated and/or stressed T lymphocytes. In parallel with the upregulation of costimulatory and HLA class II expression mature DC possess increased antigen-presenting function as defined by enhanced allostimulatory capacity (Sallusto & Lanzavecchia, 1994; Zhou & Tedder, 1996). DC play a crucial role in the control of immunity based on their ability to initially process and then present antigen to T cells in order to generate antigen-specific immunity (Avigan, 1999; Banchereau & Steinman, 1998; Barratt-Boyes *et al*, 2000; Colaco, 1999; Hart, 1997; Labeur *et al*, 1999; Macatonia *et al*, 1995; van Schooten *et al*, 1997).

As DC and leukaemia cells share the same common myeloid progenitor cells this prompted investigators to study whether leukaemia cells could be driven to differentiate to dendritic-like cells using cytokine combinations similar to those used in DC differentiation induction. The first studies involved leukaemia cells derived from patients with CML (Choudhury *et al*, 1997; Heinzinger *et al*, 1999; Smit *et al*, 1997; Wang *et al*, 1999). *In vitro* studies of the cytokine-induced differentiation of leukaemia cells from patients with AML to dendritic-like cells followed shortly after.

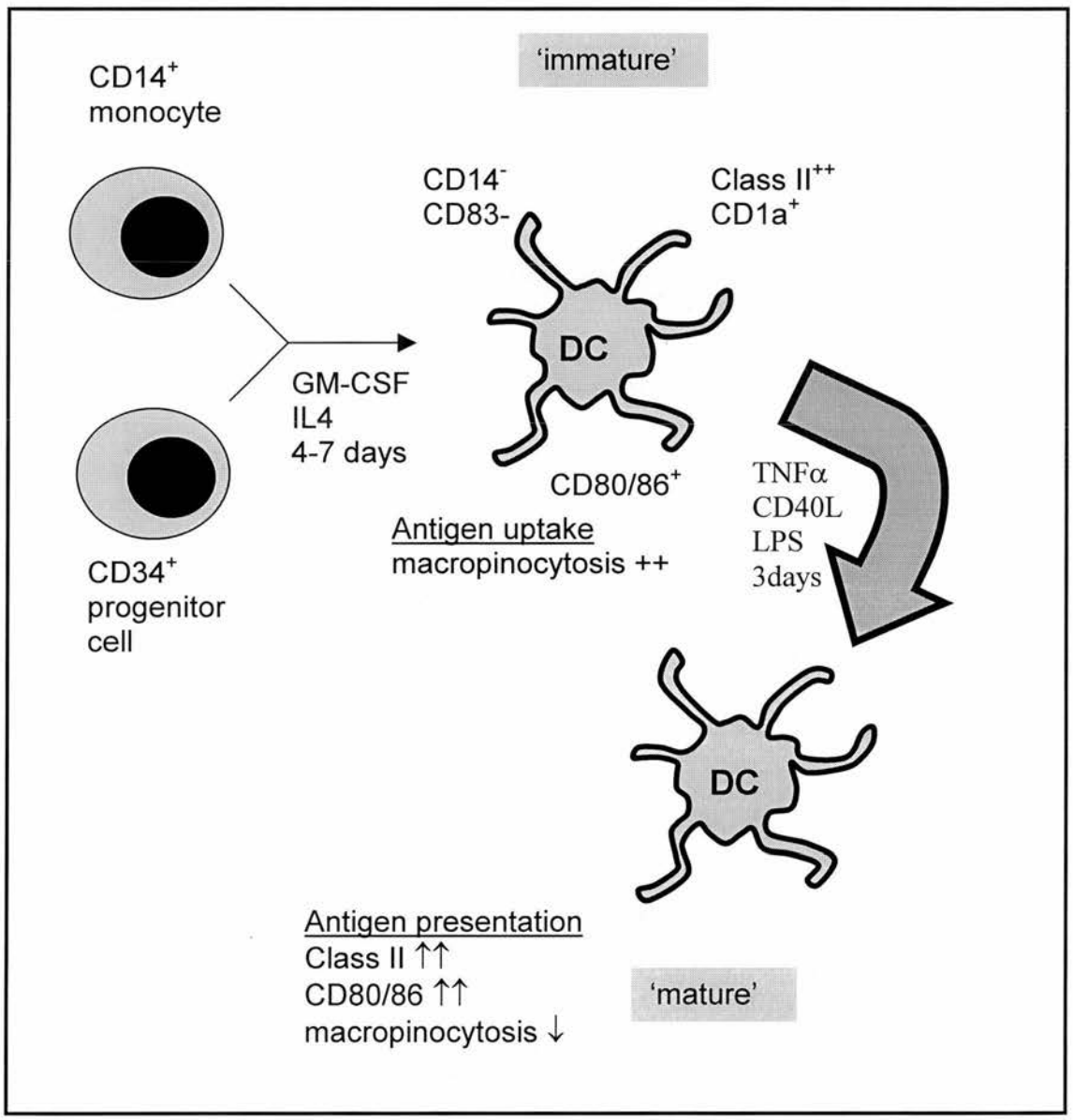


Figure 1.5 Generation of dendritic cells.
Culture of CD14+ PB monocytes or CD34+ BM progenitors with GM-CSF and IL-4 for 4-7 days induces differentiation to CD1a+ CD83- 'immature' DC. Following maturation the DC become CD83+ and show high levels of expression of CD80, CD86 and HLA class II molecules.
Reproduced (following modification), with permission, from **The biology and clinical applications of dendritic cells** *Transfusion Medicine* 1998;8:77-86

In summary these studies have shown that following culture with GM-CSF, IL-4 and TNFα/CD40L leukaemia cells derived from patients with AML developed immunophenotypic and morphological features characteristic of DC (Boyer *et al*, 2000; Brouwer *et al*, 2000a; Brouwer *et al*, 2000b; Charbonnier *et al*, 1999; Choudhury *et al*, 1999; Cignetti *et al*, 1999; Costello *et al*, 2000; Kohler *et al*, 2000; Oehler *et al*, 2000; Robinson *et al*, 1998). Immunophenotypically dendritic-like leukaemia cells (DLLC) show expression of costimulatory molecules CD40, CD80 and CD86, the cell adhesion molecule, ICAM-1, the dendritic cell-associated markers, CD1a and CD83 and both HLA class I and II molecules (Boyer *et al*, 2000; Brouwer *et al*, 2000a; Brouwer *et al*, 2000b; Charbonnier *et al*, 1999; Choudhury *et al*, 1999; Cignetti *et al*, 1999; Costello *et al*, 2000; Kohler *et al*, 2000; Oehler *et al*, 2000; Robinson *et al*, 1998). Morphologically DLLC possess the characteristic veils and processes of 'classical' DC. IL-12 production by DLLC, a characteristic of matured DC, has only been tested in one study (Charbonnier *et al*, 1999). A high level of IL-12 production by DLLC was detected in seven AML cases all of which were monocytic in nature (Charbonnier *et al*, 1999). Evidence that the DLLC are derived from the leukaemic clone, and not contaminating monocytes, has been provided by FISH analysis, which demonstrates that the DLLC retain the chromosomal rearrangement characterising the original leukaemia (Boyer *et al*, 2000; Brouwer *et al*, 2000b; Choudhury *et al*, 1999; Kohler *et al*, 2000; Oehler *et al*, 2000; Robinson *et al*, 1998). Functional studies have also confirmed the dendritic like nature of the differentiated leukaemia cells. In MLLR DLLC have been shown to be potent stimulators of allogeneic T lymphocytes (Brouwer *et al*, 2000b; Choudhury *et al*, 1999; Robinson *et al*, 1998). It has been shown that by co-culture

of autologous T lymphocytes with DLLC, CTL can be generated that can specifically recognise and destroy unmodified leukaemia cells *in vitro* (Charbonnier *et al*, 1999; Choudhury *et al*, 1999). Table 1.3 summarizes the immunophenotypic and functional similarities between monocyte derived DC and DLLC.

In the majority of these studies generation of DLLC from leukaemia blasts was possible in around a half to two thirds of AML cases (Brouwer *et al*, 2000b; Choudhury *et al*, 1999; Cignetti *et al*, 1999; Robinson *et al*, 1998). What factor(s) prevent differentiation in resistant cases has not been identified. It does not appear to be related to FAB subtype as DLLC have been generated from leukaemia cells of all seven FAB subtypes (Boyer *et al*, 2000; Brouwer *et al*, 2000a; Brouwer *et al*, 2000b; Charbonnier *et al*, 1999; Choudhury *et al*, 1999; Cignetti *et al*, 1999; Costello *et al*, 2000; Kohler *et al*, 2000; Oehler *et al*, 2000; Robinson *et al*, 1998).

1.7 Murine models of dendritic cell differentiation

Cytokine-induced dendritic cell differentiation from murine peripheral blood (PB) monocytes and bone marrow (BM) derived haematopoietic progenitors has been performed using cytokine combinations broadly similar to those used in human studies (Masurier *et al*, 1999; Schreurs *et al*, 1999; Zhang *et al*, 1997; Zhang *et al*, 1998). There has only been one study examining cytokine-induced differentiation of murine leukaemia cells to DLLC. In this study culture of murine erythroleukaemia cells with GM-CSF was reported to induce dendritic cell differentiation in the leukaemia cells (Cao *et al*, 1998).

	Monocyte-derived DC	DLLC
Immunophenotype	HLA I/II ++ CD40 ++ CD80 + CD86 ++ CD1a + CD83+ CD54 ++	HLA I/II ++ CD40 + CD80 +/- CD86 + CD1a +/- CD83+/- CD54 ++
Cytokines produced	IL-12 IL-15	IL-12 IL-15 ?
Functional properties	Potent stimulators of T cells in allogeneic MLR Generate autologous CTLs that can recognise novel and recall antigens	Potent stimulators of T cells in allogeneic MLR Generate autologous CTLs that can recognise leukaemia specific antigens

Table 1.3 A Comparison of the features of Monocyte-Derived Dendritic Cells and Dendritic-Like Leukaemia Cells

1.8 Study Objectives

The aim of this study was the development of a method for engineering leukaemia cells in order to make them suitable for use as autologous vaccines in the immunotherapy of AML. Two main approaches were to be employed to achieve this aim:

1 Gene transfer of costimulatory molecules and immunostimulatory cytokines to leukaemia cells

2 Cytokine-induced differentiation of leukaemia cells to dendritic-like cells

The first approach was to attempt the genetic modification of leukaemia cells by the gene transfer of cDNA coding for costimulatory molecules or immunostimulatory cytokines. An important prerequisite of this approach was a gene transfer system that gave sufficiently high level of gene transfer efficiency in primary leukaemia cells so as to be useful in a clinical setting. Therefore initial experiments were the evaluation of GTE, in myeloid leukaemia cell lines and in primary AML blasts, of a variety of physical, chemical and viral gene transfer systems. Whichever system proved to give the highest level of reproducible GTE was to be selected for use in the gene transfer of costimulatory molecules and/or immunostimulatory cytokines to primary leukaemia cells in order to enhance their T cell immunostimulatory properties.

The second approach involved the cytokine-induced differentiation of leukaemia cells to DLLC. Confirmation that differentiation to DLLC had taken place was to be based on the morphological and immunophenotypic characteristics of the cytokine cultured leukaemia cells.

The functional properties of these engineered leukaemia cells in terms of their antigen presenting capacity was to be tested for by measurement of their allostimulatory properties in MLLR. The ability of these cells to prime autologous T cells to recognise and destroy unmodified leukaemia cell targets was to be evaluated by means of cytotoxicity assays.

Finally whichever of these two approaches proved to be the more promising in terms of potential clinical utility was to be translated into a murine AML model in order to determine whether a vaccination strategy based on modified leukaemia cells would generate significant *in vivo* anti-leukaemia responses.

It was hoped that these pre-clinical studies would pave the way for subsequent phase I/II clinical trials of DLLC vaccination for the immunotherapy of AML.

Chapter 2

MATERIALS AND METHODS

2.1 Cells and cell culture techniques

2.1.1 Culture conditions

All cultures were incubated at 37^0C in a humidified incubator (Heraeus) containing 5% CO_2.

2.1.2 Origin and maintenance of cell lines and primary leukaemia cells

2.1.2.1 Myeloid leukaemia cell lines

Four myeloid leukaemia lines were used in the study; KG1a, U937, K562, and HL60 (all ATCC, Rockville, MD, USA). Cells were cultured in RPMI 1640 medium with 10% hiFCS (GlobePharm, Guildford, UK), L-glutamine (2mM/L), penicillin/ streptomycin (50U/ml) and amphoteracin B 250 μg/ml (all GIBCO BRL, Paisley, UK) (cRPMI) in $25cm^2$ flasks, unless otherwise specified. Medium was replaced every 3-4 days with cell density adjusted to between $1x10^5$-$1x10^6$/ml in order to maintain the cells in exponential growth phase.

2.1.2.2 Retroviral vector packaging cell lines

The three retroviral packaging cell lines used TeflyMLV-A, TeflyRD114 and TeflyGALV (Genethon, Evry, France) differ based on the specificity of the envelope protein present on the virions (Cosset *et al*, 1995; Porter *et al*, 1996). TeflyMLV-A carries the gene for the murine amphotrophic envelope protein, TeflyRD114, the feline endogenous retrovirus envelope protein and TeflyGALV the gibbon ape leukaemia virus envelope protein (Eglitis & Schneiderman, 1997; Porter *et al*, 1996). These cell lines were cultured in DMEM with 10% hiFCS, L-glutamine (2mM/L), Glucose 4.5gm/l (GIBC0 BRL), penicillin/ streptomycin (50U/ml) and amphoteracin B 250 μg/ml (cDMEM) in 25cm^2 flasks unless otherwise specified. Medium was replaced every 3-4 days. Once the monolayer was confluent cells were split. The medium was aspirated and the monolayer washed with 5mls sterile tissue culture grade Phosphate buffered saline (PBS) (GIBCO BRL). The PBS was then aspirated and 2mls of 0.1% trypsin (GIBCO BRL) was added. After 30 seconds 1.5mls of the trypsin solution was aspirated and cells were incubated for a further 2 minutes at which point the monolayer was starting to lose adherence. The flask was then tapped lightly to fully detach the cells. 5mls of cDMEM was added and cells were pelleted by centrifugation at 400g for 4 minutes. 1ml of cDMEM was added and a cell count was performed. A new 25cm^2 flask was reseeded with 10^5 cells with the remainder being frozen for later use.

2.1.2.3 Adenoviral propagating cell line

The HEK 293 cell line (F. Farzaneh, King's College School of Medicine and Dentistry, London), a fibroblast-like cell line was cultured and maintained under identical conditions to the retroviral packaging cell lines.

2.1.2.4 Gene transfer target cell lines

The TE6.71, CHO and COS (all ATCC, USA) target cell lines were cultured and maintained under identical conditions to the retroviral packaging cell lines.

2.1.4 Primary human leukaemia cells

Primary AML cells were obtained, with written informed consent using a study protocol that had received prior approval from Lothian research ethics committee, from BM marrow or PB samples of patients with newly diagnosed or relapsed AML. Diagnosis was established both by cytological criteria based on the FAB classification and by immunophenotypic analysis following staining of leukaemia cells with a panel of mAb. This acute leukaemia diagnostic panel comprised of mAb directed against CD13, CD14, CD15, CD33, CD34, and HLA class II molecules. Karyotypes were defined according to criteria of the International System for Human Cytogenetic Nomenclature. Samples were taken into tubes containing 100u/ml of preservative free heparin (Leo, Risborough, UK). An equal volume of RPMI was added to the PB or BM samples and the resulting cell suspension was overlain over Lymphoprep (Nycomed, Amersham, UK) at a 4:1 ratio in 50ml conical tubes. The tubes were then centrifuged at 650g for 25 minutes. Cells at the interface were aspirated and transferred to 15mls conical tubes and washed

with 4mls cRPMI. Following centrifugation at 400g for 4 minutes cells were resuspended in 1ml cRPMI and a viable cell count performed. Cell density was adjusted to 10^7/ml and cells were then frozen for later use.

2.1.3 Cryopreservation

All primary cells and cell lines, with the exception of the HL60 cell line, were cryopreserved in cRPMI to which was added 10% tissue culture grade DMSO (Sigma-Aldrich, Gillingham, UK). The HL60 cells were cryopreserved in cRPMI and 10% glycerol (Fisher Scientific, Loughborough, UK) because of the differentiating properties of DMSO in this cell line. Cells suspensions in the freeze mixture were added in 1 ml volumes to cryovials (cryotube™, Nunc, Denmark) and placed in a -70^0C freezer. After 24 hours they were transferred to a -140^0 freezer for storage prior to use.

2.1.4 Thawing

Cryopreserved cells were thawed rapidly at 37^0C in a water bath. The cell suspension was washed in 5 mls cRPMI and then centrifuged at 400g for 4 minutes. The resulting supernatant was then discarded and the pellet was resuspended in medium the nature of which was dependent on the cell type and the purpose for which the cells were to be used.

2.1.5 Counting of viable cell numbers

10μl of cell suspension to be counted was mixed with 90μl of 0.4% trypan blue. 10μl of the subsequent dilution was placed in a Neubauer haemocytometer. The numbers of cells excluding trypan blue in 16 squares were counted. Viable cell count/ml = number of cells in 16 squares x 10^4 x10.

2.2 Gene transfer methods

2.2.1 Qualitative comparison of GFP containing plasmid vectors

The calcium phosphate precipitation method was used for a qualitative comparison of a variety of gene transfer vectors encoding GFP as a means of selecting the most suitable for use in the comparative evaluation of gene transfer methods in myeloid leukaemia cells. Five GFP containing vector plasmids were studied as shown in Table 2.1

Plasmid vector	Promoter
pCMV-GFP-1	CMV
pCDWS GFP-w2	CMV+intron
pBabePuro.EGFP+PA	MLV
pBabe.Puro.EGFP-PA	MLV
pEGFP-1	SV40

Table 2.1 A qualitative comparison of five different GFP containing plasmid vectors

CHO and COS-7 cells were harvested when in log growth phase and seeded into 3mls cDMEM and added to the wells of a 6-well plate. Gene transfer by $CaPO_4$ precipitation was performed once the monolayer had achieved two-thirds confluence. TE buffer (1mM Tris HCL + 0.05mM EDTA, pH 7.5) was added to 5μg of plasmid DNA in a bijoux tube to give a final volume of 187.5μl. Aliquots of 250μl 2xHBS (16gm NaCl, 10gm HEPES, 0.23gm $NaPO_4$ in 1l distilled H_20, pH 7.1) were then dispensed into another sets of bijoux tubes. 62.5μl 1M $CaCl_2$ was added drop wise to the DNA containing tubes whilst vortexing at the same time. The DNA-$CaCl_2$ solution was then added drop wise to tubes containing 2x HBS again vortexing at the same time. The precipitate was allowed to form for 30 minutes at room temperature. The mixture was then added drop wise to the media, in the wells containing the target cell line, and distributed evenly by gently rotating the plate. The cells were incubated with the DNA-$CaPO_4$ precipitates for 24 hours at 37^0C, 5%CO_2. The supernatant was then aspirated and the cells were washed with 3mls of sterile tissue culture grade PBS. 3 mls cDMEM were then added and the plates were incubated at 37^0C, 5%CO_2 for 48 hours. A qualitative analysis of the efficiency of gene transfer for each of the plasmid vectors was performed by examination of the wells under fluorescence microscopy (Zeiss Axiovent 25, Zeiss, Germany). Wells corresponding to different vectors were graded according to the degree of green fluorescence exhibited by the target cells.

2.2.2 Plasmid DNA preparation

A Mo-MLV vector encoding enhanced GFP was constructed by T. Paterson by cloning the cDNA for enhanced GFP (Clontech, Basingstoke, UK) into the pBabe.puro vector plasmid (H.Land, ICRF, London)(Morgenstern & Land, 1990). The transgene is expressed from the Mo-MLV 5' LTR promoter. This vector, pBabe.puroEGFP, was used in all subsequent physical, chemical and retroviral transfection experiments.

From frozen stocks of DH5α E.coli containing pBabe.puroEGFP plasmid a single colony was picked from freshly streaked selective plate and inoculated into a starter culture of 5mls of Luria Bertani (LB) medium (Tryptone 10gm + 5gm Yeast extract +10gm NaCl in 1l distilled H_20) containing ampicillin 100μg/ml. This was placed into an orbital incubator at 200rpm 37^0C for 6 hours. 5mls was then transferred to a 2l conical flask containing 500 mls LB medium with 100μg/ml ampicillin and then placed in an orbital incubator at 32^0C at 200 revs/minute overnight. Cells were harvested by centrifugation at 6000g for 15 minutes at 4^0C and the supernatant decanted. Purification of plasmid DNA was performed using Qiagen-tip 500 kits (Qiagen, Crawley, UK) according to manufacturers instructions. The cells were suspended in 10mls of buffer P1 (RNAase pre-added). 10mls buffer P2 was then added and mixed gently by inverting four times and incubated at room temperature for 5 minutes. 10mls chilled buffer P3 was added and mixed gently by inverting four times and then incubated on ice for 20 minutes. The suspension was then centrifuged at 15000G for 30 minutes at 4^0C. The supernatant containing plasmid DNA was then removed promptly. A qiagen-tip was equilibrated by applying 10mls of buffer QBT and the column allowed to empty by

gravity flow. The supernatant was then applied to a Qiagen-tip and allowed to enter the resin by gravity flow. The qiagen tip was washed twice with 30ml of buffer QC. The DNA was eluted with 15mls buffer QF. DNA was precipitated by adding 10.5 mls isopropanolol to the eluted DNA. This was mixed and centrifuged at 15,000g for 30 minutes at 4^0C. The supernatant was carefully decanted without disturbing the pellet. The pellet was air-dried for 10 minutes and then solubilised in 1ml of TE buffer. Quantification of plasmid DNA was performed as follows. 10μl of DNA was diluted in 100μl of distilled water and placed into quartz cuvette. The absorbence at 260nm was measured in a spectrophotometer. DNA quantity (μg/ml) = absorbence at 260nm x 50 x 10. Plasmid DNA was stored at –20°C prior to use.

2.2.3 Physical/chemical gene transfer techniques

2.2.3.1 Electroporation

Cells were harvested when the leukaemia cell lines were in the exponential growth phase. The cells were pelleted by centrifugation at 400g for 4 minutes and then washed with 5 mls of room temp RPMI. Following further centrifugation for 4 minutes at 400g the supernatant was discarded and 1 ml of AIM-V (Gibco-BRL) medium was added. A viable cell count was performed. A cell suspension of 10^6 cell/ml was prepared in 2400μl of AIM-V. 50μg of plasmid DNA was added to give a final concentration of 20μg/ml. 800μl of the resulting cell/plasmid DNA mixture was added to 0.4 cm gap electroporator cuvettes (EquiBio, Monchelsea, UK). Electroporation was performed

using the Easyject Plus electroporator (EquiBio) with the resistance set at infinite. Conditions were varied as shown in Table 2.2. Immediately following completion of the pulse the cell suspension was transferred to the wells of a 6-well plate and 3 mls of pre-warmed cRPMI was added. Plates were incubated for 48 hours prior to analysis of reporter gene expression.

DNA (μg)	0	10	10
Voltage (V)	260	260	260
Capacitance (μF)	1500	1500	1800

Table 2.2 Conditions for electroporation of leukaemia cell lines

2.2.2.3 Commercial transfection reagents

Evaluation of gene transfer efficiency of commercial transfection reagents was performed with cells from the four myeloid leukaemia cell lines. Cells were harvested when the cell lines were in the exponential growth phase. The cells were pelleted by centrifugation at 400g for 4 minutes and the resulting supernatant decanted. 1ml of cRPMI was added and a viable cell count performed. Gene transfer experiments for each of the commercial reagents was performed according to the manufacturers instructions. This included performing optimisation of the quantity and proportions of the transfection reagent and plasmid DNA (see Appendix I).

i/ Transfast (Promega, Southampton, UK)

A cell suspension of $5x10^5$ cells in 500µl AIM-V was prepared for each of the myeloid leukaemia cell lines. Transfast/DNA mixtures were made up in 6ml Bijoux tubes. AIM-V medium was added to the Transfast/DNA mixture to give a total volume of 500µl. The mixture was then vortexed for 10 seconds and incubated for 10 minutes. This mixture was added drop wise to the leukaemia cell suspension that had been previously seeded to the wells of a 6-well plate. After 60 minute incubation at 37^0C, 5% $C0_2$, the cells and Transfast/DNA complex were overlain with 5mls of prewarmed cRPMI. The plates were then incubated for a further 48 hours prior to analysis of reporter gene expression.

ii/ Fugene 6 (Boehringer Mannheim, Lewes, UK)

A cell suspension was prepared of $5x10^5$ cells/ml in 1600µl cRPMI. Fugene 6 was diluted to 400µl in AIM-V. The resulting mixture was then incubated for 5 minutes at room temperature. Plasmid DNA was added to a second bijoux tube. The diluted Fugene 6 was then added drop wise onto the plasmid DNA. The tube was tapped gently to mix contents and incubated for 15 minutes at room temperature. This mixture was then added drop wise to the cell suspension previously seeded to the wells of a 6-well plate. The plates were then incubated for 48 hours with the Fugene 6/DNA complex left with cells until analysis of reporter gene expression.

iii/ Effectene (Qiagen)

A cell suspension was prepared of $5x10^5$ cells/ml in 1600µl cRPMI. Plasmid DNA was diluted in 100µl buffer EC in a bijoux tube. Enhancer was added at a 1:8 ratio and the resulting solution was mixed by vortexing for 10 seconds. Following incubation at room temperature for 2-5 minutes the Effectene reagent was added to DNA/enhancer solution and mixed by vortexing for 10 seconds. The resulting mixture was then incubated for 10 minutes at room temperature. 600 µl AIM-V was added to transfection complexes and mixed by pipetting up and down twice. Transfection complexes were than added drop wise onto the cell suspension that had been previously seeded into wells of a 6-well plate. The plates were then incubated for 48 hours with the transfection complexes left in contact with cells until analysis of reporter gene expression.

iv/ Superfect (Qiagen)

A cell suspension was prepared of $5x10^5$ cells/ml in 2mls cRPMI. Plasmid DNA was diluted in 100µl AIM-V medium in a bijoux tube. The Superfect transfection reagent was then added to the DNA and the tube vortexed for 10 seconds. The mixture was incubated for 5 minutes at room temperature to allow complex formation. The Superfect/DNA complex was then added drop wise onto the cell suspension that had previously been seeded into the wells of a 6-well plate. The cells and Superfect/DNA complex were than incubated for 3 hours. The cells were then pelleted by centrifugation at 400g for 4 minutes and resuspended in 3 mls cRPMI. The cell suspension was

transferred to the wells of a 6-well plate, which was incubated for 48 hours prior to analysis of reporter gene expression.

2.2.4 Retroviral vector system

2.2.4.1 Derivation of retroviral producer cell lines

The first step in the generation of stable producer cell lines was the transfection of the packaging cell lines with the desired retroviral vector, either pBabe.puroEGFP or the 'empty' pBabe.puro vector. This was performed using the commercial transfection reagent, Superfect. The three packaging cell lines TeflyRD114, TeflyGA and TeflyMLV-A were grown to 40% confluence in a 25cm^2 flask containing 5 mls of cDMEM. Prior to transfection the monolayer was washed with 5 mls of sterile tissue culture grade PBS. The plasmid DNA/Superfect transfection complex was prepared as follows; 10 μg of plasmid DNA (the pBabe.puroEGFP vector or the 'empty' pBabe.puro vector) was diluted in 150μl of AIM-V medium. To this was added 30μl of Superfect and the resulting mixture was vortexed for 10 seconds. Following 5 minutes incubation at room temperature to allow complex formation, 2mls of cDMEM was added to tube and mixed by vortexing. The Superfect/DNA complex was then added to 25 cm^2 flasks containing the packaging cell lines. The flasks were then incubated for 4 hours. The supernatant was then decanted and the monolayer washed twice with sterile tissue culture grade PBS. 5mls of cDMEM was added to the flask and the cells were allowed to grow until they had reached confluence. Once this was achieved, usually 48-72 hours

following the initial transfection, the medium was replaced with cDMEM containing puromycin 1μg/ml (Sigma-Aldrich, Gillingham, UK). Medium was replaced every 3-4 days with cDMEM containing puromycin 1 μg/ml. Cells were maintained in selective medium until a confluent monolayer of puromycin resistant cells was achieved. The monolayer was then washed with 5 mls of sterile tissue culture PBS. The PBS was then aspirated and 2mls of 0.1% trypsin was added. After 30 seconds 1.5mls of the trypsin solution was aspirated and cells were incubated for a further 2 minutes at which point the monolayer would be starting to lose adherence. The flask was then tapped lightly to fully detach the cells. 5mls of cDMEM was added and the resulting cell suspension was centrifuged at 400g for 4 minutes. 1ml of cDMEM was added and a viable cell count was performed. These cells were used to reseed a fresh $25cm^2$ flask containing 5 mls of cDMEM and puromycin 1 μg/ml. Once cells were approaching confluence the supernatant was decanted and the monolayer washed twice with PBS. 4 mls of cDMEM was added. The supernatant was collected daily and replaced with 4 mls cDMEM. The harvested supernatant was centrifuged at 400g for 4 minutes and then passed through a 45μm filter. This was then stored at -70^0 C. This supernatant containing retroviral virions was used to infect newly generated producer cell lines carrying one of the other two envelope proteins specificities as a means of enhancing the titre, a process known as shuttling i.e. retroviral supernatant from TeflyGALV.EGFP was used to infect TeflyRD114.EGFP. This was performed by replacing the medium for a 25-40% confluent producer cell line with neat retroviral supernatant (as prepared above) to which was added polybrene 8 μg/ml (Sigma-Aldrich). After 4 hours incubation the

medium was replaced with cDMEM and the flask was incubated for a further 48 hours. Medium was then replaced with cDMEM containing puromycin 1 μg/ml. This whole process was then repeated using retroviral supernatant from the other producer cell line in order to generate high titre retroviral producer cell lines (Figure 2.1).

2.2.4.2 Production of retroviral vector stock

High titre producer cell lines were used for generation of retroviral vector stock. 10^5 cells were used to seed a 25 cm^2 flask containing 5 mls of cDMEM with puromycin 1 μg/ml. Medium was replaced every 3-4 days with cDMEM containing 1μg/ml puromycin. Once the monolayer was fully confluent the cells were harvested as described above and a viable cell count was performed. 5 x 10^6 cells were used to reseed a 75 cm^2 flask in 10 mls cDMEM. Once the monolayer was approaching 70-80% confluence the medium was replaced with 5mls cDMEM. The flasks were incubated for 24 hours and the supernatant was decanted into a 15 mls conical tube and replaced with 5mls cDMEM. The harvested supernatant, containing the retroviral particles, was centrifuged at 400g for 4 minutes and then passed through a 45μM filter. The retroviral supernatant was either used fresh or stored at -70^0C prior to use. Small volume aliquots of supernatant were taken for subsequent titration. This process was repeated on a daily basis until full confluence was reached.

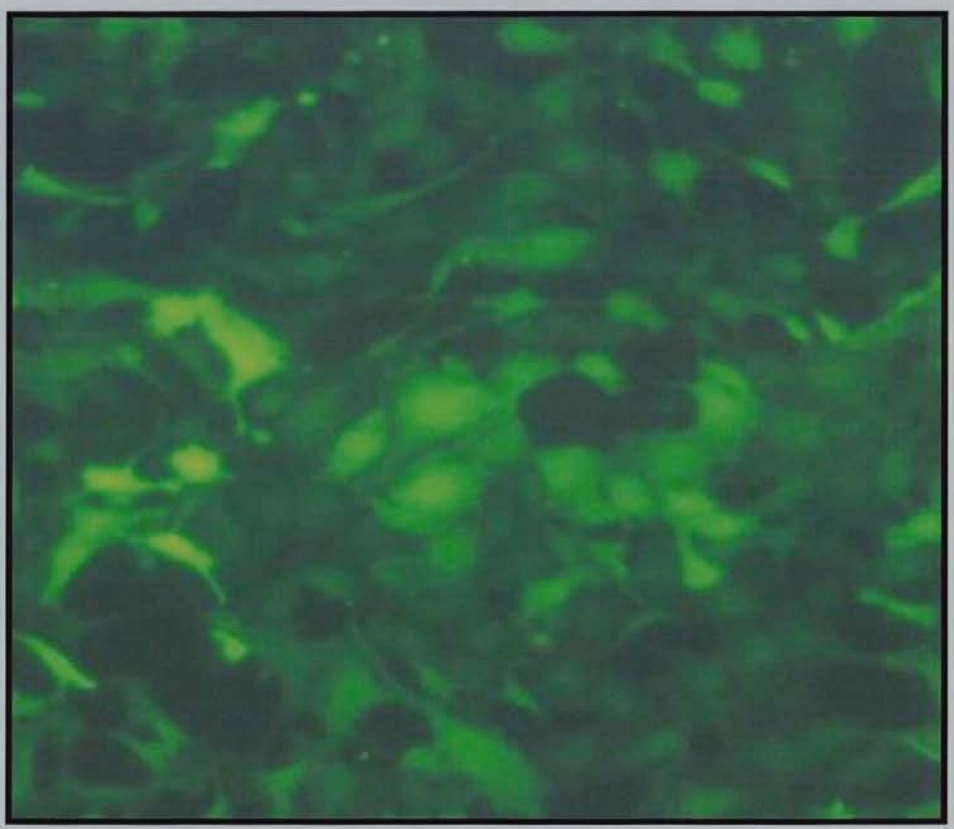


Figure 2.1 Retroviral producer cell line.
Photographs taken under fluorescence microscopy demonstrating enhanced GFP expression by the retroviral producer cell line, Tefly.RD114 packaging the retroviral vector pBabe.puroEGFP

2.2.4.3 Concentration of retroviral vector stock

In order to obtain higher titres of retroviral vectors two methods of concentration were used. The first of these employed was the conventional method of concentration, by ultracentrifugation. The second was a novel concentration method that had been suggested by David Darling (King's College School of Medicine and Dentistry, London) following his observations that mixing retroviral particles with Pansorbin lead to an enhancement in their titre.

i/ Ultracentrifugation

Retroviral vector stock was produced as described above. 25mls of freshly harvested pooled retroviral vector stock was added to 40ml sterile round bottomed tubes. The side of the tube where the retroviral particles were expected to pellet was marked. The tubes were then centrifuged for 2hrs 20 minutes at 20,000 rpm (SW-27 rotor) at 4^0C. The supernatant was decanted whilst carefully avoiding discarding the pellet. The pellet was then resuspended in 500µl DMEM. The resulting solution was stored at -70^0C. Titration was performed as described below by the gene transfer unit assay on pooled unconcentrated and concentrated retroviral vector stock.

ii/ Pansorbin Method

Retroviral vector stock was produced as described above. Freshly harvested pooled vector stock was used for concentration. 50µl of Pansorbin 5% (Pansorbin 5% in RPMI) (CN Biosciences, Nottingham, UK) was added to 10mls of retroviral supernatant. The

mixture was incubated for 3 hours at 4^0C on a roller. The Pansorbin and retroviral particles were then harvested by centrifugation at 500g for 10 minutes. The resulting pellet was resuspended in 500µl DMEM and stored at -70^0C until required. Titration of pooled vector stock and of pansorbin concentrated vector stock was performed as described below using the gene transfer unit assay.

2.2.4.4 Titration of retroviral vector stock

For titration of retroviral vector stock two main assays were used. The colony forming assay is based on the stable transduction of the retroviral vector into cells leading to the expression of a selectable marker; puromycin resistance in the case of the vectors used in this study. Following a period of culture, under the selective pressure of puromycin, new colony growth appears. The titre is calculated by the number of transduced colonies at a given dilution of retroviral stock, expressed as colony forming units (CFU)/ml. This assay proved to be laborious and poorly reproducible. Therefore for titration of EGFP containing retroviral vector stock an assay based on the flow cytometric analysis of reporter gene expression was adopted. This assay proved to be reliable and reproducible and had the added advantage of given rapid titration results. MOI calculations were based upon the results of titrations performed by this gene transfer unit (GTU) assay. Both assays are described in more detail below.

i/ Colony-forming unit assay

The target cell line TE6.71 was grown in cDMEM until confluence in a $25cm^2$ flask. Once at confluence the tissue culture supernatant was decanted and the monolayer was washed with tissue culture grade PBS. 2mls of trypsin 0.1 % were then added. After 30 seconds 1.5 mls of the trypsin solution was aspirated. The flask was then returned to a 37^0 C incubator. When the cells were started to lose adherence the flask was tapped on the bench to detach the monolayer and 3.5 mls of cDMEM were added. From the resulting cell suspension 100μl was seeded into the wells of a 6-well plate to which 2mls of cDMEM was then added. The plate was then incubated for 24hrs. Retroviral vector stock for titration was thawed at 37^0C and serial dilutions were made in cDMEM supplemented with 8μg/ml polybrene. 500μl of each dilution were added to wells of the 6-well plate containing the TE6.71 target cell, which was now at 40-60% confluence. The plate was then incubated for 3 hours. 2 mls of cDMEM was overlain onto the retroviral supernatant. Plates were incubated for a further 2 days until the monolayer had reached confluence. The medium was then aspirated and 2mls of cDMEM supplemented with 1μg/ml puromycin was added. The plates were returned to the incubator. Every 2-3 days the medium was replaced with fresh cDMEM supplemented with puromycin 1μg/ml. When colonies became readily identifiable by microscopy the wells were stained with Giemsa (BDH, Poole, UK). The number of colonies in each well were counted with the retroviral vector stock titre (CFU/ml) = number of colonies per well x dilution factor x 2

ii/ Gene transfer unit assay

The cells from the target cell line were seeded into the wells of a 6-well plate as described above in the CFU assay. Once the monolayer had reached 40%-60% confluence infection with the retroviral vector stock was performed. From two of the wells the cells were harvested, following trypsinisation, and a viable cell count performed. Retroviral vector stock for titration was thawed at 37^0C and serial dilutions were made in cDMEM supplemented with 8μg/ml polybrene. 500μl from each of three dilutions was added to the wells of the 6-well plate containing the TE6.71 target cell. To the final well was added neat retroviral supernatant harvested from the producer cell lines containing the 'empty' pBabe.puro vector. The plate was then incubated for 4 hours at 37^0 C. 4 mls of cDMEM was then overlain over the retroviral supernatant and the plates incubated at 37^0C, 5%CO_2. Following 48 hours incubation cells were harvested and analysed for GFP expression by flow cytometry. Cells harvested from the well that had been infected with the 'empty' pBabe.puro vector acted as a negative control. Titre (GTU/ml) = % cells expressing GFP/100 x dilution factor x 2 x average cell number from two wells

2.2.4.5 Fibronectin coating of plates

Enhancement of retroviral vector GTE into haematopoietic cells has been achieved by using recombinant fibronectin fragments, which leads to co-localisation of the retrovirus with the target cells (Hanenberg *et al*, 1996). In an attempt to improve the GTE into the leukaemia cell lines 6-well plates were coated with plasma-derived fibronectin. This was performed as follows: 1ml of RPMI was added to the wells of 6-well plate. To this was added 100µl of fibronectin (cold immununoglobulin fraction PFC, SNBTS, Edinburgh) at a concentration of 500µg/ml. The plates were left for 30 minutes at room temperature. Medium was then aspirated from the wells and the plates were wrapped in cellophane and stored at -40^0C prior to use.

2.2.4.6 Infection of myeloid leukaemia cell lines

Cells were harvested when leukaemia cell lines were in exponential growth phase. Cells were pelleted by centrifugation at 400g for 4 minutes and then washed with 5 mls RPMI. Following further centrifugation at 400g for 4 minutes cells were resuspended in 1 ml of RPMI and a viable cell count was performed. 2.5 x10^5 cells were suspended in retroviral supernatant, at a MOI of 10, with added polybrene (4µg/ml) and then added to the wells of a 6-well plate. For control infections cells were resuspended in retroviral supernatant containing the 'empty' pBabe.puro vector. After 4 hours incubation cells were pelleted by centrifugation at 400g for 4 minutes and resuspended in 3 mls complete RPMI and reseeded into the wells of a 6-well plate. Following 48 hours incubation cells were harvested for analysis of reporter gene expression by flow cytometry.

2.2.4.7 Infection of primary AML blasts

Freshly thawed primary AML blasts were washed with 5mls RPMI and then centrifuged at 400g for 4 minutes. The cells were resuspended in 1ml cRPMI and a viable cell count was performed. Cells were then resuspended in cRPMI to which was added GM-CSF 200ng/ml (Schering-Plough, Welwyn Garden City, UK) and IL-3 20ng/ml (R&D systems, Abingdon, UK). Following culture for 48 hours cells were harvested by centrifugation at 400g for 4 minutes and resuspended in 1ml RPMI. A viable cell count was performed. 2.5 $x10^5$ cells were taken and resuspended in retroviral supernatant, at a MOI of 10, with added polybrene (4μg/ml) and then added to the wells of a 6-well plate. For control infections cells were resuspended in retroviral supernatant containing the 'empty' pBabe.puro vector. After 4 hours incubation cells were pelleted by centrifugation at 400g for 4 minutes and resuspended in 3 mls complete RPMI and reseeded into the wells of a 6-well plate. Following 48 hours incubation cells were harvested for analysis of reporter gene expression by flow cytometry.

2.2.5 Adenoviral vector system

2.2.5.1 Production of adenoviral vector stock

HEK 293 cells were seeded into a $25cm^2$ flask in 5 mls cDMEM. Medium was replaced every three to four days. When the monolayer was at 90-95% confluence cells were infected with either the adenovirus vector AdGFP (D.Curiel, University of Alabama, USA) or the control 'empty' vector, Ad5ΔE1a, (F.Farzaneh, King's College School of Medicine and Dentistry, London) diluted in 2 mls cDMEM to give a MOI of 20. Viral absorption was performed for 90 minutes at 37^0C, 5% CO_2. The medium then replaced with cDMEM. After 24-48 hours cells began to show the typical cytopathic effect caused by intracellular adenoviral replication (Figure 2.2). Cells and supernatant were harvested once greater than 50% of the monolayer had become detached. The cell suspension was transferred in 14 ml round bottomed tubes to a -70^0 freezer. The tubes were left for one hour and then thawed rapidly to 37^0C in a water bath. The tubes were then returned to the freezer and the process repeated again for a total of three freeze thaw cycles. The lysed cells were pelleted by centrifugation at 400g for 4 minutes. The supernatant was aspirated and then passed through .45μm filter. Small volume aliquots of supernatant were taken for subsequent titration. The adenoviral vector stock was stored at -70^0C prior to use.

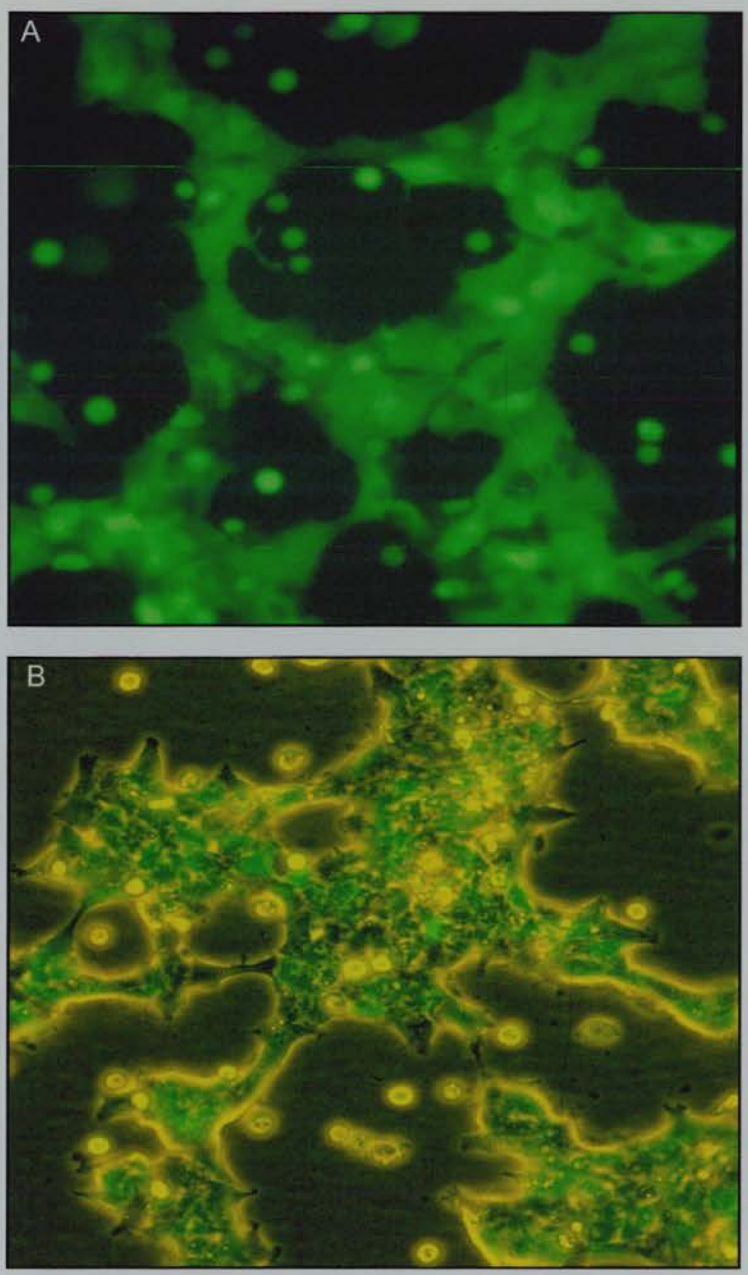


Figure 2.2 Adenoviral vector propagation in the HEK 293 cell line.
A. Photograph taken under fluorescence microscopy demonstrating GFP expression by the infected 293 cells
B. In combination with low white light levels demonstrating the cytopathic effect induced by the intracellular adenoviral multiplication

2.2.5.2 Titration of adenoviral vector stock

The plaque-forming unit (PFU) assay was used in order to confirm the presence of adenovirus in vector stock harvested from HEK293 cells infected with the control Ad5ΔE1a vector and to ensure that the titre was broadly comparable with the titres obtained using the same assay for AdGFP vector stock. For the purposes of establishing MOI for comparative gene transfer experiments using AdGFP vector stock the GTU assay was used.

i/ Plaque-forming unit assay

The target cell line HEK 293 was grown in cDMEM until confluence in a $25cm^2$ flask. Once at confluence the tissue culture supernatant was decanted and the monolayer was washed with tissue culture grade PBS. 2mls of trypsin 0.1 % were then added. After 30 seconds 1.5 mls of the trypsin solution was aspirated and the flask returned to the incubator. When the cells were started to lose adherence the flask was tapped on the bench to detach the monolayer and 3.5 mls of cDMEM were added. From the resulting cell suspension 100µl was seeded into the wells of a 6-well plate to which 2mls of cDMEM was then added. The plate was then incubated 48 hours. Serial dilutions of adenovirus vector stock were made in cDMEM. 500µl of each dilution was overlain onto HEK 293 cells at 70% confluence. Plates were incubated for 2 hours at 37^0C following which the viral inoculum was removed by aspiration. Molten 1% agarose/DMEM was prepared by microwaving Seaplaque (3% in distilled H_2O) (BioWhittaker, Wokingham, UK) for 5 minutes, which was then allowed to cool to

37^0C. This was then mixed with prewarmed cDMEM at a ratio of 1:3. 2mls of the agarose/DMEM mixture was then overlain over the HEK 293 cells. The agarose/medium was allowed to set at room temperature for 20 minutes following which it was overlain with 2mls cDMEM. The plates were then incubated for between 4-7 days to allow formation of cytopathic plaques. 1ml of neutral red stain (0.1% in distilled H_20)(BDH) was added into the medium and plates left for 2 hours. The supernatant was then decanted and the plates were left overnight at 4^0C. Viral plaques were counted for each well with the adenoviral vector stock titre (PFU/ml) = number of plaque per well x 2 x dilution factor.

ii/ Gene transfer unit assay

The cells from the target cell line, TE6.71, were seeded into the wells of a 6-well plate. Once the monolayer had reached 40%-60% confluence infection with the AdGFP vector stock was performed. From two of the wells the cells were harvested, following trypsinisation, and a viable cell count performed. AdGFP vector stock for titration was thawed at 37^0C and serial dilutions were made in cDMEM. 500µl from each of three dilutions was added to the wells of the 6-well plate containing the TE6.71 target cell. To the final well was added neat vector stock containing the 'empty' adenovirus vector, Ad5ΔE1a. The plate was then incubated for 90 minutes at 37^0 C. The supernatant was then aspirated and 4 mls of cDMEM overlain over the cells. The plates were incubated at 37^0C, 5%CO_2 for 48 hours following which the cells were harvested (following trypsinisation) and analysed for GFP expression by flow cytometry. Cells harvested

from the well that had been infected with the 'empty' adenovirus vector acted as a negative control. Titre (GTU/ml) = % cells expressing GFP/100 x dilution factor x 2 x average cell number from two wells

2.2.5.3 Infection of myeloid leukaemia cell lines

Cells were harvested when leukaemia cell lines were in exponential growth phase. Cells were pelleted by centrifugation at 400g for 4 minutes and then washed with 5 mls RPMI. Following further centrifugation at 400g for 4 minutes cells were resuspended in 1 ml of RPMI and a viable cell count was performed. 2.5 x 10^5 cells were taken and suspended in 500μl complete RPMI to which was added AdGFP at MOI 40, or control adenovirus, with polybrene (4μg/ml) and placed in 12x75mm plastic tubes. After 90 minutes incubation at 37^0 C cells were washed with 5mls RPMI and then pelleted by centrifugation at 400g for 4 minutes. The cells were then resuspended in 3 mls cRPMI and transferred to the wells of a 6-well plate which was incubated for a further 48 hours prior to analysis of reporter gene expression by flow cytometry.

2.2.5.4 Infection of primary AML blasts

Freshly thawed primary AML blasts were washed with 5mls RPMI and then centrifuged at 400g for 4 minutes. The cells were resuspended in 1ml cRPMI and a viable cell count was performed. Cells were then resuspended in cRPMI to which was added GM-CSF 200ng/ml and IL-3 20ng/ml or cRPMI with added GM-CSF 200ng/ml, IL-4 20ng/ml (R&D systems) and CD40L 1μg/ml (Alexis corporation, Nottingham, UK). Primary

AML blasts were cultured for 48 hours with the GM-CSF/IL-3 cytokine combination and 7 days with the GM-CSF/IL-4/CD40L combination prior to infection. The cells were then harvested by centrifugation at 400g for 4 minutes and resuspended in 1ml RPMI. A viable cell count was performed. 2.5 x 10^5 cells were taken and suspended in 500µl complete RPMI to which was added AdGFP at a MOI of 40, or control adenovirus, with polybrene (4µg/ml) and placed in 12x75mm plastic tubes. After 90 minutes incubation at 37^0 C cells were washed with 5mls RPMI and then pelleted by centrifugation at 400g for 4 minutes. The cells were then resuspended in 3 mls cRPMI and transferred to the wells of a 6-well plate which was incubated for a further 48 hours prior to analysis of reporter gene expression by flow cytometry.

2.2.6 Flow cytometric analysis of gene transfer efficiency

Transfected cells either target cell lines, myeloid leukaemia cells or primary AML blasts, were analysed for GFP expression by flow cytometry in an identical manner. Cells were harvested and then centrifuged at 400g for 4 minutes. The supernatant was discarded and the cells resuspended in 1 ml PBS. A viable cell count was performed. Cell density was adjusted to 2 x 10^6/ml with PBS and 100µl of the resulting cell suspension was added to 12x75mm plastic tubes. Cells were then analysed using either a FACSort or a FACSCalibur flow cytometer (Becton Dickinson, USA). Control cells that had been transfected either in the absence of plasmid DNA for the purposes of the physical and chemical transfection methods, or with 'empty' vectors for the viral systems were run in parallel. Non-viable cells were excluded from analysis on the basis

of forward and side scatter characteristics. Data was then analysed using WinMDI software. For experiments using enhanced GFP containing vectors, markers were set on the FL1 channel to include 99.8% of the control cell population. The GFP contained in the AdGFP vector was not enhanced and the use of such a stringent exclusion criteria for positivity generated an unacceptably high false negative rate. Therefore for this vector markers were set to include 99% of the control cell population. GFP expression was recorded as the percentage of the viable cell population exceeding the upper boundary of negative control marker.

2.2.7 Generation of stably transduced cell lines

2.2.7.1 Puromycin dose response curves

In order to determine the optimal concentration of puromycin for selection of retrovirally transduced AML cell line clones puromycin dose response curves were generated based on cell viability following treatment with puromycin. A MTT (Sigma-Aldrich) stock solution of 5mg/ml in RPMI-1640 was prepared, filtered through a .45μm filter and stored at -40^0C prior to use. The MTT conversion at various cell numbers was established before performing the puromycin dose response assays. Serial $\log_{10}$ dilutions of cells (10^6-10^3) from each of the four leukaemia cell lines were made. In triplicate 100μl was added to wells of a 96-well plate. 10μl MTT solution was added to each well and the plate incubated at 37^0C, 5% CO_2 for 4 hours. 100 μl of acidic isopropanolol (0.1M HCL) was added to wells and titurated to solubilise the converted dye.

Absorbence of converted dye was measured at 540nm with subtraction at 620nm using an ELISA plate reader (multiskan MCC/340 P version 2.20). A graph of MTT conversion according to cell number was generated by plotting cell number versus absorbence on a linear scale. For the puromycin dose response curves 10^7 leukaemia cells in cRPMI were seeded into the wells of a flat-bottomed 96-well plate. Doubling dilutions of puromycin ranging from 20 to 0.625μg/ml were added to the wells. The plates were then incubated for 48 hours at 37^0C. 10μl MTT was added to each well and the plates were incubated for a further 4 hours. 100 μl of acidic isopropanolol (0.1M HCL) was added to each well and tituration was performed in order to solubilise the converted dye. Absorbence of the converted dye was measured at 540nm with subtraction at 620nm using an ELISA plate reader. Plotting puromycin concentration versus cell number on a linear scale generated dose response curves. The lowest puromycin concentration that achieved a three-$\log_{10}$ reduction in cell number was used for selection of retrovirally-transduced clones. For the K562 cell line the experiment was repeated with higher concentrations of puromycin, as a three-$\log_{10}$ reduction in cell number was not achievable over the initial dose range.

2.2.7.2 Infection and selection of retrovirally transduced clones

Transduction of leukaemia cells was performed with the pBabe.puro vectors containing cDNA for CD40, CD80 and IL-2 respectively with the vectors packaged by the TeflyRD114 cell line. Cells from the leukaemia cell lines were harvested when in exponential growth phase. $5x10^5$ cells were suspended in 1 ml of retroviral supernatant

supplemented with polybrene at 8μg/ml and added to the wells of a 6-well plate. Following 4 hours incubation at 37^0C the cells were transferred to a 15mls conical tube and centrifuged at 400g for 4 minutes. The supernatant was removed and 3mls of cRPMI was added. After a further 48 hours culture the cells were harvested and then centrifuged at 400g for 4 minutes. The supernatant was decanted and replaced with 1 ml of cRPMI. A viable cell count was performed and cell suspensions of 5×10^4 and 5×10^3 cells/ml were made up in universal containers in 20mls cRPMI supplemented with puromycin at the predetermined optimal concentration. 200μl of the cell suspension were added to the wells of a flat-bottomed 96-well plate, with each plate corresponding to a different cell concentration. The plates were then incubated for 7-10 days until colony formation became apparent. The contents of wells containing single colonies were then transferred to a 24-well plate for further expansion in puromycin containing medium. Following further expansion in selective medium the transduced clones were transferred to wells of 6-well plates. Individual clones were then tested for the success of retroviral vector transduction by flow cytometric analysis following staining with appropriate mAb. Clones with high level of expression of the desired gene were selected for further expansion.

2.2.7.3 Analysis of transgene expression

i/ Intracellular staining for IL-2

In order to establish the methodology for measurement of intracellular IL-2 production experiments were initially performed using activated PBMC as a positive control.

PBMC derived from 10mls of PB taken from a healthy volunteer donor were separated by centrifugation over Lymphoprep using the method as previously described for harvesting of primary AML blasts. Cells at the interface were aspirated and transferred to a 15mls conical tube, washed with 5 mls cRPMI and then centrifuged at 400g for 4 minutes. The supernatant was decanted and cells washed again with 5mls cRPMI. Following further centrifugation at 400g for 4 minutes cells were resuspended in 5 mls cRPMI in a $25cm^2$ flask and incubated overnight at 37^0C. Non-adherent cells were harvested, centrifuged at 400g x 4 minutes and resuspended in 5 mls cRPMI to which was added phytohaemagglutinin (PHA) 1μg/ml, and phorbol myristate acetate (PMA) 50ng/ml (both Sigma-Aldrich) and added to a $25cm^2$ flask which was incubated for 24 hours. Monensin 2μM (GolgiStop™, Pharmingen, High Wycombe, UK) was added for the final 4 hours of culture period. Cells were then harvested, centrifuged at 400g for 4 minutes and the supernatant decanted. The cell pellet was resuspended in 3 mls PBS, centrifuged again at 400g for 4 minutes. The supernatant was decanted and 50μl PBS was added. The cells were permeabilised using a commercial permeabilisation solution (Fix and Perm™, Caltag, Buckingham, UK) according to the manufacturers instructions. Briefly 100μl reagent A was added to 50μl cell suspension in 12x75mm plastic tube.

Following 15 minutes incubation at room temperature the cells were washed with 5 mls PBS and pelleted by centrifugation at 400g for 4 minutes. The supernatant was decanted and 100µl reagent B added together with 20µl anti-IL2-PE (Sigma-Aldrich) and 10µl anti-CD69-FITC (Caltag) and isotype matched controls. Following 30 minutes incubation at room temperature the cells were washed with 5 mls PBS. The cell suspension was centrifuged at 400g x 4 minutes, supernatant decanted and the cell pellet resuspended in 200µl PBS. Cells were analysed by flow cytometry on a FACSort flow cytometer. Negative controls were run in parallel with conditions altered as summarised in Table 2.3

Unstimulated control	Activation control	Intracellular IL-2
No PMA/PHA	PHA/PMA	PHA/PMA
+ Monensin	No monensin	+ monensin
Fixed and permeabilised	Fixed only	Fixed and permeabilised
Isotype controls	Isotype controls	Isotype controls
CD69-FITC/CD3-PE	CD69-FITC/CD3-PE	CD69-FITC/CD3-PE
CD69-FITC/IL2-PE		CD69-FITC/IL2-PE

Table 2.3 Experimental controls for the intracellular IL-2 assay

ii/ Cell surface expression of costimulatory molecules

Expression of cell surface molecules by stably transduced cell lines was confirmed by flow cytometric analysis following staining of cells with the appropriate mAbs.

2.3 Immunophenotypic analysis of human myeloid cells

Myeloid cells underwent immunophenotypic analysis using a panel of mAb directed against costimulatory molecules, HLA molecules, dendritic cell-associated markers, CD54 (ICAM-1) and CD95 (Fas) prior to and following attempted dendritic cell differentiation (Table 2.4). Cells were harvested and pelleted by centrifugation at 400g for 4 minutes. The supernatant was decanted and blocking solution made up of 0.5% human normal immunoglobulin solution (SNBTS, Edinburgh, UK) with 0.1% sodium azide (Fisher Scientific, Loughborough, UK), was added to give a cell suspension of 5 x 10^6 cells/ml. Following 30 minutes incubation at 4^0C cells were pelleted by centrifugation at 400g for 4 minutes and 1 ml of PBS was added. A viable cell count was performed and $2x10^5$ cells were taken and added to 12x75mm plastic tubes. Cells were either single stained by adding 10 µl of mAb directed against CD1a, CD54, CD80, CD86, CD95, HLA class II all fluorescein isothiocyanate (FITC)-conjugated (all Serotec, Oxford, UK) or dual stained by adding 10 µl of mAb directed against FITC-conjugated CD40 (Serotec) and 20 µl of phycoerythrin (PE)-conjugated CD83 (Beckman Coulter, High Wycombe, UK). PBS was added to give a final volume of 100µl. Tubes were then incubated for 30 minutes at 4^0C. The cells were washed with 3 mls PBS and then pelleted by centrifugation at 400g for 4 minutes. The supernatant was decanted and 100µl phosphate buffered saline (PBS) containing 2µg/ml propidium iodide (PI)(Bender MedSystems, Middlesex, UK) was added. After 5 minutes incubation analysis was performed on a FACSCalibur flow cytometer. Viable cells were defined with a forward scatter (FS)/PI gate.

CD marker or name	Brief description of function
CD1a	Dendritic cell associated marker-involved in presentation of lipid antigen to T cells
CD83	Dendritic cell associated marker-highly restricted to mature DC populations. May have a role in mediating adhesion to monocytes and a subset of activated and/or stressed T cells
CD40	Costimulatory molecule-when bound by CD40 ligand, present on T cells, induces the upregulation of costimulatory and HLA molecules
CD54 (ICAM-1)	Cell adhesion molecule involved in binding to T cells via LFA-1
CD80 (B7.1)	Costimulatory molecule-binding to CD28 provides second signal for T cell activation
CD86 (B7.2)	Costimulatory molecule-binding to CD28 provides second signal for T cell activation
CD95 (Fas)	Receptor for Fas ligand. Induces apoptosis in susceptible cells on binding by Fas ligand
HLA I	MHC molecule involved in antigen presentation to CD8+ T lymphocytes
HLA II	MHC molecule involved in antigen presentation to CD4+ T lymphocytes

Table 2.4 Panel for the immunophenotypic analysis of leukaemia cells

2.4 Induction of differentiation in human myeloid cells

2.4.1 Cytokine-induced differentiation

2.4.1.1 Peripheral blood monocytes

Buffy coats were obtained from whole blood given by healthy volunteer blood donors at the SEBTS blood donor center (Edinburgh). The buffy coat was mixed at a 1:1 ratio with cRPMI and then centrifuged at 650g for 25 minutes over Lymphoprep at a 4:1 ratio. The cells at the interface were aspirated and resuspended in 5 mls cRPMI medium in 15mls conical tubes. The cells were pelleted by centrifugation at 400g for 4 minutes and washed again with 5 mls cRPMI medium. Following further centrifugation at 400g for 4 minutes the supernatant was decanted and 10 mls of cRPMI added. The resultant cell suspension was transferred to a $75cm^2$ flask and incubated overnight. The non-adherent cells were decanted and 10mls of cRPMI supplemented with IL-4 20ng/ml and GM-CSF 200ng/ml was added. The flask was then incubated for a further 4 days at which point there was morphological evidence of DC differentiation. Cells were harvested and washed with 5 mls cRPMI. Cells were pelleted by centrifugation at 400g for 4 minutes and resuspended in 1 ml of cRPMI. A viable cell count was then performed. 5×10^6 cells were taken and used for immunophenotypic analysis as described above. The remaining cells were resuspended at 10^6/ml in cRPMI supplemented with GM-CSF 200 ng/ml and IL-4 20 ng/ml. Analysis of DC maturation was performed by seeding 10^6 cells in 1 ml of medium into the wells of a 24-well plate.

To each well was then added various concentrations of CD40L ranging from 1-1000 ng/ml either with or without a proprietary enhancer (Alexis corporation). The plate was then incubated for 4 days. Cells were harvested and pelleted by centrifugation at 400g for 4 minutes. Immunophenotypic analysis of the expression of CD40, CD54. CD80, CD86 and HLA class II molecules was performed as described above.

2.4.1.2 Myeloid leukaemia cell lines

Cytokine-induced differentiation was attempted in the four leukaemia cell lines and in the CD40 expressing leukaemia cells generated by the retroviral transduction of the K562, KG1a and U937. Cells were harvested when leukaemia cell lines were in exponential growth phase. Cells were pelleted by centrifugation at 400g for 4 minutes and then washed with 5 mls RPMI. Following further centrifugation at 400g for 4 minutes cells were resuspended in 1 ml of RPMI and a viable cell count was performed. 3 x 10^6 cells were added to the wells of a 6-well plate to which was added cRPMI supplemented with GM-CSF 200 ng/ml and IL-4 20 ng/ml and CD40L 1 μg/ml Following incubation for 4 days cells were harvested and pelleted by centrifugation at 400g for 4 minutes. The supernatant was discarded and the cells were resuspended in 3 mls of cRPMI supplemented with GM-CSF 200 ng/ml, IL-4 20 ng/ml and CD40L 1μg/ml or TNF-α 15 ng/ml. The resulting cell suspension was returned to the wells of the 6-well plate. Following 3 days culture cells were harvested and analysed for evidence of dendritic cell-like differentiation by flow cytometry as described above.

2.4.1.3 Primary AML blasts

Freshly thawed primary AML blasts were washed with 5mls RPMI and then centrifuged at 400g for 4 minutes in a 15mls conical tube. The cells were resuspended in 1ml cRPMI and a viable cell count was performed. 3 x 10^6 cells were then resuspended in 3 mls cRPMI supplemented with GM-CSF 200 ng/ml and IL-4 20 ng/ml and transferred to the wells of a 6-well plate. The plate was incubated for 4 days. The cells were then harvested and pelleted by centrifugation at 400g for 4 minutes. The cells were resuspended in 3mls cRPMI supplemented with GM-CSF 200 ng/ml and IL-4 20 ng/ml and CD40L 1μg/ml or TNFα 15ng/ml. The resulting cell suspension was returned to the wells of the 6-well plate and incubated for a further 3-7 days. Cells were harvested and used either for flow cytometric analysis, as described above, or as stimulators in allogeneic mixed leukaemia lymphocyte reactions (MLLR), or to generate leukaemia-specific CTL or for FISH analysis, as described below. Supernatant from cultures in which CD40L had been given for the final 3 days of the culture period were aspirated and stored at -70^0 C prior to measurement of IL-12(p70) concentration.

Analysis of various maturational agents was performed using leukaemia cells from a single AML case. Leukaemia cells were cultured in an identical manner for the first 4 days of the culture period. Cells were then harvested and then resuspended in 3mls cRPMI supplemented with either GM-CSF/ IL-4 alone or with various combination of Polyriboinosinic polyribocytidylic acid (polyI:C) (Sigma-Aldrich), CD40L and a proprietary enhancer. After a further 72 hours culture the cell suspension was harvested and centrifuged at 400g for 4 minutes. Supernatant was aspirated and stored at -70^0 prior

to measurement of IL-12(p70) concentration and the cells were used for flow cytometric analysis of costimulatory molecule expression.

2.4.2 FISH analysis

To determine whether the DLLC were derived from the leukaemic clone two AML cases that possessed chromosomal rearrangements detectable by FISH were selected for further study. Primary AML blasts were induced to differentiate to DLLC as described above. Confirmation of DLLC differentiation was performed by flow cytometric analysis following staining with the appropriate mAb as described above. In one case unselected leukaemia cells were used to make cytospin preparations. In the second AML case DLLC were positively selected based on their expression of CD83 as follows: $5x10^6$ cells were incubated with 20µl of anti-PE microbeads (Miltenyi Biotec, Surrey, UK) in 100µl PBS for 30 minutes at room temperature. Cells were then washed with 5 mls PBS and centrifuged at 400g for 4 minutes. The supernatant was decanted and the cells resuspended in 500µl PBS. The resulting cell suspension was run through a miniMACS column placed within a miniMACS magnet (Miltenyi Biotec). 1ml of PBS was run through the column to ensure complete removal of negative cells. The column was then removed from the magnet and the positively selected cell fraction was recovered by adding 3mls of PBS to the column and collecting the resulting cell suspension in a 15ml conical tube. The cells were centrifuged at 400g for 4 minutes and the supernatant decanted. 1ml of PBS was added and a viable cell count was performed. The volume was adjusted with PBS to give a cell density of $2 x10^5$/ml and 200µl of the

resulting cell suspension was added to pre-assembled cytospin cups. These were then centrifuged at 200rpm for 5 minutes in a cytospin centrifuge (Shandon Southern, Shandon, Runcorn, UK). The slides were then fixed in a 3:1 mix of methanol/acetic acid solution for 10 minutes and allowed to air dry at room temperature. Cytospin slides were pre-treated in 2 x sodium saline citrate at 37°C for 30 minutes and dehydrated in an ethanol series. They were denatured in 70% formamide (Fluka, UK)/2xSSC at 72°C for 2 minutes and again dehydrated in a cold ethanol series. The slides were hybridised overnight at 37°C. For identification of inv(16)(p13;q22) a CBFβ dual colour probe (Vysis (UK), Ltd., GB-Richmond, Surrey, UK.) was used. The CBFβ gene is located at 16q22. A 150Kb 5' region of the CBFβ gene centromeric to the breakpoint region of the gene is directly labeled with SpectrumRed fluorochrome. A 170Kb 3' sequence telomeric to the breakpoint region is directly labeled with SpectrumGreen fluorochrome. For identification of t(8;21) a AML1/ETO probe was used. This is mixture of the AML1 probe directly labeled with SpectrumGreen fluorochrome and the ETO probe directly labeled with SpectrumOrange fluorochrome (Vysis (UK), Ltd.). The 1.3 Mb AML1 probe hybridises to the AML1 gene and encompasses the breakpoint region at intron 5. The 87Kb ETO gene is centrally located within the 480Kb ETO probe and encompasses the breakpoint region at introns 1a and 1b. The slides were counterstained with 4',6-diamidino-2-phenylindole-dihydrochloride (Sigma-Aldrich) and mounted in antifade medium (glycerol: PBS solution). Fluorescent signals were detected using a Zeiss Axioskop 50 microscope with a triple band pass filter. Images were captured using a digital image analysis system. 200 cells were scored for each sample.

2.4.3 IL-12(p70) production by leukaemia cells following differentiation

IL-12(p70) concentration in tissue culture supernatants was measured by ELISA assay. Microwells (Medisorb ELISA plate, Greiner) were coated with 100µl per well of capture antibody, mouse anti huIL-12 (Pharmingen), diluted 1:250 in coating buffer (0.2M $NaPO_4$, pH 9.0). The plate was then sealed and incubated overnight at 4^0C. The wells were aspirated and washed three times with 300µl/well of wash buffer (PBS with 0.05% Tween-20). After the last wash the plate was inverted and blotted on absorbent paper to remove any residual buffer. The plate was then blocked with 200µl/well of assay diluent (PBS with 10% FCS). Following incubation for 1 hour at room temperature the wells were aspirated and washed with 300µl of wash buffer two times. Standards were then prepared from a stock solution of 500pg/ml recombinant IL-12 (Pharmingen). Serial dilutions of IL-12(p70) were prepared by adding 300µl of each standard to a series of tubes containing 300µl of assay diluent. The test samples were tissue culture supernatants collected from the final three days of culture of primary leukaemia cells (10^6 cells/ml) with GM-CSF, IL-4 and CD40 ligand. These were stored at -20^0 and thawed at 37^0C immediately prior to use. 100µl of each test sample and standard were added to the appropriate wells, in duplicate, and the plate was sealed and incubated for 2 hours at room temperature. The plate was then washed with wash buffer for a total of 5 washes. 100µl of the working detector, biotinylated mouse anti-huIL-12 at 1:250 dilution with streptavidin-horseradish peroxidase conjugate at 1:250 dilution in assay diluent, was added to the wells and the plate was sealed and incubated for 1 hour at room temperature. The wells were then washed and aspirated as before for a total of 7

washes. 100µl of the substrate solution, Sigma fast OPD (Sigma-Aldrich) 1 tablet in 20mls dH_2O, was added to each well. The plate was then incubated at room temperature in the dark for 30 minutes. Absorbance was then read at 490nm with an ELISA reader (V max kinetic microplate reader, Molecular devices). A standard curve was generated by plotting the IL-12 concentration on the x-axis and absorbance on the y. Using Microsoft Excel software an equation of the curve was calculated and IL-12(p70) concentration of the test samples were determined by using this equation.

2.4.4 Immunostimulatory properties of leukaemia Cells

2.4.4.1 Allogeneic MLLR

Mononuclear cells were prepared by lymphoprep separation of PB taken from healthy donors as previously described. Responder T cells were obtained by harvesting non-adherent cells from cell suspensions, following overnight plastic adherence in $25cm^2$ flasks. Stimulator cells, either differentiated leukaemia cells or unmodified leukaemia cells, were treated with 25µg/ml mitomycin C (Sigma-Aldrich) for 2 hours prior to use in MLLR. Stimulator cells were then washed three times with cRPMI and added in graded doses to 1×10^5 allogeneic T cells in 96-well U-bottomed tissue culture plates in cRPMI with 5% hiABS.

Initially the read-out of proliferation was performed using a commercial cell proliferation assay, Cell titre 96® (Promega). This is a non-radioactive, colorimetric based assay. The assay was performed according to the manufacturers instructions.

Briefly, following 5 days culture, 20μl of combined MTS/PMS solution was added to each well. The plates were then incubated for 4 hours at 37^0C. Absorbance was read at 490nm using an ELISA reader (V max kinetic microplate reader, Molecular devices).

Because the colorimetric assay did not appear to be sensitive enough to detect cell proliferation in the MLLR subsequent experiments were performed using a radioisotope based assay. Cells were incubated for the last 18 hours of 5 days culture with 1 μCi titriated thymidine (ICN Biochemicals, Basingstoke, UK). The plates were then frozen at -20^0C prior to harvesting. On the day of harvesting the plates were thawed and the wells were harvested onto filter paper using a cell harvester (Tomtec harvester 96♦ Mach III M, Wallac, Finland). The filter was air-dried for 1 hour. Wax paper was warmed on a heating block and the filter paper was then overlain on top of molten wax. Following setting the wax embedded filter was loaded into cassette and read with a β scintillation counter (1450 microbeta trilux liquid scintillation and luminescence counter, Wallac).

2.4.4.2 Generation of autologous leukaemia reactive CTL

Autologous T cells from AML patients were obtained when patients had achieved documented morphological remission. PBMC were separated by centrifugation over lymphocyte separation medium (Lymphoprep) as previously described. Following overnight culture in cRPMI adherent cells were decanted and centrifuged at 400g for 4 minutes. The supernatant was decanted and the cells resuspended in cRPMI supplemented with anti human CD3 mAb (Serotec) at 1μg/ml and IL-2 50u/ml (R&D systems). After 3 days the medium was replaced with cRPMI containing IL2 50u/ml and

IL-7 10u/ml (Peprotech, London). Following a further 4 days culture T cells were harvested and co-cultured with freshly thawed autologous unmodified leukaemia cells or DLLC in cRPMI with 5% hiABS at a ratio of T cells to unmodified leukaemia cells, or DLLC, of 3:1. Unmodified leukaemia cells and DLLC were treated with mitomycin C 25μg/ml for 2 hours and then washed three times prior to co-culture with T cells. Control T cells were cultured with cRPMI supplemented with IL-2 and IL-7 alone. At the end of the co-culture period the T cells were harvested, centrifuged at 400g for 4 minutes and the supernatant discarded. The cells were then resuspended in cRPMI containing IL-2 and IL-7 and cultured for a further 4-7 days prior to performing cytotoxicity assays.

2.4.4.3 Cytotoxicity assays

Initially cytotoxicity assays were performed with freshly thawed unmanipulated AML blasts as targets. However it became apparent that a significant proportion of the AML cells were apoptotic therefore giving a high background spontaneous cell death in both assays. Therefore in later assays apoptotic cells were removed by MACS separation with annexin-V microbeads as follows: Freshly thawed AML blasts were washed and resuspended in 200μl of annexin-V buffer (Bender MedSystems). 10μl of annexin-V microbeads (Miltenyi Biotec) were added and mixed with cells by vortexing for 10 seconds. Cells and beads were incubated for 15 minutes at room temperature. The cells were then pelleted by centrifugation at 400g for 4 minutes and resuspended in 500μl of AIM-V medium. The cell suspension was then added to a miniMACS column placed

within a miniMACS magnet. The negatively selected fraction was collected, washed with 2mls cRPMI and centrifuged. These cells were then used as targets in the cytotoxicity assay

i/ LDH release assay

The LDH release assay was performing using a commercial kit, CytoTox 96® (Promega), according to the manufacturers instructions. The AML targets cells were adjusted to a cell density of 10^5/ml in cRPMI with 5% hiABS. 100µl of the cell suspension was added to wells of a U-bottomed 96-well plate. Serial dilutions of effectors, either T cells cultured with IL-2/IL-7 containing medium alone or with autologous unmodified AML blasts or DLLC, were made up in cRPMI with 5% hiABS to give final effector:target ratios of 25:1, 10:1 and 5:1. 100µl were added to the wells of the 96-well plate. Controls were set up for target cell maximum and spontaneous release, effector cell spontaneous release and culture medium background as in the Figure 2.3 below. The plate was incubated for 4 hours at 37^0C. 45 minutes prior to the completion of the incubation period 20µl of cell lysis (9% Triton® X-100) solution was added to the wells containing targets alone to establish target cell maximum release and to wells for volume correction control. The plate was then centrifuged at 250g for 4 minutes. 50µl of supernatant removed from each well and transferred to the wells of a flat-bottomed 96-well plate. 50µl of substrate mixture was added. The plate was then covered in tin foil and incubated for 30 minutes at room temperature. 50µl of STOP solution (1M acetic

acid) was added to the wells. The absorbence was read at 492nm with an ELISA reader (V max kinetic microplate reader, Molecular devices).

$$\% \text{ cytotoxicity} = \frac{\text{Experimental-Effector Spontaneous-Target spontaneous}}{\text{Target Maximum-Target Spontaneous}} \times 100$$

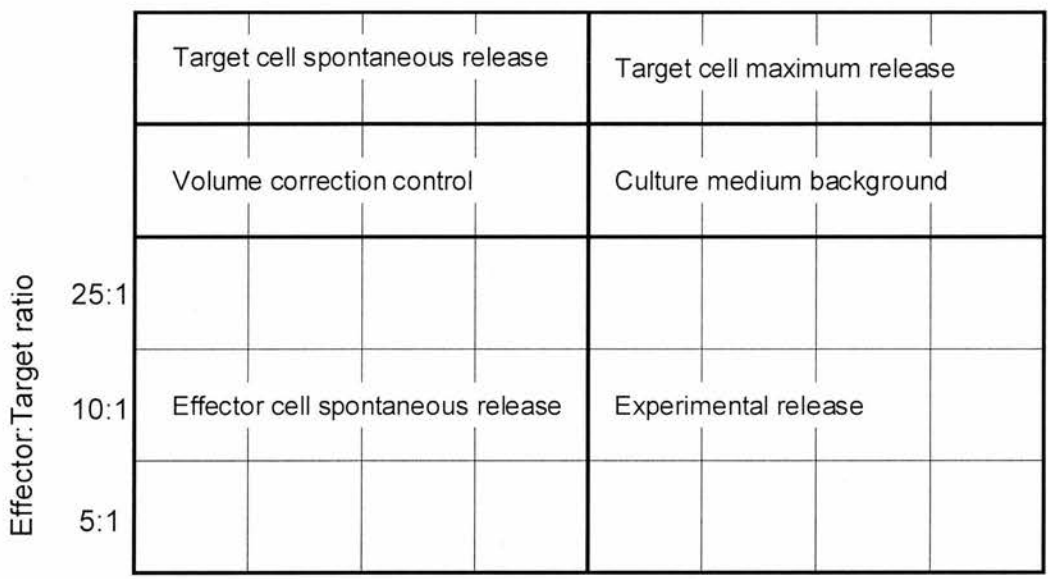


Figure. 2.3 Plate layout for LDH release cytotoxicity assay

ii/ Flow cytometric based assay

The principle of flow cytometric assay was based on the identification of apoptosis in target cells by annexin-V and PI staining with elimination of PKH26 stained effectors achieved by a FL2 high fluorescence exclusion gate (Aubry *et al*, 1999). Prior to performing the cytotoxicity assay the cytometer settings were optimised for three colour staining as follows: 10^6 primary AML blasts were stained singly with annexin V-FITC (10µl), PKH26 (4mM) and PI (2µg/ml). FITC labelled cells were run through a FACSCalibur flow cytometer using settings already optimised for the forward and side scatter characteristics of leukaemia cells. On a FL1 versus FL2 dot plot the FL-2-%FL-1 compensation was adjusted so that the FL1 positive population was horizontally aligned with the FL1negative population. The PKH26 labelled cells were then run through the cytometer. The FL1-%FL2 compensation was adjusted so that the FL2 positive population was vertically aligned with the FL2 negative population on the FL1 versus FL2 dot plot. Finally the PI stained AML cells were run through whilst monitoring the FL3 versus FL2 plot. FL2-%FL3 adjusted so that PI was undetectable in the FL2 channel. These settings were used for all dual and triple colour analysis of primary AML blasts.

For performing the cytotoxicity assays $5x10^6$ T cells, either following co-culture with DLLC or undifferentiated leukaemia blasts or cultured with IL-2 and IL-7 alone, were stained with 1 ml of 4µM PKH26-GL solution (Sigma-Aldrich) and then incubated for 3 minutes at room temperature. 1 ml of cRPMI was then added to stop the reaction. Cells were then pelleted by centrifugation at 400g for 4 minutes, the supernatant discarded and

the cells washed with 4 mls cRPMI. This was repeated three times to completely remove any unbound PKH26. The cells were then resuspended in cRPMI with 5% hiABS. PKH26 labelled T cells were made up in various dilutions in cRPMI with 5% hiABS. Freshly thawed target cells were resuspended in cRPMI with 5% hiABS at cell density $5x10^5$/ml and 200µl was added to 12x75mm plastic tubes. 200µl of the effector cell suspensions was then added to the 12x75mm plastic tubes containing the targets to give effector:target ratios of 25:1, 10:1 and 5:1. The tubes were then centrifuged at 400g for 2 minutes. Following 4 hours incubation at 37^0 cells were pelleted by centrifugation at 400g for 2 minutes and the supernatant was discarded. The cells were resuspended in 100µl of annexin-V buffer (Bender MedSystems). 10µl of annexin-V-FITC was added and the tubes were incubated for 15 minutes at 4^0C. Cells were washed with 2 mls PBS and then pelleted by centrifugation at 400g for 4 minutes. The supernatant was discarded and the cells resuspended in 100µl annexin-V buffer containing 2µg/ml of PI. Analysis was then performed within 15 minutes on a FACSCalibur flow cytometer using instrument settings pre-optimised for three colour analysis of primary AML blasts.

2.4.5 Differentiating agents

2.4.5.1 Calcium Ionomycin induced differentiation

Attempts to induce DLLC differentiation with calcium ionophore A23187 (Sigma-Aldrich) were performed with leukaemia cells from the HL60 cell line and primary AML blasts from selected AML cases. Cells were harvested and resuspended at 10^6 cells/ml in cRPMI. 3 mls of the cell suspension was transferred to the wells of a 6-well plate and calcium ionophore A23187 was added to give a final concentration varying from 0 ng/ml to 400ng/ml. Plates were incubated at 37^0C, 5% CO_2 for 48 hours following which the cells were harvested and analysed for expression of costimulatory molecule expression by flow cytometry.

2.4.5.2 Differentiating agents in combination with cytokines

In those AML cases which had shown resistance to cytokine-induced differentiation to DLLC the culture was repeated but with differentiating agents given in combination with the cytokine regimen in an attempt to induce differentiation. AML blasts from nine differentiation resistant cases were cultured at 10^6 cells/ml in the wells of a 6-well plate in cRPMI supplemented with GM-CSF, IL-4 (as described previously), with differentiating agents trichostatin-A (TSA) 75ng/ml in combination with azacytidine (AZA) 1μM, or bryostatin-1 (BRYO) 10nM alone, added for the first 4 days of the culture period. The cells were then harvested and pelleted by centrifugation at 400g for 4 minutes. The supernatant was discarded and the cells were resuspended in cRPMI

containing GM-CSF and TNF-α. The cells suspension was reseeded into the wells of a 6-well plate and the plate was incubated for a further 3 days. Cells were then harvested and used for either for immunophenotypic analysis of costimulatory molecule expression, or as stimulators in allogeneic MLLR, as described previously.

2.5 Induction of differentiation in murine myeloid leukaemia cells

2.5.1 Murine AML model

The murine AML (mAML) model is a radiation induced leukaemia in CBA mice (Fennelly *et al*, 1997; Major, 1979; Wright, 1991). Cryopreserved mAML cells were obtained for Eric Wright, Dundee Medical School.

2.5.2 Maintenance of animals

All mice were housed within the animal facilities at ICAPB, Edinburgh, according to the provisions of the animals (Scientific Procedures) Act (UK) 1986.

2.5.3 Passage of murine AML

A frozen aliquot of murine AML cells (aliquot MC7.3 spleen 14/09/95) was thawed at 37^0C. The cells were diluted slowly in 20mls of cRPMI. The cells were then pelleted by centrifugation at 400g for 7 minutes and the medium replaced with 20mls cRPMI. This process was then repeated. The cells were centrifuged at 400g for 7 minutes and the resulting cell pellet was resuspended in 2 mls PBS. A viable cell count was performed.

A total of 11x 10^6 murine AML cells were recovered. 10 female CBA/Ca mice were irradiated 200R each and injected intraperitoneally with 10^6 cells in 200µl of PBS. A further passage of tumour was performed using spleen cells from a first passage mouse that had developed overt signs of leukaemia. A single cell suspension was prepared from homogenised spleen and a further 10 female CBA/Ca mice were injected with 2 x 10^6 cells in 200µl PBS following 300R irradiation.

2.5.4 Harvesting of murine AML cells

To obtain peripheral blood samples the following procedure was performed. The mouse was anaesthetised with halothane. Using a heparinised capillary tube (Hawksley, Sussex, UK) 10-15 drops blood were collected by venepuncture of the retro-orbital venous sinuses and added to 10mls of PBS and then mixed by inversion. The cell suspension was centrifuged at 400g for 4 minutes and the supernatant removed by aspiration. The cell pellet was then resuspended by flicking tube. 9ml of distilled H_2O was added and mixed by inversion for 12 seconds. 1ml of 10x PBS was then added and again mixed by inversion. The cells were then centrifuged at 400g for 4 minutes. The supernatant was removed by aspiration and the pellet was resuspended by flicking tube. 10 ml of PBS was added and mixed by inversion. The cells were centrifuged at 400g for 4 minutes. The supernatant was removed and the pellet was resuspended by flicking tube. 1 ml of cRPMI was added. A viable cell count was performed and cRPMI with 10% DMSO was added to give a final cell density of 2 x 10^7/ml. The cells were then frozen.

When mice began to develop overt signs of leukaemia they were sacrificed and leukaemia cells were harvested from peritoneal exudates and spleen. Mice were killed by CO_2 method and then washed down with 70% alcohol. The mouse was lain on the right side and the skin pinched up to allowing a skin incision and subsequently peeling off of skin to be performed whilst leaving the peritoneal membrane intact. With a 5ml syringe and 21g needle 5mls of PBS was injected into the abdominal cavity. Following this the hind legs were picked up and the mouse shaken gently. Using the same syringe and needle the fluid from the peritoneal cavity was carefully removed and transferred to a 15ml conical tube. Injection of PBS into the peritoneal cavity and its subsequent aspiration was repeated between 4-6 times more until the total volume in the conical tube was 15mls. The cells were pelleted by centrifugation at 400g for 4 minutes. The supernatant was discarded and the cell pellet resuspended by flicking the tube. 1 ml of cRPMI added and a viable cell count performed. Cell density was adjusted to $5x10^7$ by addition of cRPMI with 10% DMSO and cell were frozen.

Spleen obtained from a freshly dissected mouse was placed in a glass homogeniser. 1 ml of PBS was then added. A glass rod was placed into homogeniser and turned 3-4 times in order to disrupt the tissue. The resulting cell suspension was transferred to a 15ml conical tube. This was left to stand for 3-5 minutes to allow tissue debris to settle to the bottom of the tube. The cells were then decanted into a fresh tube. PBS was then added to give a final volume of 10mls. The cells were pelleted by centrifugation at 400g for 4 minutes. The supernatant was discarded and the cell pellet resuspended by flicking tube. 1ml of cRPMI was added and a viable cell count was performed. Cell density adjusted to $5x10^7$ by addition of cRPMI with 10% DMSO and cell were frozen.

2.5.4 Immunophenotypic profile of leukaemia cells

Murine AML cells were analysed for expression of costimulatory molecule expression before and after attempted differentiation by staining 2x 10^5 cells in 12x75mm plastic tubes with 10 µl rat mAb directed against mouse CD40, CD80, CD86, HLA class II (all Serotec), CD11b (Pharmingen) (all FITC-conjugated) and Gr-1 (Pharmingen) (PE-conjugated). Cells were also stained with isotype matched control rat antibodies (Serotec). The tubes were then incubated for 30 minutes at 4 ^{0}C. Cells were washed with 2mls PBS and then pelleted by centrifugation at 400g for 4 minutes. The supernatant was discarded and 100µl of PBS containing 2µg/ml PI was added. After 5 minutes incubation analysis was performed on a FACSCalibur flow cytometer using the pre-optimised 3-colour settings modified for the FSC and SSC characteristics of murine cells. Viable cells were defined with a forward scatter (FS)/PI gate.

2.5.6 Cytokine-induced differentiation

Freshly thawed murine AML cells were cultured at 5x10^6 cells/ml in the wells of a 6-well plate in cRPMI with 5% hiABS and 100µM 2-Mercaptoethanol (Sigma) to which was added murine GM-CSF 10ng/ml and murine IL-4 50ng/ml for 4 days at 37^0 C. The cell suspension was then harvested and the cells pelleted by centrifugation at 400g for 4 minutes. The medium was replaced with fresh cRPMI to which was added GM-CSF 10ng/ml and murine TNFα 25ng/ml and the cell suspension reseeded into the wells of a 6-well plate. Following further culture for 3 days the cells were harvested and were used for immunophenotypic analysis.

2.5.7 Bryostatin-1 in combination with cytokines

In an attempt to overcome cytokine-induced differentiation resistance murine AML cells were cultured under identical conditions except that for the first 4 days of the culture period BRYO 10nM was used in combination with the cytokine regimen.

2.6 Photomicroscopy

Phase contrast photomicrographs were taken using a Nikon F-301 camera attached to a Nikon Diaphot phase contrast microscope (Nikon, Japan).

Cytospin preparations for photography purposes were prepared as follows. Cells were resuspended in PBS to give a cell density of 10^6/ml. Cytospin cups were assembled with Shandon glass slides (Cytoslide™, Shandon) and filter cards. 200µl of the cell suspension was then transferred to the cytospin cups placed within a cytospin centrifuge (Shandon, Southern). These were then centrifuged at 200rpm for 5 minutes. The slides were then removed and allowed to air dry for 10 minutes. The slides were then stained with May-Grunwald-Giemsa stain (BDH, Poole, UK) using an automatic slide stainer (Varistain 24-2, Shandon). Photomicrographs were taken with a Leica Wild MPS52 camera attached to a Leica Periplan (Leica,Germany) microscope.

2.7 Statistical analysis

Statistical analysis was performed using the student's t test.

Chapter 3

RESULTS

3.1 Comparative experiments of gene transfer efficiency

3.1.1 Selection of a GFP containing plasmid vector

A qualitative comparison in the GTE of six GFP containing plasmid vectors, following calcium phosphate precipitation, into COS and CHO target cell lines was performed. The pBabe.puroEGFP vector was selected for subsequent experiments, as under fluorescence microscopy it appeared to infect a greater proportion of cells and gave higher fluorescence intensity than the other vectors.

3.1.2 Myeloid leukaemia cell lines

3.1.2.1 Electroporation

Electroporation, under the range of conditions tested, gave a low transfection efficiency in the four leukaemia cell lines tested, <1%, and was associated with a high degree of cell death (>60%) in all four of the leukaemia cell lines.

3.1.2.2 Commercial transfection reagents

For all the commercial transfection reagents optimisation was performed to establish the conditions that gave the highest efficiency of gene transfer. An example of this is illustrated in Figure 3.1. Optimisation of the commercial transfection reagent

Transfast into the K562 cell lines was performed by varying the plasmid DNA quantity at two separate charge ratios. GTE was determined by the measuring the percentage of cells expressing GFP, in comparison to cells transfected in the absence of plasmid DNA, by flow cytometry.

Despite optimisation the level of gene transfer using commercial transfection reagents was very low. A GTE of >5% was only achieved in the K562 cell line and only with the transfection reagents Transfast and Effectene (Fig 3.2). In the remaining cell lines the level of GTE with all of the commercial transfection reagents was $\leq 1\%$.

3.1.2.3 Retroviral vectors

Three packaging cells lines, TeflyMLV-A, TeflyRD114 and TeflyGALV containing the pBabe.puroEGFP were created (as well as three lines containing the 'empty' pBabe.puro vector for the purposes of generating control retroviral vectors). A comparison of the GTE of these packaging cell lines was performed with the four leukaemia cell lines. The results from this experiment is shown in Table 3.1

Envelope protein	% GFP positive			
	K562	KG1a	U937	HL60
MLV-A	5.8	7.1	10.3	0.2
GALV	26.5	6.2	9.2	0.4
RD114	72.7	10.8	30.8	0.9

Table 3.1 Comparison of gene transfer efficiency of a retroviral vector packaged with three different envelope specificities

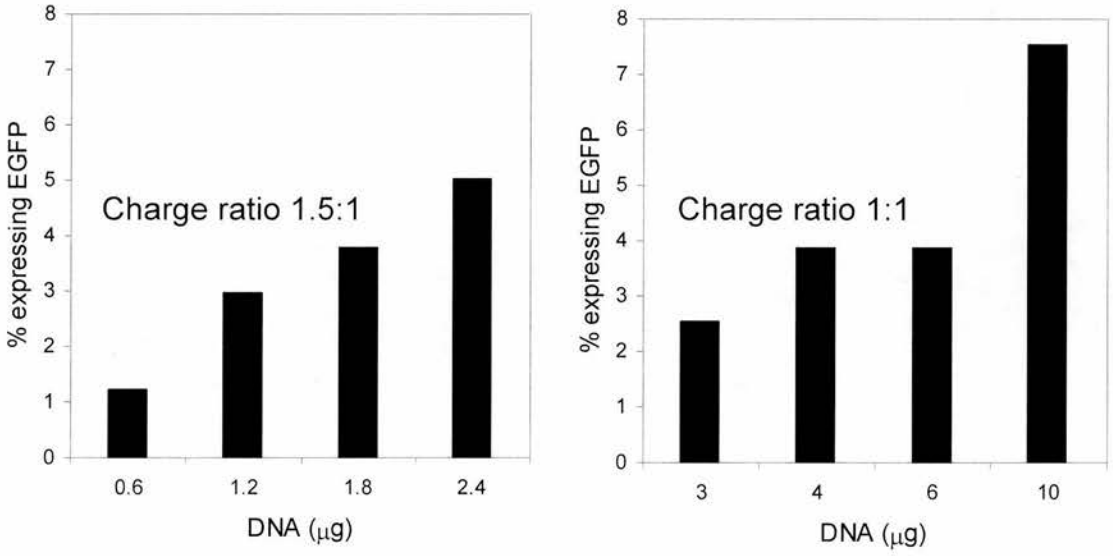


Figure 3.1 Optimisation of gene transfer to K562 cell line with Transfast. Plasmid DNA quantity varied at two separate charge ratios

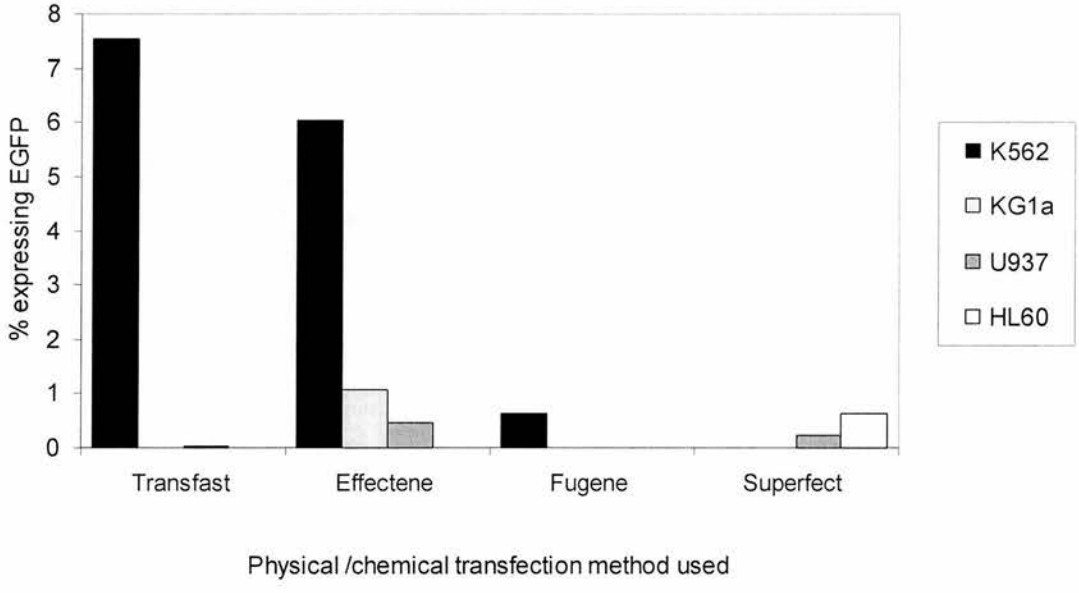


Figure 3.2 Comparison of gene transfer efficiency with commercial transfection reagents

Packaging of the retroviral vector by the TeflyRD114 cell line gave consistently higher levels of GTE than with the other two packaging cell lines. This may have been due to the fact that the RD114 envelope protein increased the efficiency of retroviral vector attachment to the myeloid leukaemia cells in comparison with vectors possessing the other two envelope protein specificities. However as no physical evaluation of the total number of retroviral particles in the individual vector stocks was made another possible explanation could be that the TeflyRD114.pBabe.puroEGFP producer cell line generated higher concentrations of retroviral particles than the other producer cell lines. However as, for whatever reason, relatively high tire retroviral vector stock could be generated by the TeflyRD114.pBabe.puroEGFP producer cell line all subsequent comparative experiments of GTE with the retroviral vector were performed with vector stock harvested from this producer cell line.

Although retroviral stock of reasonable titre could be generated attempts were made to perform further concentration of this stock in order to allow experiments on leukaemia cells to be performed at the highest possible MOI. Two methods were used, either concentration by ultracentrifugation or by mixing with pansorbin. Concentration of retroviral vector stock by ultracentrifugation led to around a 10-fold increase in titre based on the GTU assay when compared with pooled unconcentrated vector stock. However when comparisons were made between the GTE of neat pooled unconcentrated vector stock with vector stock concentrated by ultracentrifugation there was no apparent enhancement of GTE despite the higher titre (data not shown). A similar pattern was observed in experiments comparing unconcentrated vector stock with vector stock concentrated by the pansorbin method

Table 3.2 shows the titres obtained at various dilutions for unconcentrated vector stock and vector stock concentrated by the pansorbin method.

Dilution	Titre (GTU/ml)	
	Pooled	Pansorbin
10^{-3}	-	$1x10^7$
10^{-2}	$5x10^5$	$4x10^6$
10^{-1}	$9x10^5$	$3x10^6$

Table 3.2 Comparison of the titres obtained with pansorbin concentrated versus unconcentrated retroviral vector stock

These experiments showed that enhancement of titre, as measured by the GTU assay, was highest at the lowest dilution and progressively fell as the degree of dilution diminished. A possible explanation for this observation is that the pansorbin itself had a detrimental effect on the GTE, this effect being most marked at the highest pansorbin concentration. When a comparison of pooled retroviral vector stock was made with neat pansorbin concentrated vector stock in the four leukaemia cell lines it was found that there was no significant enhancement of GTE with the pansorbin concentrated vector stock (Table 3.3).

Cell line	% GFP positive	
	Pooled (titre $4x10^5$)	Pansorbin concentrated (titre $2x10^6$)
K562	46.7	38.5
KG1a	1.3	1.1
U937	29.2	28.2
HL60	0.3	0.2

Table 3.3 Comparison of gene transfer efficiency of pansorbin concentrated versus unconcentrated retroviral vector stock

Because concentration of retroviral vector stock led to no apparent benefit in terms of enhancement in GTE unconcentrated retroviral vector stock was used in all subsequent experiments.

An alternative approach to increasing the GTE of retroviral vectors, other than by using a higher MOI, is to enhance the binding of the retrovirus to the target cells by co-localisation on recombinant fibronectin (Hanenberg *et al*, 1996). Because of the availability of plasma-derived fibronectin, sourced from the SNBTS Protein Fractionation Centre (Edinburgh), fibronectin-coated plates were evaluated as a means of improving GTE. In parallel infection of human leukaemia cell lines was performed using 6-well plates that were either uncoated or coated with plasma-derived fibronectin. High titre retroviral vector stock from the TeflyRD114.pBabe.puroEGFP producer cell line was used for infection of the leukaemia cells. The results of these experiments showed that there were no apparent differences between fibronectin-coated and non-coated plates in terms of GTE (Table 3.4).

Cell line	% GFP positive	
	No Fibronectin	Fibronectin
K562	21.1	19.9
KG1a	7.3	9.7
U937	49.7	35.8
HL60	0.5	1.2

Table 3.4 Comparison of gene transfer efficiency using uncoated and fibronectin coated plates

Because of this failure to identify methods to enhance GTE either by concentration of retroviral vector stock, or by using fibronectin-coated plates, work focused on generating sufficiently high titre retroviral vector stock to allow a reasonable level of MOI. The highest titre obtainable, based on the GTU assay, was $2x10^6$ GTU/ml, which meant that the highest practicable MOI for infection of the myeloid leukaemia cell lines was 10. Figure 3.3 shows the mean GTE from three independent experiments in the four leukaemia cell lines. The K562 cell line was the most permissive of the cell lines to retroviral vector infection (mean GTE 78.3%), followed by U937 (mean GTE 39.2%) and then the KG1a cell line (mean GTE 8.8%). The HL60 cell line was relatively resistant to retroviral vector infection with a mean GTE of only 0.96%.

3.1.2.4 Adenoviral vectors

One of the main advantages of using adenoviral vectors is that higher titres of vector stock can be obtained, in comparison with retroviral vectors, therefore making it possible to perform infections at higher MOI. AdGFP vector stock titres, based on the GTU assay, were in the order of $2x10^7$-$5x10^7$ GTU/ml. In preliminary experiments it was found that very high MOI ($\geq$100) was associated with an excessive loss of cell viability, presumably due to the toxic effects of the adenovirus. Therefore subsequent infections were performed at a MOI of 40, which appeared to give good levels of GTE but was not associated with too much toxicity. Because there was no difficulty in achieving sufficiently high MOI no purification of adenoviral vectors was performed. As a number of studies had demonstrated that combining polycationic lipids with adenoviral vectors enhanced GTE (Clark *et al*,

1999; Dietz & Vuk-Pavlovic, 1998; Fasbender *et al*, 1997) some comparative experiments of AdGFP with and without polybrene 4μg/ml were performed. These showed that there was significant enhancement of GTE with polybrene (data not shown), so in all subsequent infections polybrene was used in combination with the adenoviral vectors. The GTE of the AdGFP vector in the four leukaemia cell lines is shown in Figure 3.4. Results represent the mean of three independent experiments. Three of the myeloid leukaemia cell lines were relatively permissive to adenoviral infection with mean GTE for the K562, U937, and KG1a cell lines of 51.1%, 63.1% and 56% respectively. The HL60 cell line again proved difficult to transfect although GTE was significantly higher than was achieved with retroviral vectors at 6.5%.

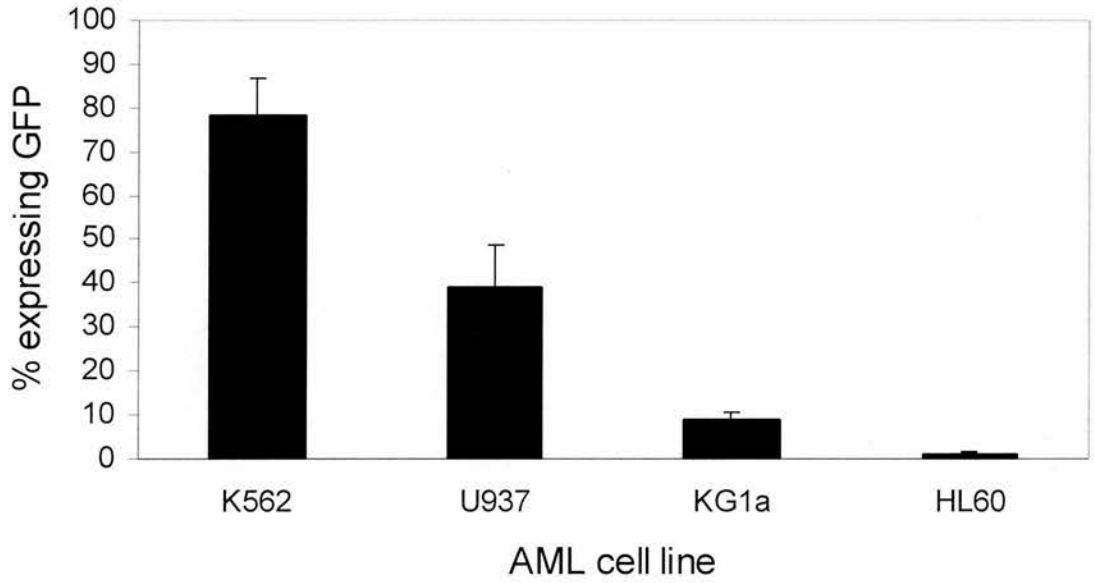


Figure 3.3 Comparison of gene transfer efficiency of a retroviral vector, in leukaemia cells lines.
Experiments performed with the pBabe.puroEGFP vector packaged by the TeflyRD114 packaging cell line (Represents mean of three independent experiments. Error bars set at one standard deviation from the mean)

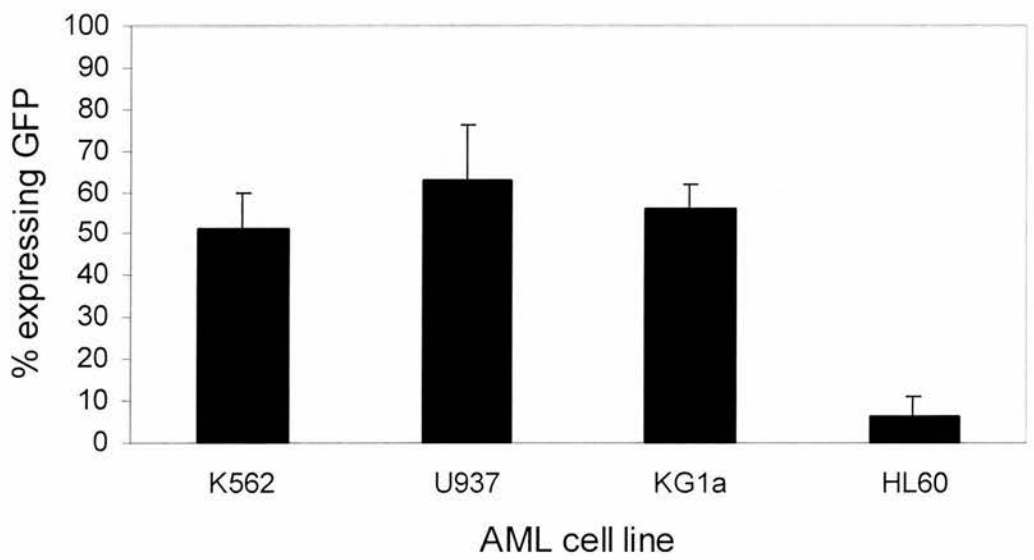


Figure 3.4 Comparison of gene transfer efficiency of an adenoviral vector in leukaemia cell lines.
Experiments performed with the AdGFP vector. (Represents mean of three independent experiments. Error bars set at one standard deviation from the mean)

3.1.3 Primary AML blasts

3.1.3.1 Retroviral vector

Infections of primary AML blasts were performed with TeflyRD114 vector stock, at MOI of 10, following 48 hours culture of leukaemia cells with GM-CSF and IL-3 given in an attempt to stimulate cell division. Leukaemia cells from 14 AML cases were tested. These primary leukaemia cells were resistant to retroviral vector infection with a mean GTE of only 0.8% (range 0-2.5%).

3.1.3.2 Adenoviral vectors

Infections of primary AML blasts were performed with AdGFP vector stock at a MOI of 40. Viral absorption was performed either after 48 hours culture with GM-CSF/IL-3 or after 7 days culture with GM-CSF/IL-4/CD40L, given in an attempt to increased viral binding and internalisation through upregulation of integrin expression. The GTE of the AdGFP vectors into primary leukaemia cells showed a high degree of variability between individual AML samples. For leukaemia cells that had been cultured with GM-CSF/IL-3 prior to infection the range of GTE was 0.7%-67.1% (mean GTE 12.1%). AdGFP infections performed after prior culture of the leukaemia cells with GM-CSF/IL-4/CD40L gave a GTE ranging from 0.7-56.2% (mean GTE 11%). Overall there was no statistically significant difference between GTE in leukaemia cells cultured with either of the cytokine combinations ($p=0.4$). However if subgroup analysis was performed in the cases that had shown a GTE of <5% following prior culture with the GM-CSF/IL-3 combination then there did appear to be a significant enhancement in GTE, in these cases, following culture with GM-CSF/IL-4/CD40L (mean GTE 9.4%)($p<0.005$).

AML case	% of cells expressing GFP	
	GM-CSF/IL-3	GM-CSF/IL-4/CD40L
UPN1	1.6	5.8
UPN2	0.7	0.7
UPN4	12.0	7.8
UPN5	4.4	24.7
UPN9	16.1	10.4
UPN12	3.8	12.6
UPN13	67.1	56.2
UPN14	1.5	7.5
UPN15	4.0	8.9
UPN17	6.7	7.2
UPN20	4.2	6.3
UPN22	25.3	8.9
UPN23	41.0	8.3
UPN30	4.5	4.0
UPN31	4.6	20.3
UPN32	3.1	3.6
UPN33	1.1	8.8
UPN39	6.8	2.3
UPN40	22.1	4.7
Mean	**12.1**	**11.0**

Table 3.5 Comparison of gene transfer efficiency of an adenoviral vector in primary leukaemia cells cultured with two different cytokine combinations.
Leukaemia cells derived from 19 AML cases were cultured for either 48 hours with GM-CSF/IL-3 or for 7 days with GM-CSF/IL-4/CD40L prior to infection with AdGFP

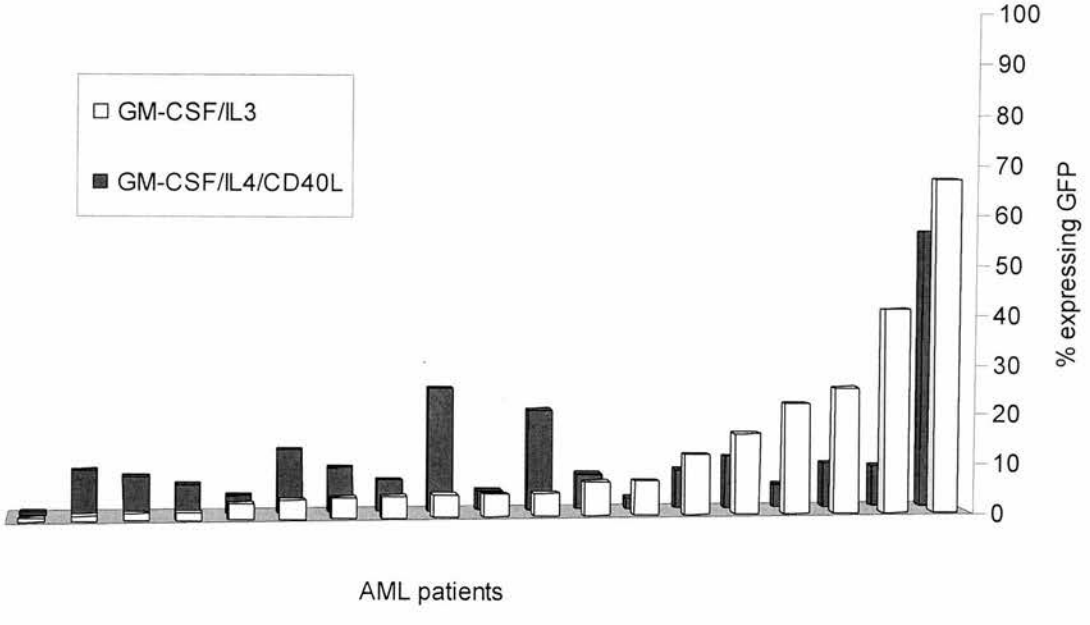


Figure 3.5 Comparison of gene transfer efficiency of an adenoviral vector in primary leukaemia cells cultured with two different cytokine combinations.
Data represented in histogram form

3.2 Generation of stably transduced cell lines

Stably transduced cell lines were created initially for the purpose of testing whether gene transfer of costimulatory molecules or immunostimulatory cytokines would lead to an increase in the immunostimulatory properties of the genetically modified leukaemia cells. For this purpose CD80 and IL-2 transduced cell lines were created. However when it became apparent that gene transfer to primary AML blasts, with the vectors available, was not of a sufficient level to be clinically useful the focus switched to generating CD40 transduced leukaemia cell lines for the purposes of their attempted differentiation induction with CD40L.

With the K562 cell line one of the major difficulties in selecting stably transduced clones was that within the leukaemia cell population there appeared to be a sub-clone of puromycin resistant cells. This is illustrated by the puromycin response curves that had been generated prior to the stable transduction of the cell lines. In Figure 3.6 the puromycin response curve for the K562 cell line is shown alongside that for U937. Even at very high puromycin concentrations there appears to be a population of K562 cells that are resistant to the puromycin. It was still possible to generate K562 cell lines transduced with the cDNA of interest by screening multiple clones and selecting and expanding those that showed high levels of transgene expression. (Figure 3.7).

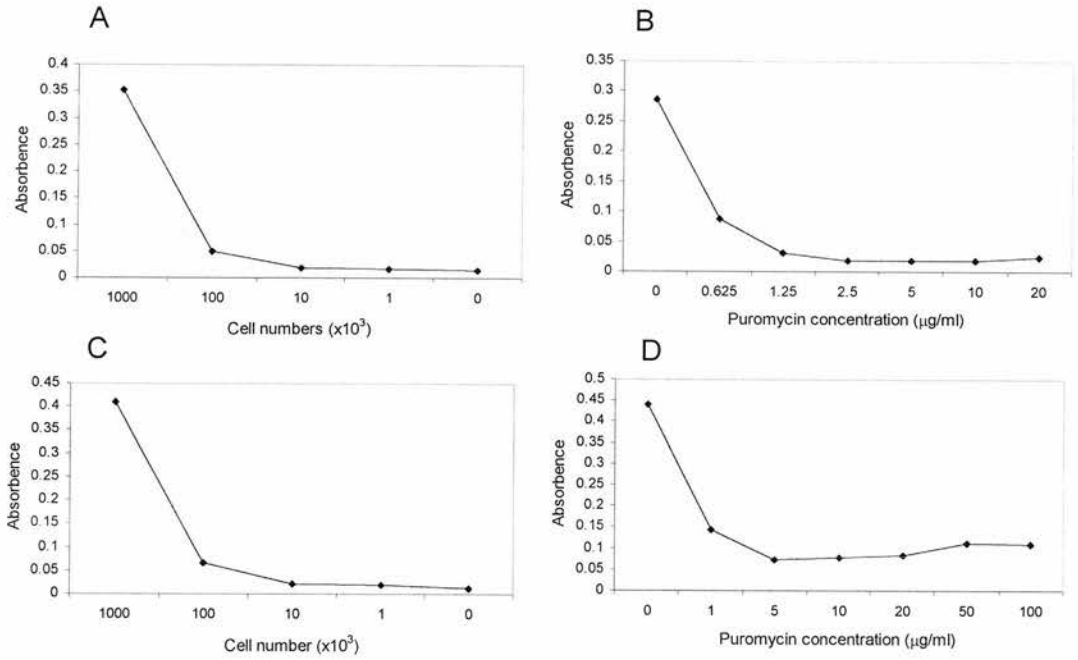


Figure 3.6 Puromycin dose response curves in leukaemia cell lines
A. and B. represent the MTT conversion at various cell number and the puromycin dose response curve respectively for U937 C. and D. are the corresponding graphs for K562

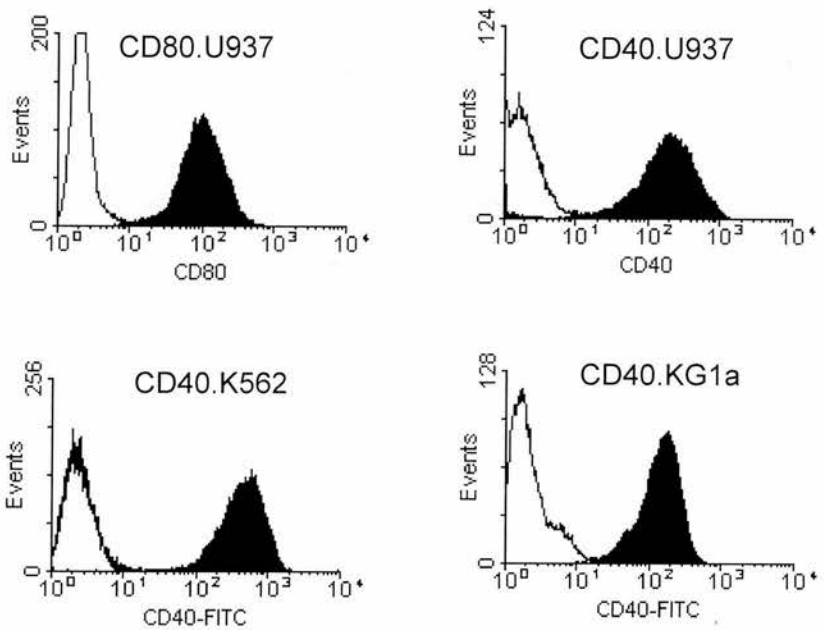


Figure 3.7 Stable transduction of leukaemia cell lines with retroviral vectors

3.2.1 Cell lines expressing costimulatory molecules

As previously described stably transduced cell lines were produced by selecting clones that showed high levels of transgene expression. For the costimulatory molecules CD40 and CD80 this involved staining a sample of the cells derived from individual clones with murine mAb and then performing flow cytometric analysis (Figure 3.7). Clones with high transgene expression were selected for further expansion in puromycin containing medium. Transgene expression appeared to be stable over time with no evidence of transcriptional silencing in any of the retrovirally transduced cell lines generated.

3.2.2 Cell lines expressing IL-2

Leukaemia cell lines transduced with an IL-2 containing retroviral vector were produced in an identical manner to that previously described for the cell lines transduced with cDNA encoding for costimulatory molecules. Screening for high transgene expressing clones was performed using a flow cytometric method designed to detect intracellular cytokine production (Prussin & Metcalfe, 1995). However despite the appearance of puromycin resistant clones no IL-2 production by apparently transduced leukaemia cells could be detected by this method. Therefore in order to establish that the methodology was effective at detecting intracellular IL-2 production, activated T cells, derived from healthy volunteer donor PBMC, were used as an IL-2 producing positive control. PBMC were stimulated with PMA and PHA and the presence of T cell activation confirmed by dual staining with mAb for CD3 and the T cell activation marker CD69. Staining of monensin treated PBMC with anti-IL-2, following permeabilisation, revealed that there was an IL-2 secreting

population (Figure 3.8). This confirmed the validity of this method for detecting intracellular cytokine production. However repeated testing of various supposed IL-2 transduced clones, using this method, failed to detect any intracellular IL-2 production. The reason why this method was unsuccessful was not established. A possible explanation could be that despite successful transduction of leukaemia cells with cDNA coding for the IL-2 the amount of IL-2 produced by individual cells was below the level of detection of this particular method. If this were the case then a more sensitive assay system such as ELISA would probably be better suited for the detection of cytokine production by retrovirally transduced leukaemia cells.

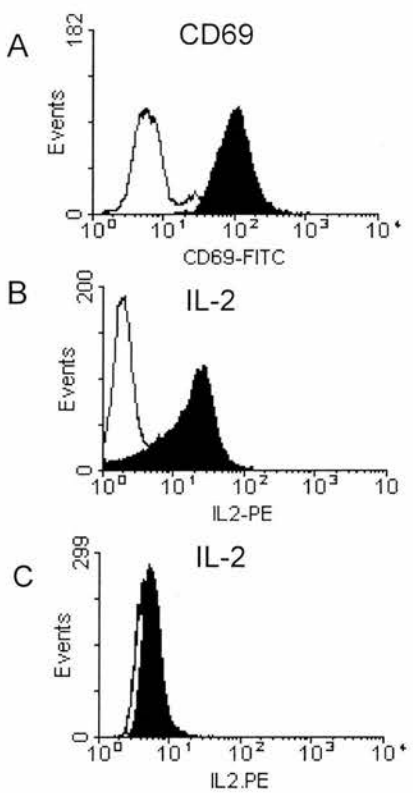


Figure 3.8 Measurement of IL-2 production.
Analysis performed by flow cytometric method of detection of intracellular cytokines
A. Expression by PBMC of the T cell activation marker CD69 following stimulation with PHA and PMA (Gated on the CD3 positive population)
B. Expression of intracellular IL-2 by PBMC following treatment with monensin (Gated on the CD69 positive population)
C. Expression of intracellular IL-2 by an apparently stably transduced IL2.U937 cell line using the same method

3.3 Discussion

The physical and chemical transfection methods evaluated in this study gave a low efficiency of gene transfer to leukaemic cells and in the case of electroporation was associated with a high degree of cell death. They therefore do not represent suitable vectors for gene transfer in a clinical setting. Despite the high transduction efficiency in the majority of leukaemia cell lines achieved with the pBabe.puro retroviral vector packaged by the TeflyRD114 cell line, GTE in primary AML blasts was low. The most probable reason for the low GTE observed using the retroviral vector, in primary leukaemia cells, is that retroviral vectors require the target cell to be in cycle in order for transgene expression to take place. Blast cells from patients with AML tend, however, to be characterised by a low overall proliferation rate due to cell cycle arrest at G1 (Ferrari *et al*, 1988). As it is possible to force AML blasts to re-enter the cell cycle by incubation with GM-CSF and IL-3 leukaemia cells were cultured for 48 hours with these cytokines, prior to infection, in an attempt to improve the efficiency of retroviral vector transduction (Pebusque *et al*, 1989). The GTE despite using cytokines to stimulate cell proliferation was low. Because of the requirement for cell cycling and the relatively low titres of infectious retroviral particles that can be obtained, Mo-MLV based retroviral vectors are unlikely to fulfil the requirements of a clinically useful gene transfer system in this context.

Adenovirus based vectors, in contrast to retroviral vectors, are able to infect non-dividing cells. In addition it is possible to generate vector stock of much higher titre, in the range of $10^{10\text{-}11}$ viral particle/ml, thereby allowing for a greater MOI. Although transgene expression for the adenovirus vector is not permanent it has been shown to be sufficiently stable over time to be suitable for use in immunotherapeutic strategies

(Dietz & Vuk-Pavlovic, 1998; Frey *et al*, 1998; Gonzalez *et al*, 1999; Zhong *et al*, 1999). Using the adenovirus vector, AdGFP, high GTE was obtained in three out of the four leukaemia cell lines tested. Only the HL60 cell line appeared to be relatively resistant to adenovirus infection. GTE of the AdGFP vector in primary AML blasts showed a high degree of variability, regardless of whether the leukaemia cells were cultured with GM-CSF/IL-3 or GM-CSF/IL-4/CD40L prior to infection. There was no apparent correlation observed between the FAB subtype of the AML cases and the individual GTE.

A possible reason for this observed variability between AML samples from different patients may reflect differential expression of integrins and CAR by the leukaemic cells. Haematopoietic cells have been shown to have low or absent expression of the primary adenovirus receptor, CAR, and it's integrins, such as $\alpha_v\beta_3$, which appear to play the major role in adenoviral viral attachment and internalisation (Huang *et al*, 1996; Wickham *et al*, 1993). Resting monocytes express low levels of α_v integrin and are relatively resistant to gene transfer by adenovirus vectors (Huang *et al*, 1995). During terminal differentiation of monocytes α_v integrins become upregulated and this is associated with an increase in GTE. Similarly during DC maturation a variety of integrins become upregulated, among them α_v, and this is believed to be the reason for the high efficiency of gene transfer by adenovirus vectors observed in this cell type (Brown *et al*, 1995; Dietz & Vuk-Pavlovic, 1998; Zhong *et al*, 1999). Based on studies showing that it was possible to induce the differentiation of primary leukaemia cells into DLLC by culture with cytokine combinations (Boyer *et al*, 2000; Brouwer *et al*, 2000a; Brouwer *et al*, 2000b; Charbonnier *et al*, 1999; Choudhury *et al*, 1999; Cignetti *et al*, 1999; Costello *et al*, 2000; Kohler *et al*, 2000;

Oehler *et al*, 2000; Robinson *et al*, 1998), it was hypothesised that DLLC might also possess the same integrin profile as DC, therefore making them permissive to adenoviral infection. Therefore a comparison was made between adenoviral infection of leukaemia cells cultured prior to infection either with the cytokine combination GM-CSF/IL-3 or with GM-CSF/IL-4/CD40L, given in an attempt to induce DLLC differentiation. Although in 10 cases GTE was enhanced by prior culture with the GM-CSF/IL-4/CD40L combination, overall there was no statistically significant difference in mean GTE between the two cytokine regimens. However if analysis was restricted to those cases that had only achieved a GTE of <5% with the GM-CSF/IL-3 combination there was a significant improvement by prior culture of the leukaemia cells with GM-CSF/IL-4 and CD40L. A possible explanation for this could be that the leukaemia cells from the AML cases that showed resistance to adenoviral infection, had low levels of expression of α_v integrins, or other integrins involved in adenoviral attachment or internalisation, and following culture with GM-CSF/IL-4/CD40L showed upregulation in expression of these integrins with consequent enhancement in GTE. The AML cases that were permissive to adenoviral infection might be expected to show higher levels of basal expression of the adenoviral receptors, either integrins or CAR, and therefore would be less likely to show enhancement of GTE following culture with cytokines aimed at upregulation in expression of these receptors. In fact in many of these adenoviral permissive cases GTE was lower with the GM-CSF/IL-4/CD40L combination, which may be as a consequence of decreasing cell viability over the culture period, itself an important factor in determining GTE. A further advantage of the GM-CSF/IL-4/CD40L combination in terms of increasing the immunogenicity of the leukaemia cells, is that

in addition to achieving genetic modification of the leukaemia cells, in many cases culture with this cytokine combination was associated with upregulation in costimulatory and cell adhesion molecule expression by the leukaemia cells. Although using AdGFP, following one or the other of the cytokine regimes, gave moderate levels of GTE in the majority of AML cases tested, this levels of gene transfer is still probably insufficient for the purposes of a clinical gene therapy strategy.

As it appears that the main limiting factor to achieving high levels of gene transfer with adenoviral vectors in haematopoietic cells is the lack of adenoviral surface receptors investigators have sought ways of retargeting adenoviral vectors to improve their attachment to haematopoietic cells (Douglas *et al*, 1996; Douglas *et al*, 1999; Smith *et al*, 1999; Wickham *et al*, 1996). Adenoviral vectors have been re-engineered in a number of ways aimed at increasing their binding to haematopoietic cells. One group has achieved this by linking the recombinant adenovirus vector to stem cell factor thereby allowing binding of the vector to the c-kit receptor present on primitive haematopoietic cells (Smith *et al*, 1999). Using a different approach Wickham et al created a recombinant adenoviral vector containing a heparin-binding domain that targets the virus to broadly expressed, heparan-containing cellular receptors (Wickham *et al*, 1996). This vector was found to give a high level of gene transfer in primary AML blasts (Gonzalez *et al*, 1999).

Lentiviral vectors were not evaluated as a part of the comparative study of gene transfer methods in AML, but evidence from recent studies suggests that these vectors might prove to be efficient at gene transfer to leukaemia cells. In contrast to Mo-MLV based retroviral vectors, lentiviral vectors are able to transduce non-

dividing cells (Naldini *et al*, 1996) and therefore would be more likely to be successful at gene transfer to the predominately cell-cycle arrested primary AML blasts. Two groups have independently evaluated a HIV-1 based lentiviral vector pseudotyped with VSV-G envelope protein in primary AML blasts. Mean GTE of 77.6% (Stripecke *et al*, 2000) and 19.5% (Biagi *et al*, 2001) for the lentiviral vector in leukaemia cells were achieved, respectively for the two groups.

In summary following a comparative analysis of a variety of physical, chemical and viral transfection systems no method was identified that could be used clinically for the transfer of immune genes to leukaemia cells for the purpose of creating a cellular vaccine. However, based on recent studies, adenoviral vectors that have been retargeted to increase their binding to haematopoietic cells or VSV-G pseudotyped lentivirus vectors do offer promise as clinically useful gene transfer vectors. This next generation of vectors may be suitable for the *ex vivo* transfer of genes encoding costimulatory molecules and cytokines to AML blasts for use in immunotherapy based clinical trials.

Chapter 4

RESULTS

4.1 Differentiation induction in human myeloid leukaemia cells

4.1.1 Immunophenotypic profile of undifferentiated leukaemia cells

Freshly thawed, unmodified, primary AML blasts from 42 patients with newly diagnosed or relapsed AML were analysed for expression of costimulatory molecules CD40, CD80 (B7.1) and CD86 (B7.2), cell adhesion molecule CD54 (ICAM-1), CD95 (Fas), HLA class I and II molecules. The results of these studies, in addition to the clinical features of the AML cases and the routine diagnostic leukaemia immunophenotype, is summarised in Table 4.1. There was no significant expression of CD80 by leukaemia cells found in any of the AML cases. In 9 of the AML cases there was some degree of CD86 expression with 6 of these cases being of FAB subtypes M4 or M5 i.e. with a monocytic component. CD40 expression was present in 6 cases, again the majority of these being M4 or M5 AML. Significant Fas expression was present in 13 cases (31%). All the AML cases were HLA class I positive with 35 (83%) also class II positive. Primary AML blasts were also analysed for expression of the dendritic cell associated markers CD1a or CD83 prior to attempted differentiation. No cases were found to be positive for either of these markers.

UPN	Age (yrs)	De novo or secondary	AML SUBTYPE	KARYOTYPE	FISH	CD13	CD14	CD15	CD33	CD34	CD40	CD54	CD80	CD86	CD95	HLA I	HLA II
UPN1	82	de novo	M0	46XX		++	-/+	-/+	++	+	-	+	-	-	-/+	++	++
UPN2	49	de novo	M1	Fail		++	-	-/+	++	+	-	-	-	-	-	++	++
UPN3	26	de novo	M1	47XY,+19		-/+	-	++	++	-	+	-	-	-	-	++	++
UPN4	64	de novo	M1	46XY		+	-	-	++	-	-	-	-	-	-	++	-
UPN5	77	de novo	M1	Complex karyotype		++	-	+	++	-	-	-/+	-	-	+	++	+
UPN6	73	de novo	M1	Fail		++	-	+	++	++	-	-	-	-	+	++	++
UPN7	62	de novo	M1	46XY,del(12p)		++	-	+	-	++	-	-	-	-/+	-	++	++
UPN8	81	de novo	M1	47XY,+8		++	-	+	+	++	-/+	+	-	-	+	++	++
UPN9	11	de novo	M1	47XY,+ 8		++	nd	nd	++	++	-/+	+	-/+	+	++	++	++
UPN10	50	de novo	M2	Fail		-	-	++	++	-	-	-	-	-	-	++	-
UPN11	81	de novo	M2	46XX		++	-/+	+	+	-	-	-/+	-	-/+	-	++	+
UPN12	56	de novo	M2	46XX		+	-	++	+	+	-	-	-	-	-	++	+
UPN13	29	de novo	M2	46XX, del 11(q23)	mll rearrangement	++	-	+	++	+	-	-	-	-	-	++	-
UPN14	78	de novo	M2	46XX		-	-	-	++	++	-	++	-	-	+	++	++
UPN15	61	de novo	M2	46XX		++	-	-/+	++	-	-	-	-	-/+	-/+	++	-/+
UPN16	65	de novo	M2	46XY;t(7;11)(q32;q23)	mll rearrangement	++	-	-/+	+	++	-	nd	-	-	nd	++	+
UPN17	58	de novo	M2	46XY		+	-	+	+	+	-	-	-	-	-	++	-
UPN18	51	secondary	M2	Fail		+	-	-/+	++	-/+	-	-	-	-	+	++	-/+
UPN19	68	secondary	M2	Complex karyotype		++	-	+	++	+	-/+	+	-	-	-/+	++	++
UPN20	67	de novo	M2	46XY,del(22q)	bcr/abl fusion signal	++	-	+	++	+	-	+	-	-	-	++	++
UPN21	39	secondary	M2	Complex karyotype		++	-	-	++	++	-	+	-	-	-	++	+
UPN22	60	de novo	M2	46XX,t(8;21)(q22;q22)	aml/eto fusion signal	++	-	++	+	++	-	+	-	+	+	++	++
UPN23	8	de novo	M2	46XY,inv(16)		++	nd	nd	++	++	-/+	++	-	++	+	++	++
UPN24	64	secondary	M2	46XY		++	-/+	+	+	+	-/+	++	-/+	+	+	++	++
UPN25	64	de novo	M2	46XY		++	-	-/+	+	-	-	-	-	-	-	++	+
UPN26	84	de novo	M2	46XX		++	-	++	++	-	+	+	-	-/+	+	++	++
UPN27	67	secondary	M2	46XX		++	-	+	++	-/+	-	-/+	-	-/+	+	++	++
UPN28	71	secondary	M2	Fail		+	-	++	+	+	-	+	-	-	-/+	++	++
UPN29	33	de novo	M3	47XX;t(15,17)	pml-rara fusion signal	++	-	-/+	++	-	-	-/+	-/+	-	+	++	-
UPN30	68	secondary	M4	46XX,interstitial del (9)		++	-	++	+	++	-	-	-	-	-	++	++
UPN31	79	de novo	M4	46,XX		++	+	++	++	-	+	+	-	-/+	+	++	++
UPN32	17	de novo	M4	46XY		++	+	++	++	+	-/+	-	-/+	-/+	-	++	++
UPN33	74	secondary	M4	46XY,del(5q),del(12p)		nd	nd	nd	nd	nd	-	-	-	-	-	++	++
UPN34	86	de novo	M4	46XX		+	-/+	++	++	-	+	++	-/+	+	+	++	+
UPN35	62	de novo	M4	46XY;inv(16)		++	-	++	+	++	-	++	-	+	+	++	++
UPN36	18	secondary	M4	Fail	mll rearrangement	++	+	++	++	-	-	+	-/+	+	+	++	++
UPN37	23	de novo	M5	45X,-Y,del(9)(q21),del(22)(q11)		+	-	-	-	++	-	-	-	-	-	++	++
UPN38	89	de novo	M5	47XX,+ 21		nd	nd	nd	nd	nd	-/+	++	-	-	+	++	++
UPN39	51	de novo	M5	46XX		-	-	++	++	-	-	-	-	-	-/+	++	++
UPN40	85	de novo	M5	Fail		++	++	++	++	-	-	++	-	+	++	++	++
UPN41	77	primary	M5	Fail		++	++	++	++	-	++	++	-	+	++	++	++
UPN42	53	de novo	M5	46XY		+	+	++	++	nd	+	-/+	-	+	++	++	++

Table 4.1 Clinical features and immunophenotypic profile of AML cases.
Scoring for immunophenotype: ≤9% -, 10-19% -/+, 20-49% +, ≥50% ++

4.1.2 Cytokine-induced differentiation

4.1.2.1 Peripheral blood monocytes

Culture of peripheral blood monocytes with GM-CSF and IL-4 for 4 days led to the appearance of a population of cells with the characteristic morphological appearance of DC. Immunophenotypic analysis of these cells showed them to have expression of the DC marker CD1a and the costimulatory molecule CD40 and to have weak expression of CD80 and CD86 (Figure 4.1). Following maturation with CD40L the DC showed an increase in their intensity of expression of CD80 and CD86 consistent with maturation induction. In order to determine the optimal dose of CD40L to use for maturation of the DC (and by inference maturation of DLLC), a comparison of the effect of increasing concentrations of CD40L in DC maturation induction performed (Figure 4.2). CD40L was use either alone or in combination with a proprietary enhancer (Alexis corporation), purported to increase the biological activity of recombinant CD40L. From this data it can be seen that whereas at a concentrations of 1000ng/ml CD40L had a significant effect on the upregulation of costimulatory molecule expression, based on the mean fluorescence intensity (MFI), when the dose was less than 1000ng/ml this effect was much less marked. The addition of enhancer did however potentiate the effects of the CD40L particularly at the dose of 1000ng/ml CD40L.

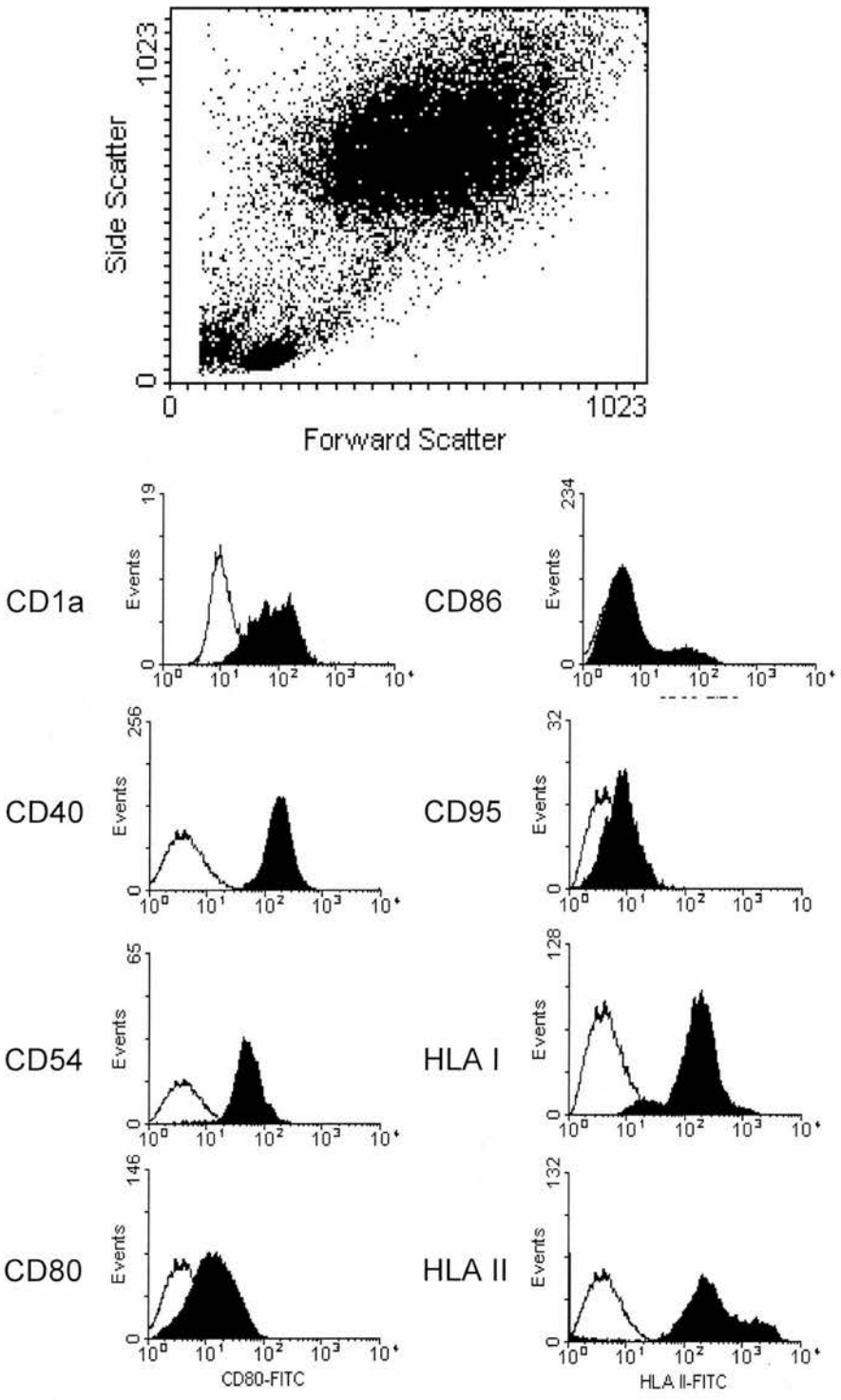


Figure 4.1 Immunophenotypic profile of immature monocyte-derived dendritic cells. Dot-plot shows the typical high forward and side scatter characteristics of DC. Histogram plots demonstrate CD1a, CD40, CD54, HLA class I and II molecule expression by DC. These Immature DC show weak CD80, CD86 and CD95 expression.

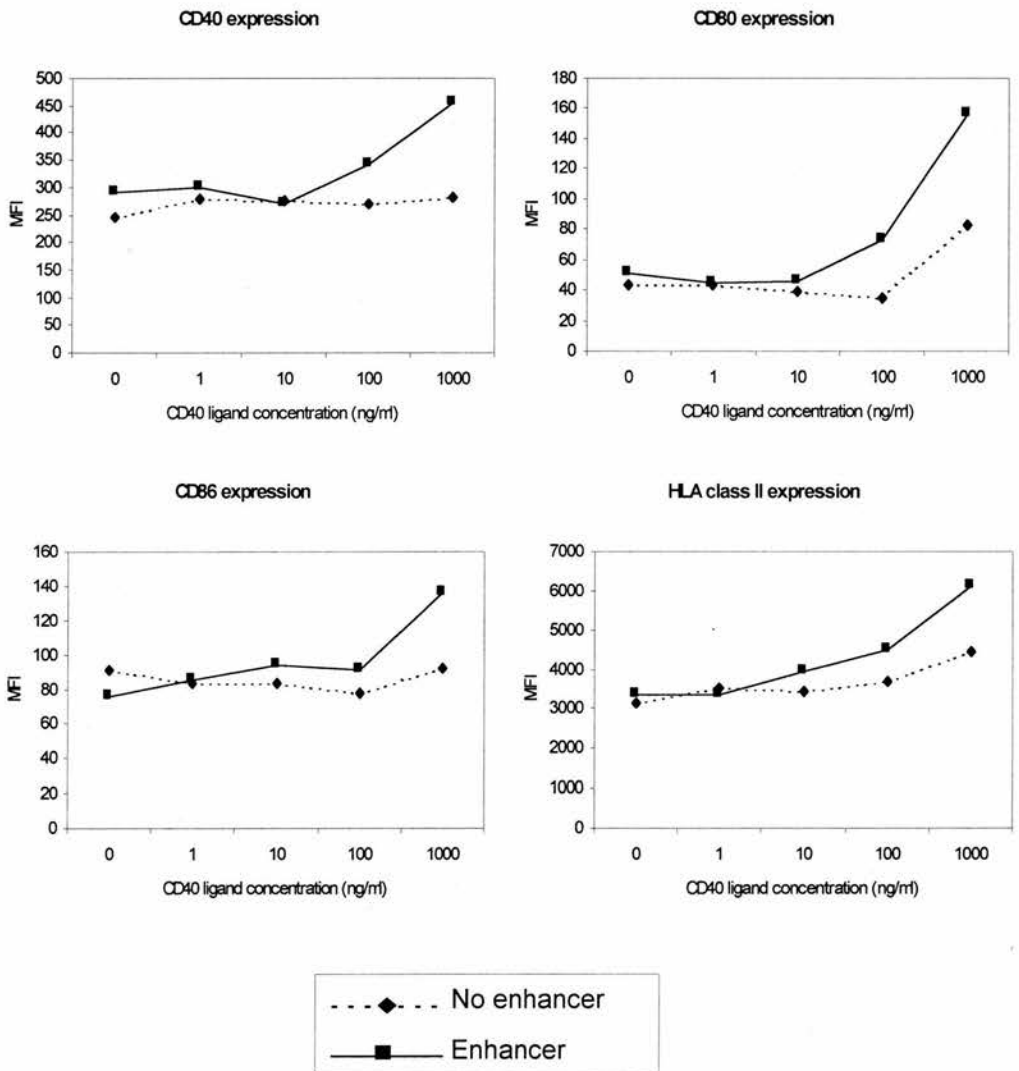


Figure 4.2 Maturation of dendritic cells.
Upregulation of costimulatory and HLA class II molecule expression by DC following maturation with CD40L +/- enhancer

4.1.2.2 Myeloid leukaemia cell lines

Cytokine differentiation to dendritic-like cells was attempted in the four leukaemia cell lines using the cytokine regimen of GM-CSF, IL-4 and CD40L for 7 days. At the end of the culture period no morphological or immunophenotypic evidence of DLLC differentiation was detected. Extending the culture period to 10 days similarly was ineffective at inducing differentiation. In order to circumvent the apparent resistance of the leukaemia cell lines to cytokine-induced differentiation, CD40 transduced cell lines were generated and these were cultured with CD40L in an attempt to induce costimulatory molecule expression. Three CD40 expressing cell lines were generated as described earlier. Of the three only a CD40 expressing KG1a subline showed evidence of differentiation. Following 7 days culture with GM-CSF, IL-4 and CD40L the leukaemia cells from the CD40.KG1a cell line showed upregulation of CD80 and HLA II expression but no CD86 expression (Figure 4.3). This experiment was repeated on three separate occasions with similar results obtained after each. Control cells from the CD40 expressing KG1a subline were culture in parallel in an identical manner except that TNFα was substituted for CD40L. The TNFα containing regimen failed to induce any upregulation of CD80 expression. Despite the immunophenotypic evidence of differentiation, morphologically the CD40.KG1a cells maintained an undifferentiated appearance throughout the culture period.

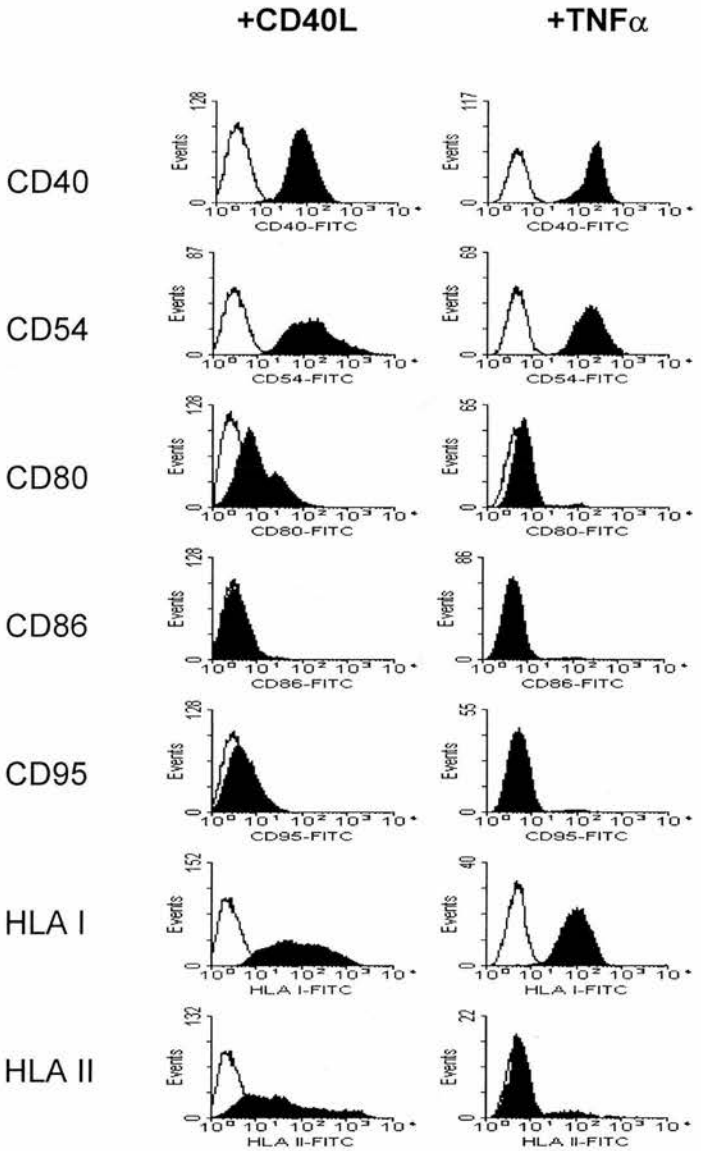


Figure 4.3 Immunophenotypic analysis of CD40.KG1a cell line following culture with CD40L or TNFα.
Expression of costimulatory molecules, CD95 and HLA I and II molecules on the cell line CD40.KG1a following 7 days culture in medium containing GM-CSF, IL4 and CD40L or TNFα.

4.1.2.3 Primary AML blasts

Leukaemia cells derived from 42 patients with AML were tested for their ability to undergo cytokine-induced differentiation. Of these 42 cases 22 were deemed to have successfully undergone DLLC differentiation based on changes in morphology and immunophenotypic profile. In 4 cases poor viability of the leukaemia cells over the culture period prevented further evaluation. The remaining 16 cases were deemed to be resistant to DLLC differentiation. The morphological and immunophenotypic evidence for DLLC differentiation is presented below. In order to confirm that the dendritic-like cells generated by cytokine-induced differentiation of leukaemia had indeed derived from the leukaemic clone FISH analysis was performed in two informative cases. IL-12(p70) production following maturation with CD40L was measured by ELISA. The functional properties of DLLC and differentiation resistant leukaemia cells were tested in allogeneic MLLR. Finally the ability of DLLC to generate leukaemia reactive autologous CTLs was assessed by cytotoxicity assays.

i/ Morphology

The morphological features that were deemed to represent successful DLLC under phase contrast microscopy were the appearance of loosely adherent clumps of cells that on high power examination had characteristic dendritic processes (Figure 4.4). Morphological features of DLLC following MGG staining of cytospin preparations were of large cells with a relatively low nuclear:cytoplasmic ratio again showing the characteristic dendritic processes (Figure 4.5). In contrast leukaemia cells from AML cases that were deemed to be resistant to DLLC differentiation maintained an undifferentiated morphological appearance.

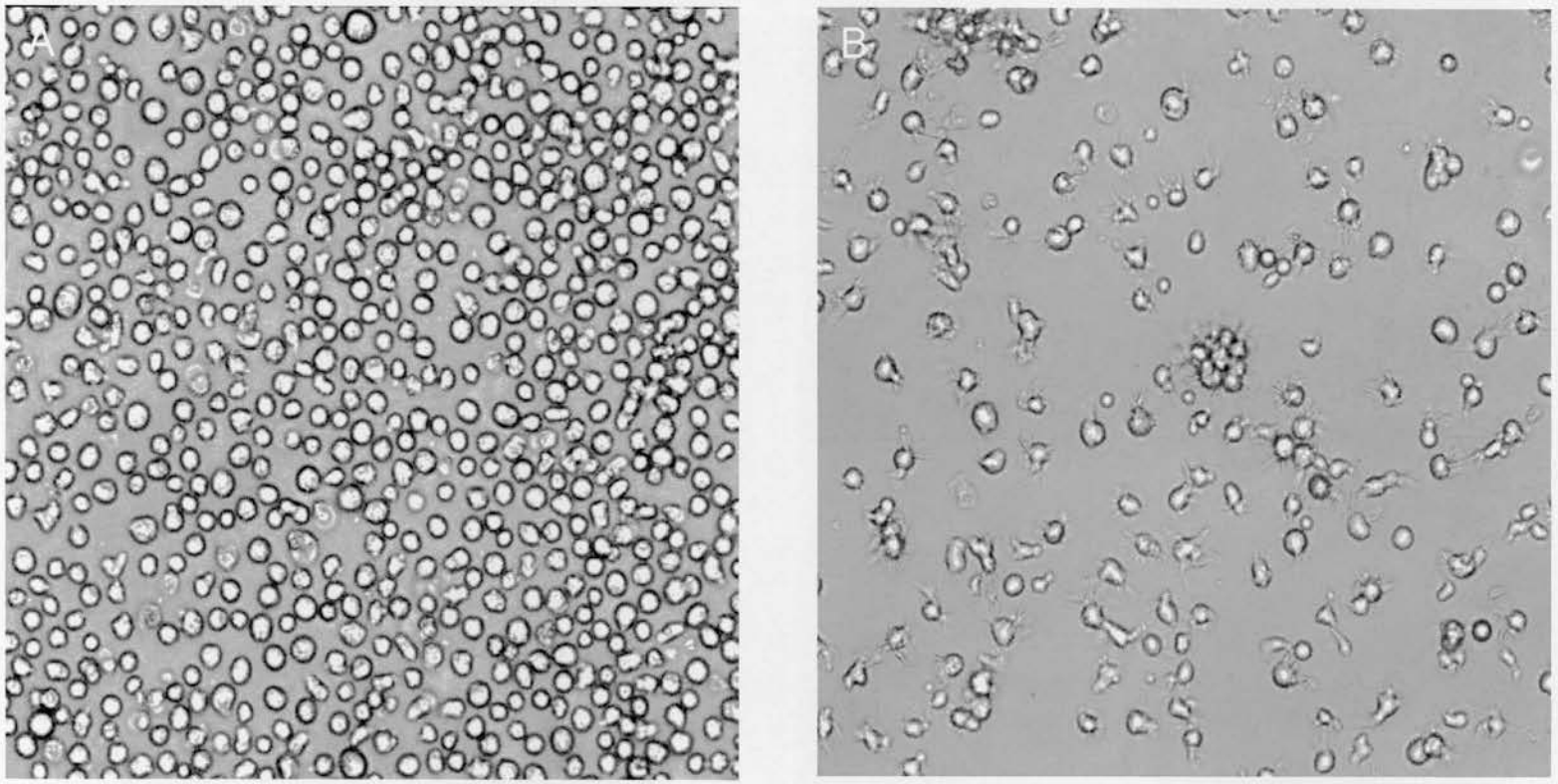


Fig 4.4 Comparison of the morphological features of undifferentiated leukaemia cells and dendritic-like leukaemia cells
Phase contrast microphotography (x1000 magnification)
A. Undifferentiated leukaemia cells
B. Following DLLC differentiation with the formation of cell clusters and cells possess characteristic dendritic processes

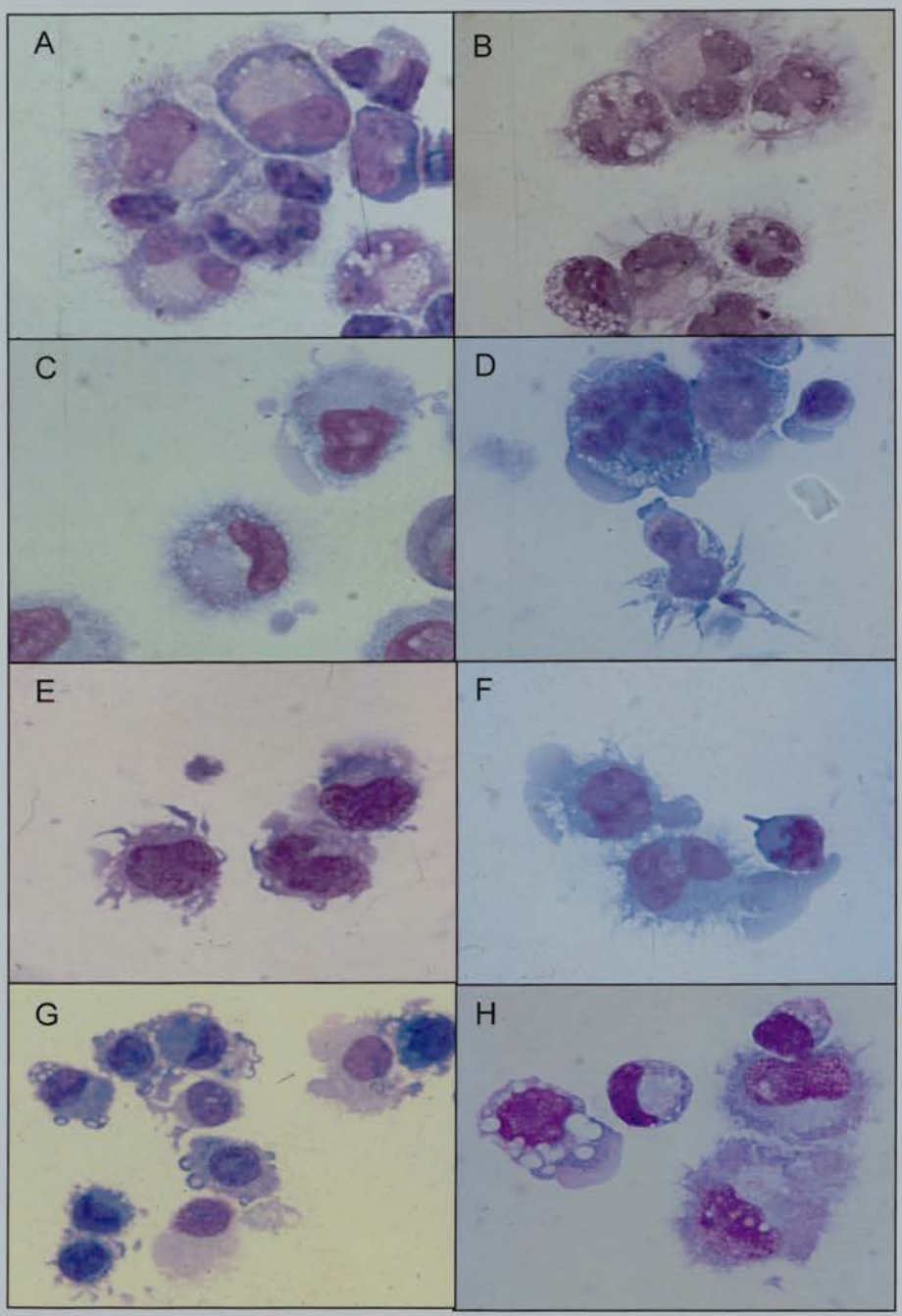


Figure 4.5 Comparison of the morphological features of dendritic cells and dendritic-like leukaemia cells.
MGG stained cytospin preparations of monocyte derived DC and DLLC generated from primary AML blasts.
A. and B. are DC derived by cytokine-induced differentiation of PB monocytes.
C., D., E., F., G. and H. are DLLC generated from primary AML blasts from AML cases UPN 3, 22, 31, 32, 38 and 41 respectively

ii/ Immunophenotypic profile

Successful DLLC differentiation of leukaemia cells was associated with the appearance of high forward and side scatter population on flow cytometric analysis. Gating on this population showed it to have expression of CD40 in combination with CD80 and/or CD86. The flow cytometric profile following gating on the high forward/side scatter population in one representative case showing DLLC differentiation is illustrated in Figure 4.6. Leukaemia cells from AML cases that failed to differentiate to DLLC, following culture with cytokines, showed no immunophenotypic evidence of DLLC differentiation. A comparison of the immunophenotypic characteristics of three AML cases that successfully underwent DLLC differentiation with three cases that were resistant to differentiation is illustrated in Figure 4.7. The DC marker, CD1a, was not found to be helpful in establishing whether DLLC differentiation had taken place as in only one patient, UPN 38, was it found to be expressed. The other DC marker CD83 was, however, a useful marker of DLLC differentiation. Dual-staining of leukaemia cells with CD40 and CD83 following their attempted cytokine-induced differentiation defined three separate populations: a CD40/CD83 dual negative population believed to correspond to undifferentiated leukaemia cells, a CD40 positive, CD83 negative population believed to correspond to leukaemia cells that were committed to differentiation but had not acquired the features of fully mature DLLC and finally a CD40/CD83 dual positive population corresponding to mature DLLC.

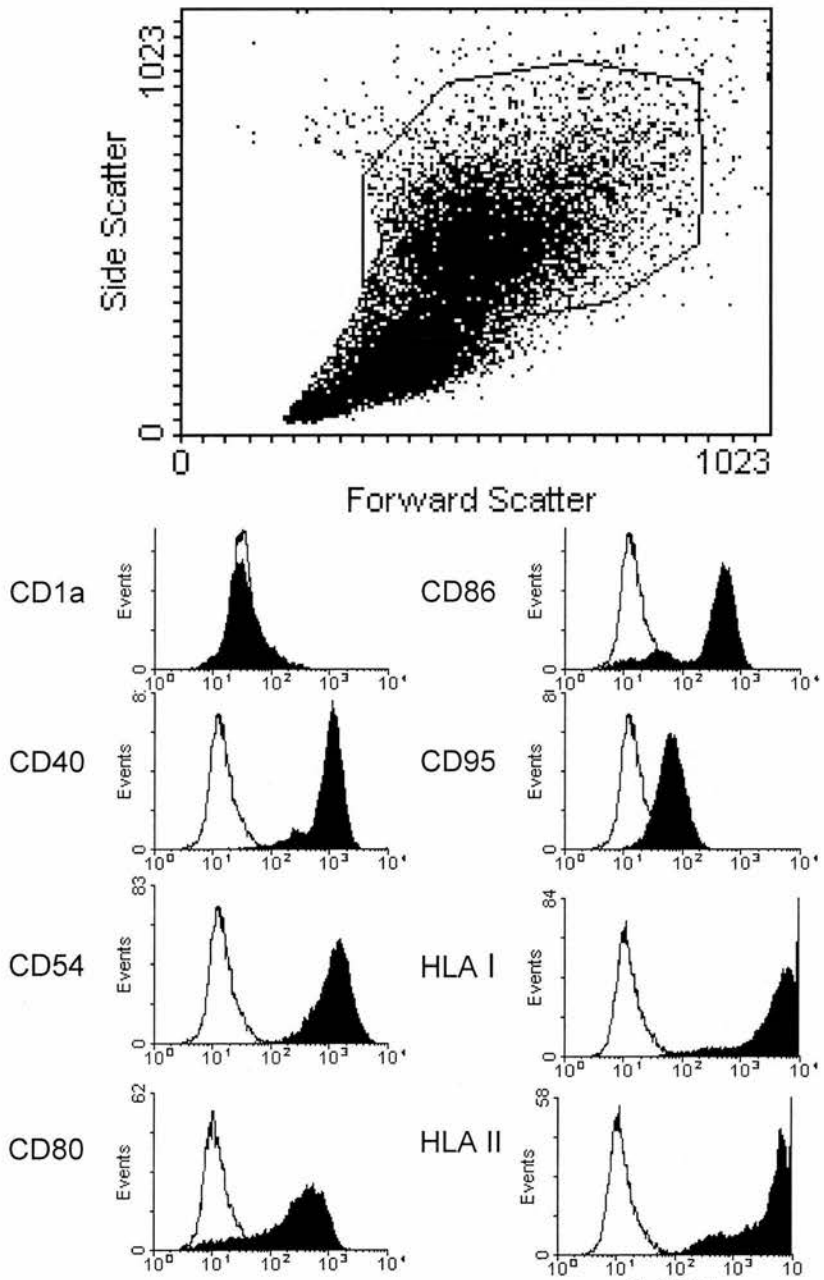


Figure 4.6 Immunophenotypic profile of dendritic-like leukaemia cells
Gated on the population with high forward and side scatter characteristics.
DLLC from one representative AML case, UPN 32.

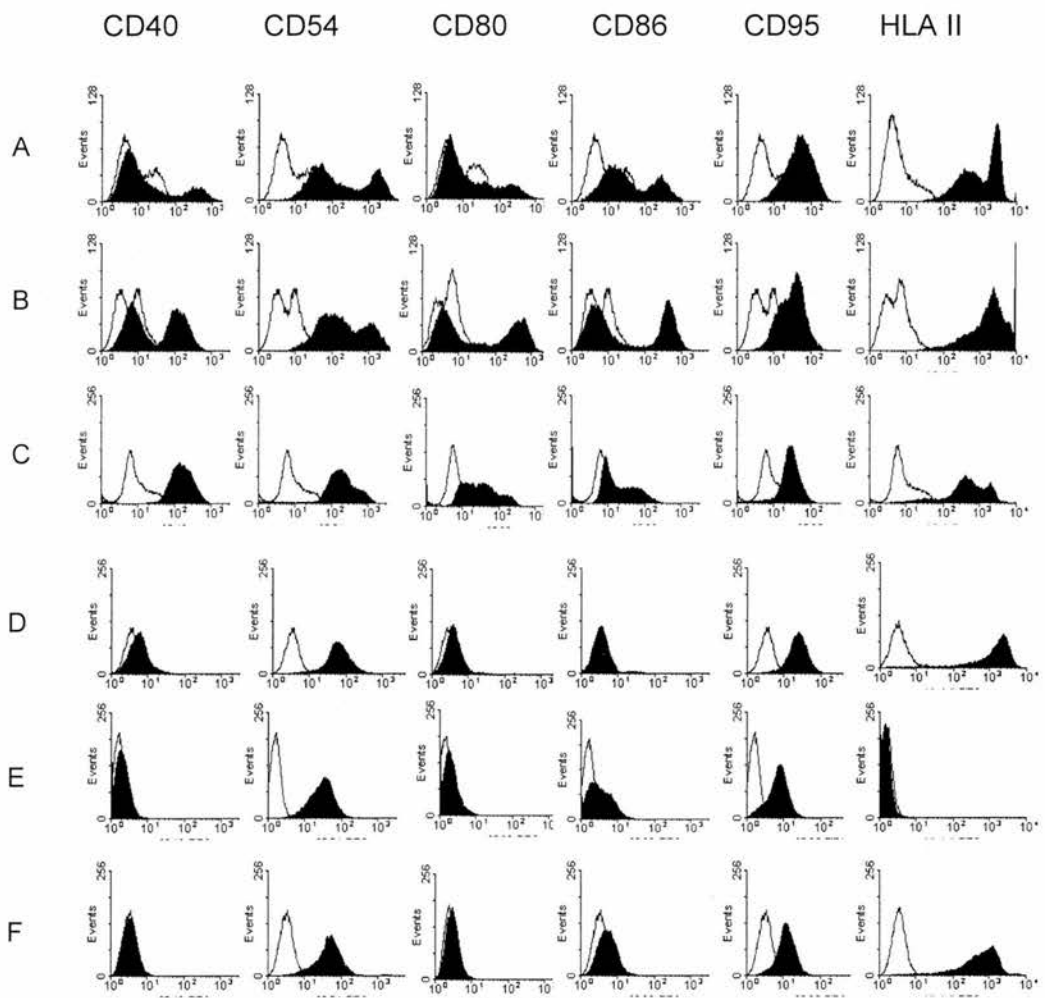


Figure 4.7 Comparison of immunophenotypic profile of dendritic-like leukaemia cells and differentiation resistant leukaemia cells.
Expression of Fas (CD95), ICAM-1 (CD54), costimulatory molecules (CD40, 80, 86), and HLA II molecules by primary leukaemia cells following attempted cytokine-induced differentiation to DLLC.
A., B. and C. are AML cases UPN 1, 32 and 38 respectively that underwent DLLC differentiation.
D., E. and F. are AML cases UPN 5, 13 and 20 respectively that failed to undergo differentiation

In AML cases where the leukaemia cells were considered to have successfully undergone DLLC differentiation, > 5% of the total population was CD40/CD83 dual positive whereas, in resistant cases, <5% of cells showed dual positivity for CD40/CD83 (Figure 4.8). Although DLLC consistently showed high expression of CD54 this was not found to be a useful marker for distinguishing differentiation permissive from differentiation resistant cases, as there was an upregulation in CD54 expression regardless of whether DLLC differentiation had taken place. DLLC did however show higher levels of CD54 expression, based on MFI, in comparison with leukaemia cells from differentiation resistant cases. A similar pattern was observed when analysing HLA class II expression. Although all the leukaemia cells that had underwent DLLC differentiation had a high levels of HLA class II expression only two of the differentiation resistant cases were HLA class II negative following attempted cytokine differentiation, with the majority of the cases showing HLA class II molecule upregulation. Culture of leukaemia cells with the cytokine regimen did not appear to have a significant effect on CD95 expression with no particular differences observed in the pattern of weak or absent expression, between differentiation permissive and differentiation resistant cases.

Using these immunophenotypic criteria for successful differentiation to DLLC it was possible to divide AML cases into whether they were permissive or resistant to cytokine-induced differentiation to DLLC. In Table 4.2 Part A. clinical features of AML cases permissive to DLLC differentiation are shown. Part B. shows the clinical features associated with the resistant cases.

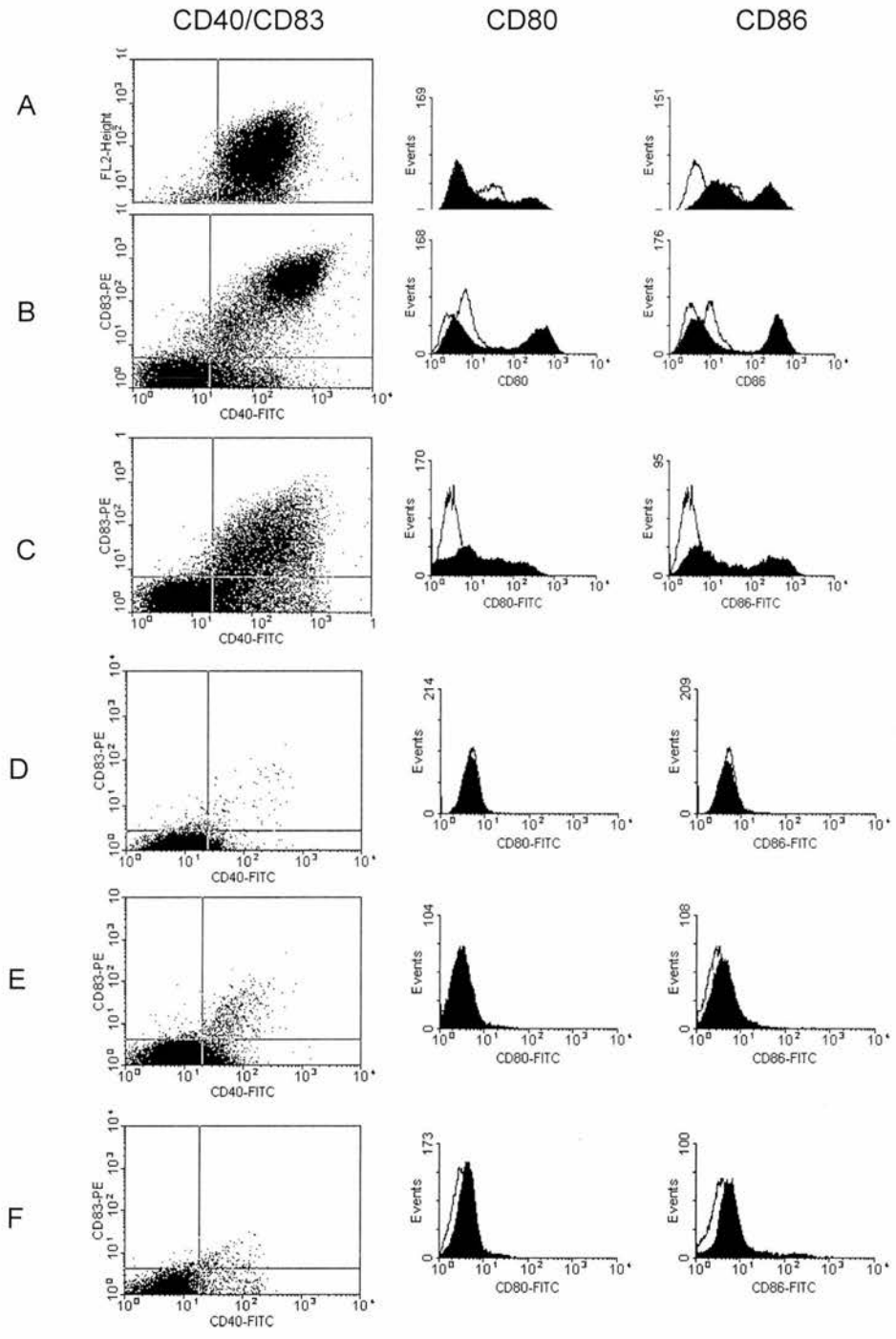


Figure 4.8 Immunophenotypic features characterising dendritic-like leukaemia cell differentiation.
A, B and C represent AML cases UPN14, UPN32 and UPN35 that underwent DLLC differentiation. Note the CD40/CD83 dual positive population and associated CD80 and CD86 expression.
D, E and F represent leukaemia cells from AML cases UPN2, UPN5 and UPN20that failed to show evidence of differentiation. Note the low numbers of CD40/CD83 expressing cells and lack of CD80 or CD86 expression (Quadrant markers set for isotype matched negative controls)

A

UPN	Age (yrs)	de novo/ secondary	AML Subtype	Karyotype
UPN1	82	de novo	M0	46,XX
UPN3	26	de novo	M1	47XY,+19
UPN4	64	de novo	M1	45XY
UPN6	73	de novo	M1	Fail
UPN9	11	de novo	M1	47XY,+ 8
UPN11	81	de novo	M2	46XX
UPN14	78	de novo	M2	46XX
UPN15	61	de novo	M2	46XX
UPN17	57	de novo	M2	46XY
UPN22	60	de novo	M2	46XX,t(8;21)
UPN23	8	de novo	M2	46XY,inv(16)
UPN24	64	secondary	M2	46XY
UPN30	68	secondary	M4	46XX,interstitial del chr9
UPN31	79	de novo	M4	46,XX
UPN32	17	de novo	M4	46XY
UPN34	86	de novo	M4	46XX
UPN35	62	de novo	M4	46XY;inv(16)
UPN38	89	de novo	M5	47XX,+ 21
UPN39	51	de novo	M5	46XX
UPN40	85	de novo	M5	Fail
UPN41	77	de novo	M5	Fail
UPN42	53	de novo	M5	46XY

B

UP No.	Age (yrs)	de novo/ secondary	AML subtype	Karyotype/ *FISH analysis*
UPN2	49	de novo	M1	Fail
UPN5	77	de novo	M1	Complex karyotype
UPN7	62	de novo	M1	46XY,del(12p)
UPN10	50	de novo	M2	Fail
UPN12	56	de novo	M2	46XX
UPN13	29	de novo	M2	46XX, partial del 11(q23)/ *mll rearrangement*
UPN16	65	de novo	M2	46XY;t(7;11)(q32;q23)/ *mll rearrangement*
UPN18	51	secondary	M2	Fail
UPN19	68	secondary	M2	Complex karyotype
UPN20	67	de novo	M2	46XY,del(22q)/ *bcr/abl fusion signal*
UPN25	64	de novo	M2	46XY
UPN26	84	de novo	M2	46XX
UPN27	67	secondary	M2	46XY
UPN28	71	secondary	M2	Fail
UPN33	74	secondary	M4	46XY,del(5q),del(12p)
UPN37	23	de novo	M5	45X,-Y,del(9)(q21),del(22)(q11)

Table 4.2 Comparison of clinical features of differentiation permissive and resistant AML cases.
A. Cases in which the leukaemia cells were deemed to have undergone cytokine-induced differentiation to DLLC
B. Cases that were resistant to DLLC differentiation

There were a range of FAB subtypes in both permissive and resistant cases suggesting that this was not a critical factor in determining differentiation potential. In respect of karyotypic features, however, there was a preponderance of poor risk karyotypic features amongst the differentiation resistant cases. Examples of these poor risk karyotypic features include the t(9;22) translocation, rearrangements involving the mll gene and complex karyotypic changes. Clinically leukaemias possessing these types of karyotypic features are characterised by poorer treatment outcomes (Grimwade *et al*, 1998). In contrast, no poor risk karyotypes were present amongst the differentiation permissive cases and three cases possessed the good risk karyotypes, either t(8;21) or inv(16), which are associated with better treatment outcomes (Grimwade *et al*, 1998).

iii/ IL-12(p70) production

Tissue culture supernatants were collected from leukaemia cell cultures that had been cultured for the first 4 days with GM-CSF and IL-4 and the final 3 days of culture with GM-CSF, IL-4 and CD40L. Figure 4.9 shows the levels of IL-12(p70) production by leukaemia cells that had undergone DLLC differentiation in comparison with those that did not. There was no significant production of IL-12(p70) by leukaemia cells taken from differentiation resistant cases. IL-12(p70) production by leukaemia cells that had undergo DLLC differentiation was restricted to five AML cases (UPN 31, 32, 34, 41, 42). All five of these cases were of AML FAB subtypes M4 or M5 i.e. having a major monocytic component.

The AML case UPN 31 that had the highest level of IL-12(p70) production was selected for further study of DLLC maturation induction. Following the initial 4 days of culture with GM-CSF and IL-4 leukaemia cells from AML case UPN 31 underwent maturation using a variety of maturational agents, either alone or in combination, for the final 3 days of the culture period. Tissue culture supernatants were measured for IL-12(p70) production by DLLC and cells were analysed for their level of costimulatory molecule expression. Figure 4.10 shows the IL-12(p70) production induced by a variety of maturational agents. These results are representative of four independent experiments. IL-12(p70) production by DLLC in the absence of any additional maturational stimulus was negligible. Although CD40L alone did induce significant levels of IL-12(p70) by DLLC there was a marked potentiation in IL-12(p70) production when the proprietary enhancer was used in combination with CD40L, and further potentiation when polyI:C was added to CD40L and enhancer. PolyI:C when used on its own was associated with minimal

levels of IL-12(p70) production by DLLC. The best combination of maturational agents, that of CD40L, enhancer and polyI:C was significantly better at inducing IL-12(p70) production than GM-CSF, IL-4 alone ($p<0.05$) or poly:IC alone ($p<0.05$). The expression of CD80 and CD86 was similarly enhanced by using the combination of CD40L, enhancer and poly:IC in comparison with no additional maturational agents or with CD40L alone (data not shown).

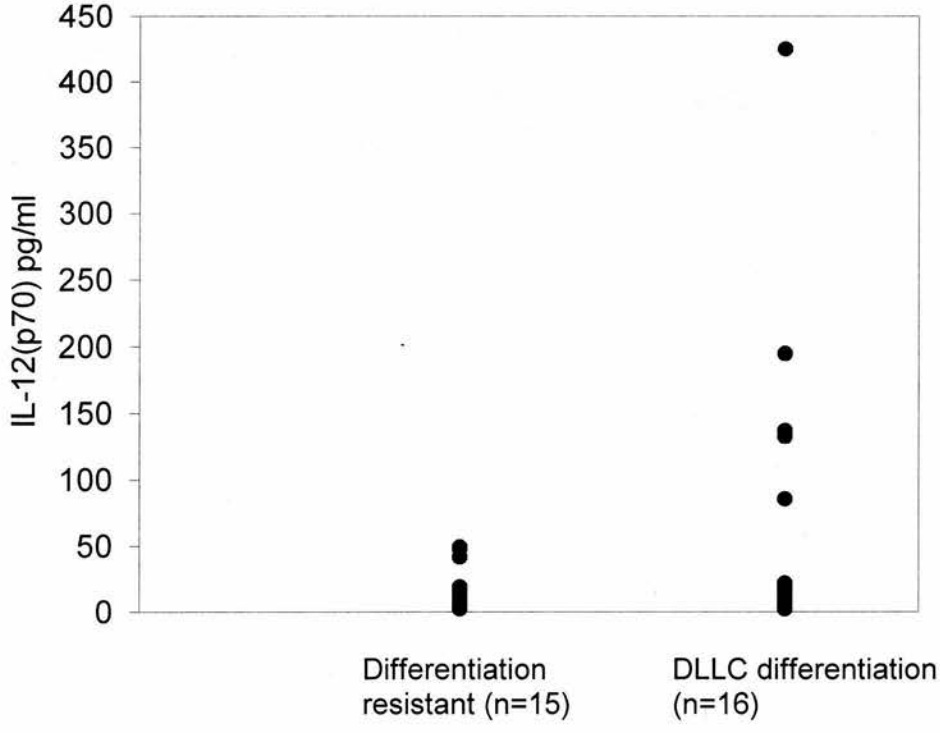


Figure 4.9 IL-12 production following cytokine-induced differentiation. Comparison between differentiation resistant and permissive leukaemia cells following 4 days culture in GM-CSF, IL-4 and then 3 days with GM-CSF, IL-4 and CD40L

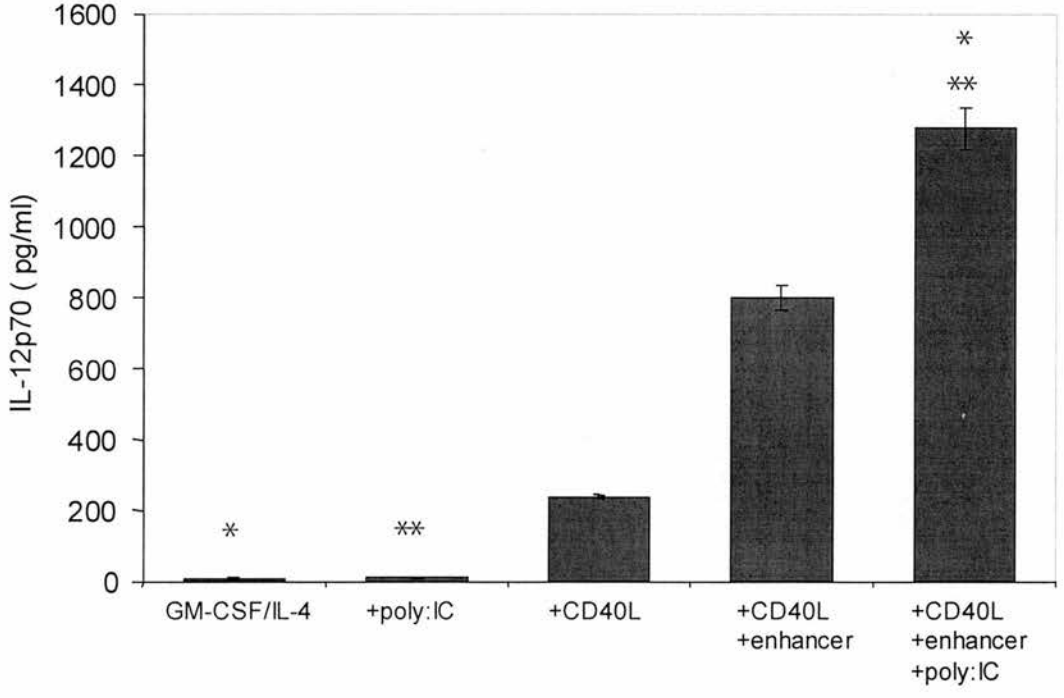


Figure 4.10 Comparison of various maturational agents ability to induce IL-12 production by dendritic-like leukaemia cells.
IL-12(p70) production by dendritic-like leukaemia cells following maturation using combinations of poly:IC, CD40L and enhancer given in addition to GM-CSF and IL-4. Dendritic-like leukaemia cells that had no additional maturation stimulus were cultured in GM-CSF and IL-4 alone. Data shown are mean +/-SD of triplicate cultures. Results are representative of four independent experiments. (*$p<0.05$)(**$p<0.05$)

iv/ FISH analysis

In order to establish that the dendritic-like cells generated by cytokine-induced differentiation of leukaemia cells had indeed derived from the leukaemic clone FISH analysis was performed in two potentially informative cases. In both cases induction of DLLC differentiation was performed by culturing leukaemia cells with the standard cytokine regimen for 7 days. Successful DLLC differentiation was confirmed on the basis of morphological changes and characteristic immunophenotypic profile. Cytospin slide preparations were made of DLLC from both cases. DLLC from AML case UPN 22 were unselected. This case was associated with the AML-associated chromosomal translocation t(8;21). FISH analysis for this rearrangement revealed that 198 of 200 cells counted (99%) possessed the t(8;21) translocation. Although the DLLC were unselected it was assumed that as the DLLC comprised greater than 25% of the total population, based on immunophenotypic analysis, and as the level of positivity by FISH analysis for t(8;21) was 99%, the DLLC must possess the rearrangement. The second case, UPN 35, had the AML-associated chromosomal rearrangement, inv(16). In this case, prior to FISH analysis, the DLLC were selected for on the basis of CD83 expression. FACS analysis of the CD83 positively selected population confirmed dual expression of CD40 and CD83. FISH analysis of the CD83 positively selected population revealed that 170 of 200 cells (85%) possessed the inv(16) rearrangement confirming that the DLLC had indeed derived from the leukaemic clone (Figure 4.11).

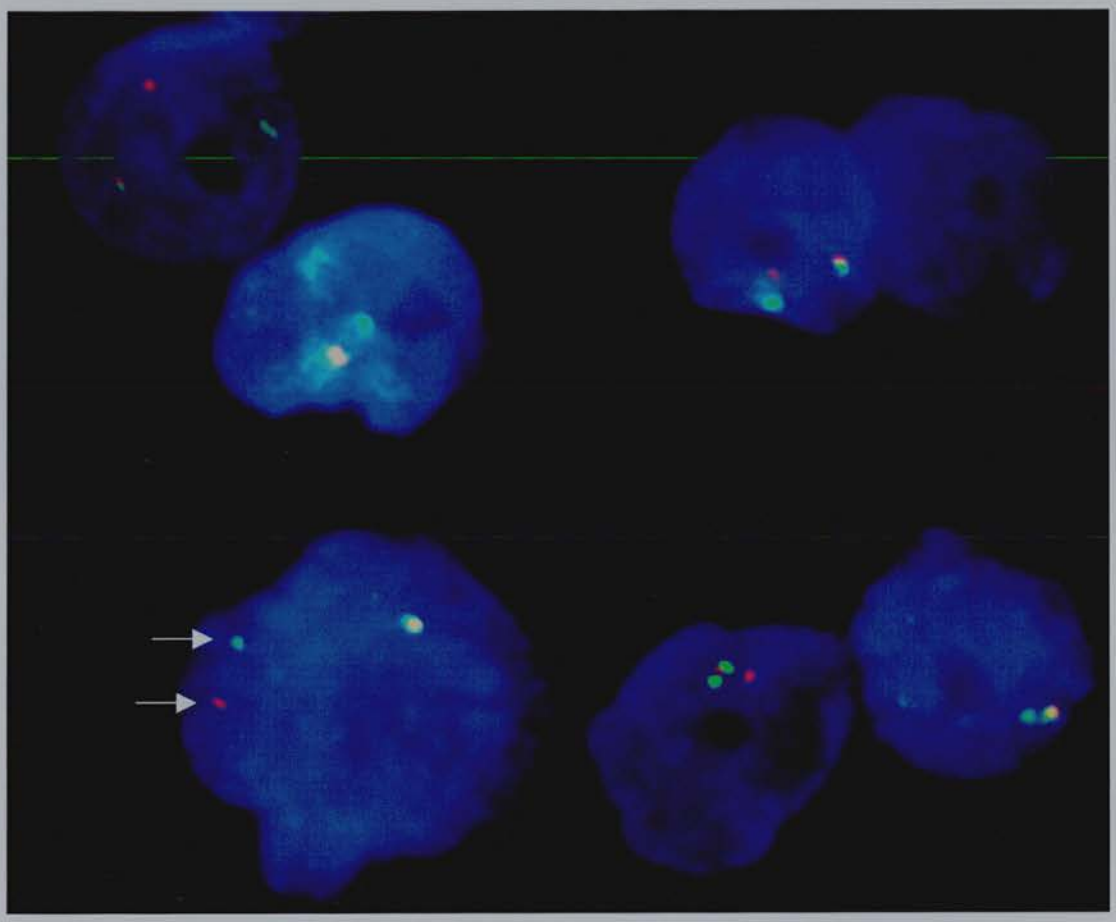


Figure 4.11 FISH analysis of dendritic-like leukaemia cells
Arrows mark the positions of the two probes that have been pulled apart by the inv(16) chromosomal rearrangement.
Analysis performed on cytospin slides of DLLC, generated from leukaemia cells from patient UPN 35, following MACS selection on the basis of CD83 expression

v/ Allogeneic MLLR

MLLR were performed to compare the ability of cytokine cultured leukaemia cells to stimulate the proliferation of allogeneic T lymphocytes in comparison with autologous unmodified leukaemia cells. Initially quantification of cellular proliferation was attempted using a colorimetric assay. This assay is based on the ability of metabolically active cells to convert a tetrazolium compound, MTS, to a formazan product. The amount of formazan generated can be determined by measuring the absorbence at 490nm and is proportional to the number of living cells in culture. Despite studies showing comparable sensitivity between this assay and the titriated thymidine assay (Russell & Vindelov, 1998), the colorimetric assay was unable to detect any differences in proliferation by responder T cells induced by various stimulators. Therefore this assay was abandoned in favour of a radioisotope-based assay. Using titriated thymidine uptake as a read out of cell proliferation DLLC were found to be potent simulators of allogeneic T lymphocyte proliferation in MLLR (Figure 4.12). In contrast freshly thawed unmodified leukaemia cells, from the same AML cases, were only weakly immunostimulatory. Leukaemia cells from differentiation resistant AML cases following attempted cytokine-induced differentiation were also found to be poor weak stimulators of allogeneic T lymphocytes (Figure 4.13).

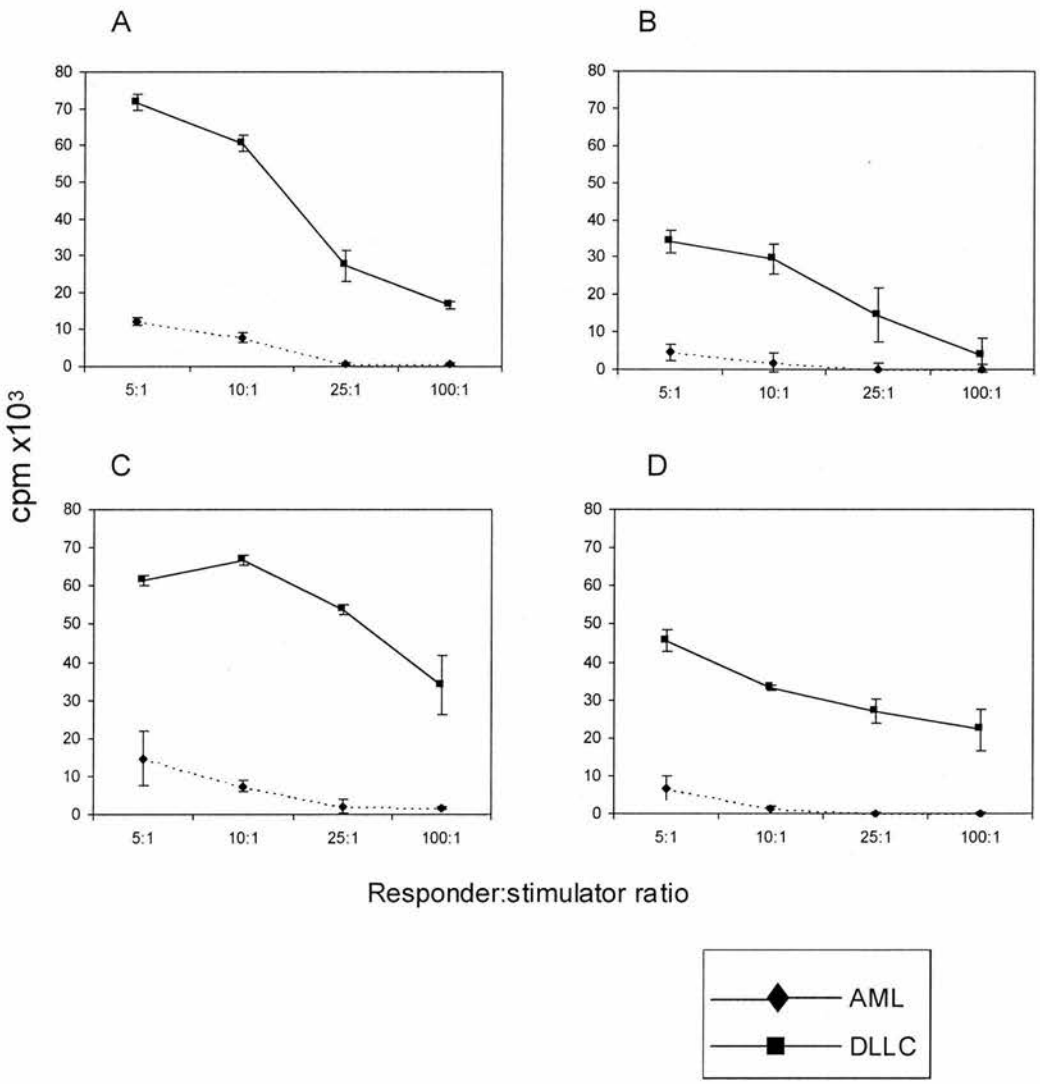


Fig 4.12 Allostimulatory properties of dendritic-like leukaemia cells.
Allogeneic MLLR. Responders: allogeneic lymphocytes. Stimulators: leukemic cells either unmodified (AML) or following DLLC differentiation (DLLC) derived from the same patient. Experiments were performed using leukemic cells from AML cases A. UPN14 B. UPN32 C. UPN35 D. UPN42

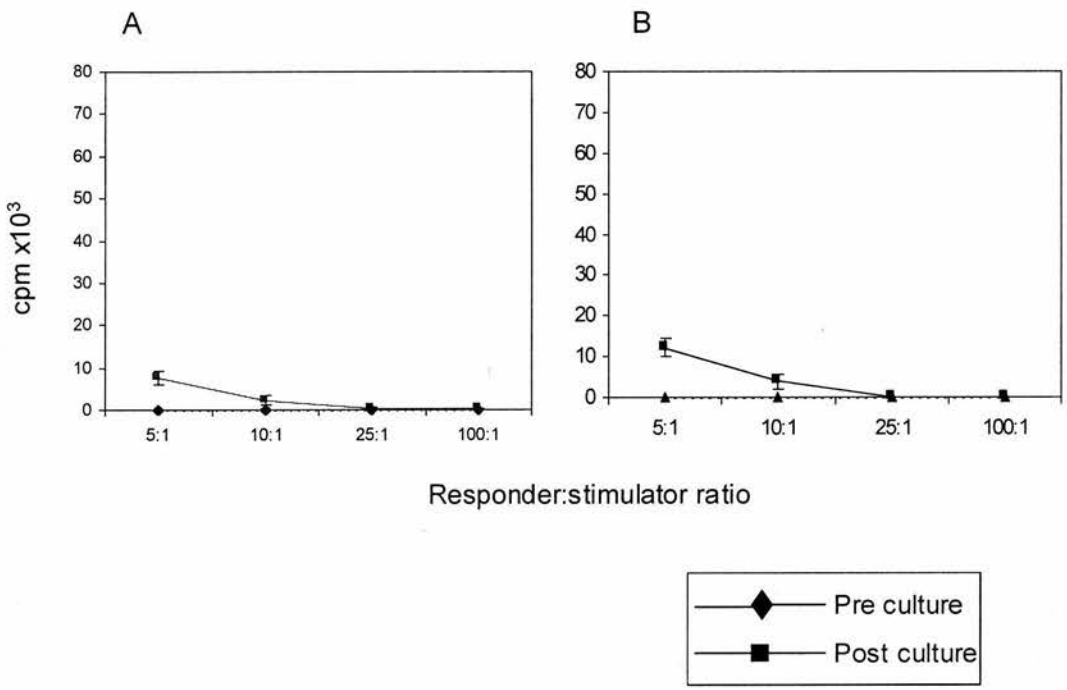


Fig 4.13 Allostimulatory properties of differentiation resistant leukaemia cells. Allogeneic MLLR. Responders: allogeneic lymphocytes. Stimulators: leukemic cells either unmodified (Pre culture) or following attempted DLLC differentiation (Post culture) from AML cases A. UPN5 B. UPN13 that showed resistance to DLLC differentiation

vi/ Generation of autologous leukaemia reactive CTL

In order to generate autologous leukaemia reactive CTL, T lymphocytes were harvested from AML patients in remission and co-cultured with mitomycin-C treated DLLC. As the number of T lymphocytes obtained from PB was insufficient to proceed directly to co-culture experiments, it was first necessary to expand T cell numbers. This was achieved by using anti-CD3 mAb to induce T cell activation followed by culture of the cells in IL-2 containing medium. Although this was an effective method of expansion it was found that after a 2-3 weeks culture period there was a loss in T cell viability. Therefore in later experiments the T lymphocyte survival factor IL-7 (Tsuda *et al*, 2000) was used in combination with IL-2 in order to prevent T cell apoptosis. Because the T cells were stimulated in this non-specific manner for the purposes of cytotoxicity assays a control was included with T lymphocytes cultured with cytokines alone acting as effectors. The first cytotoxicity experiments were performed using the LDH release assay. Although in the first two AML cases tested, apparent leukaemia specific cytotoxicity by CTL that had been generated by co-culture with DLLC was detected, there was a high degree of variability between experimental wells and the difference between target cell maximum and spontaneous release was very low (Figure 4.14). Therefore a different assay system was adopted based on flow cytometric analysis of annexin-V/PI staining as markers of apoptosis. Although it was possible to achieve a good separation between the PKH26 stained effectors and the PKH26 unstained targets on the basis of fluorescence intensity in the FL2 channel, for the purposes of analysing apoptosis in the target population it became apparent that non-target associated events were falling into the FL2 negative gate and skewing the results by increasing

the numbers of PI positive cells. This was particularly a problem at high effector:target ratios. It is likely that these FL2 negative, PI positive events represented cell fragments and debris derived from the effector cell population. Even by performing lymphoprep separation prior to the cytotoxicity assay this population still appeared within the acquisition gate. Because of this limitation to the flow cytometric assay the LDH assay was revisited. One of the positive findings from the flow cytometric based assay was that it provided information on the viability of the target cell population. It became apparent that in many cases the leukaemia cell targets were apoptotic prior to performing the cytotoxicity assay. Because a high proportion of the target cell population were non-viable this made detection of CTL induced cell death more difficult. Therefore in order to improve the assay sensitivity apoptotic cells were removed by immunomagnetically labelling then with annexin-V microbeads and running the cells through a miniMACS column. This proved a highly effective method of generating a target cell population with a very low proportion of non-viable cells and as a consequence a very low level of spontaneous LDH release for the purposes of the LDH release assay (Figure 4.15). Unfortunately very few patients with AML, in whom leukaemia cells had been harvested and stored, subsequently went on to achieve remission. Therefore in only one case was it possible to perform the LDH release assay following its optimisation. No leukaemia specific cytotoxicity was detected in that particular case (Figure 4.14).

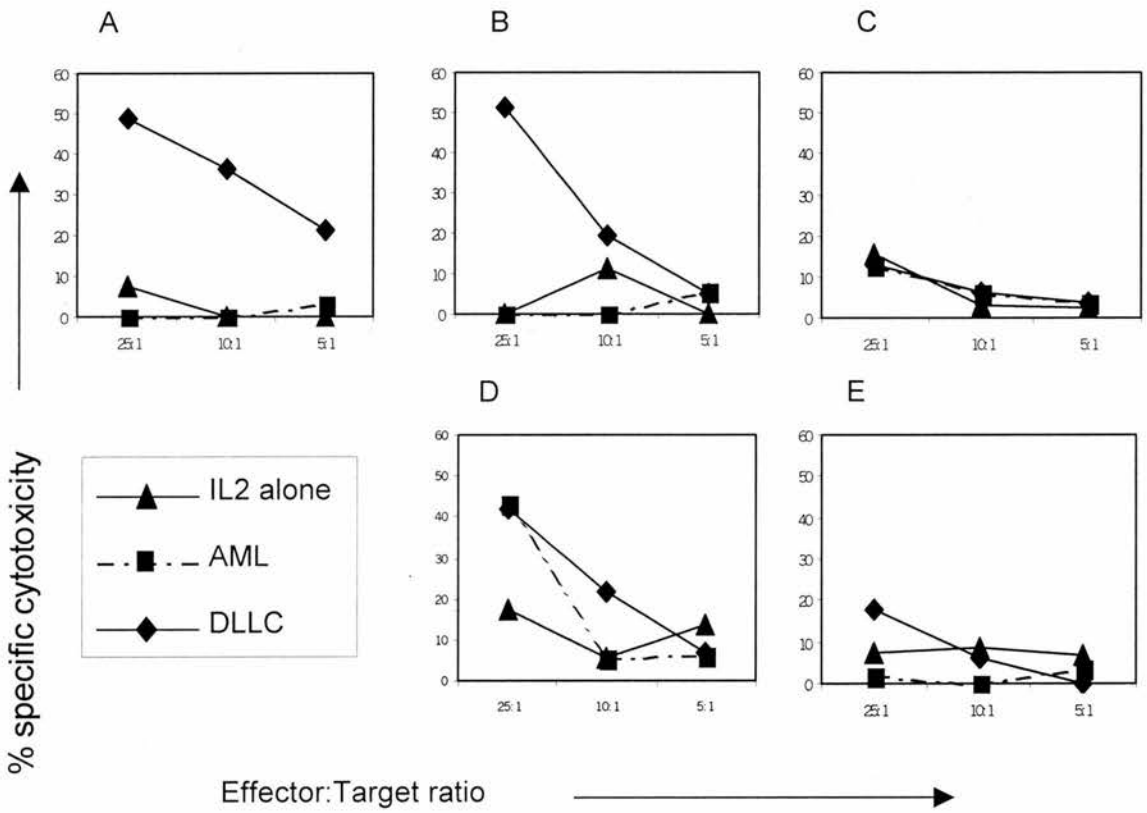
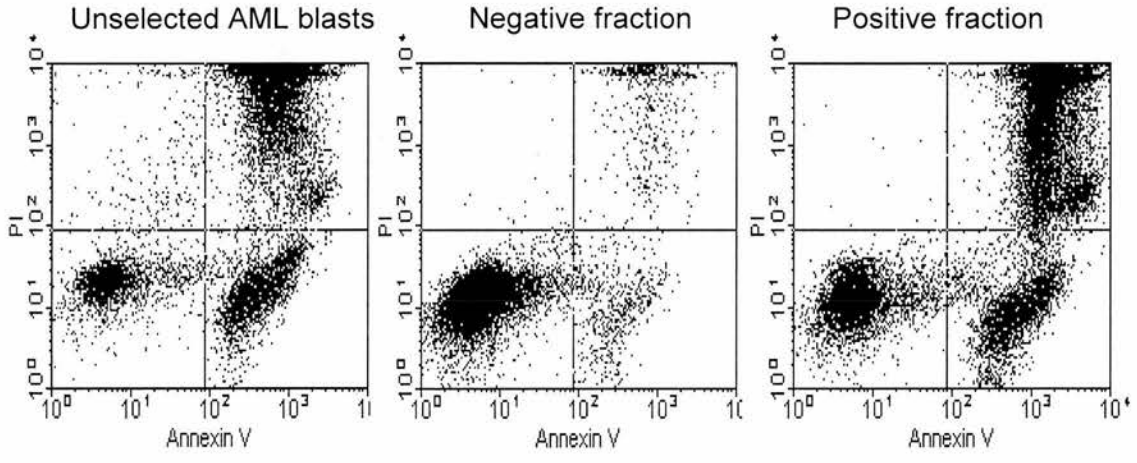


Figure 4.14 Autologous cytotoxicity assays.
Targets; freshly thawed AML blasts. Effectors; autologous lymphocytes either cultured with IL2/IL7 alone or co-cultured for 72 hours with unmodified leukaemia cells or dendritic like leukaemia cells. Assays A, B and C performed using the LDH release assay, D and E with a flow cytometric based assay. Apoptotic target cells were removed by magnetic microbead selection with annexin V microbeads prior to performing the cytotoxicity assay in C, D and E.



	Unselected AML blasts	Negative fraction	Positive fraction
Annexin V -/PI -	29%	88%	17%
Annexin V +/PI -	18%	6%	21%
Annexin V +/PI+	53%	6%	62%

Figure 4.15 Removal of apoptotic cells by MACS selection
Dot plot showing annexin V and PI staining of target cells either unmanipulated or following immunomagnetic separation with annexin-V microbeads. The relative percentages of annexin-V and PI positive and negative cells are illustrated in accompanying table. The early apoptotic population falls within the annexin-V+/PI- quadrant, the late apoptotic cells within the annexin-V+/PI+ quadrant and healthy cells in the annexin-V-/PI- quadrant.

4.1.3 Differentiating agents

4.1.3.1 Calcium Ionomycin

Despite the published studies presenting evidence that calcium ionomycin induced dendritic cell differentiation in the HL60 cell line and in primary leukaemia cells these findings could not be reproduced. After culture of the HL60 cell line with calcium ionomycin for 48 hours, at doses ranging from 25ng/ml to 400ng/ml, there was no upregulation of CD40, CD80 or CD86 expression detectable. In addition the leukaemia cells maintained an undifferentiated morphological appearance. Primary AML blasts derived from 6 patients were cultured for 3 days with calcium ionomycin at 375ng/ml in combination with GM-CSF and IL-4. Again there was no morphological or immunophenotypic evidence of differentiation detected.

4.1.3.2 Trichostatin, Azacytidine and Bryostatin

In nine of the AML cases in which the leukaemia cells were resistant to cytokine-induced differentiation to DLLC, further experiments were performed in which differentiating agents were used in combination with cytokines. TSA, AZA and BRYO were used alone or in combination for the first 72 hours of the culture period. The doses of TSA and AZA had been previously determined based on their effects on cell viability of leukaemia cells from a single AML case (UPN20). When used alone in doses exceeding 100ng/ml for TSA and 2μM for azacytidine there was a >50% decrease in cell viability, based on flow cytometric analysis following PI staining. When used in combination, doses of TSA greater than 75ng/ml and AZA greater than 1μM were associated with a >50% decrease in cell viability. The dose of

BRYO used was based on data from previously published studies on BRYO induced differentiation in leukaemia cells (Kaneki *et al*, 1999; Steube & Drexler, 1993). A comparison of the differentiating effects of TSA, AZA and BRYO, in combination with cytokines is shown in Figure 4.16. Culture with cytokines alone for the first 4 days was able to induce very little differentiation based upon the percentage of cells showing either dual expression of CD40/83 or single expression of CD40. The addition of TSA and AZA alone or in combination was ineffective at allowing differentiation to proceed. In contrast BRYO was effective at inducing DLLC differentiation with 25% of the total cell population showing dual expression of CD40 and CD83 and 31% show single expression of CD40. Using TSA and AZA in combination with BRYO did not lead to any improvement in the degree of differentiation but did considerably decrease cell viability. CD80 expression mirrored the changes in CD40 and CD83 expression. Cytokines alone or in combination with TSA and AZA failed to induce any upregulation in CD80 expression. BRYO when given alone or in combination led to significantly increased CD80 expression but was most effective when given alone. Morphologically DLLC generated by BRYO treatment of leukaemia cells differed from those generated by cytokine-induced differentiation of permissive leukaemia cells in that they remained adherent and failed to form clusters when viewed under phase contrast microscopy. However under phase contrast microscopy BRYO generated DLLC did show the characteristic dendritic processes of DLLC (Figure 4.17). On MGG stained cytospin preparations BRYO generated DLLC were indistinguishable from those generated by cytokines alone.

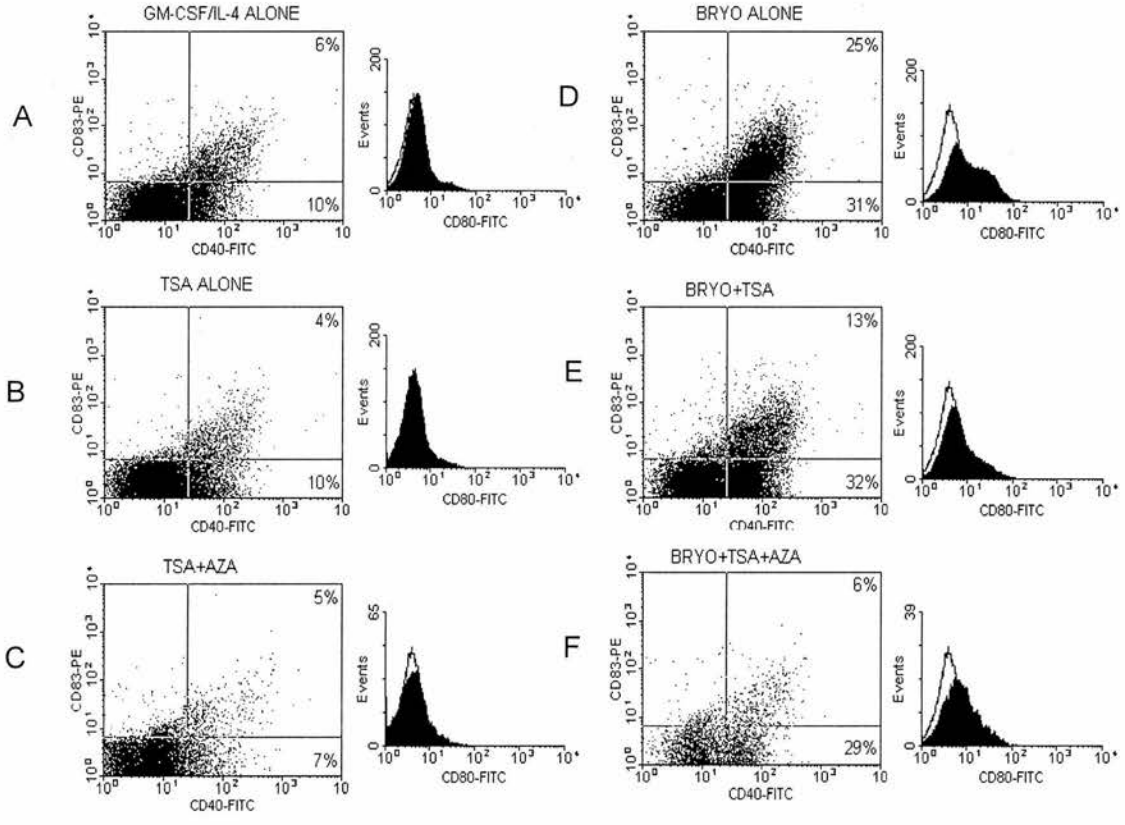


Figure 4.16 Differentiating agents given in combination with cytokines to induce differentiation.

Attempted differentiation induction of leukaemia cells from case UPN20 using differentiating agents in combination with cytokines.

A. Leukaemia cells cultured with GM-CSF and IL-4 alone for the first for 4 days of the culture period

B. TSA added to cytokines

C. TSA and AZA added to cytokines

D. BRYO added to cytokines

E. BRYO and TSA added to cytokines

F. BRYO, TSA and AZA added to cytokines

Dot plots represent dual staining for CD40 and CD83. Percentages of CD40/83 dual positive and CD40 single positive cells located in upper right and lower right quadrants respectively (Quadrant markers set for isotype matched negative controls). The histograms plots demonstrate CD80 expression by the total cell population (Closed histogram CD80 expression, open histogram staining with isotype matched negative control mAb).

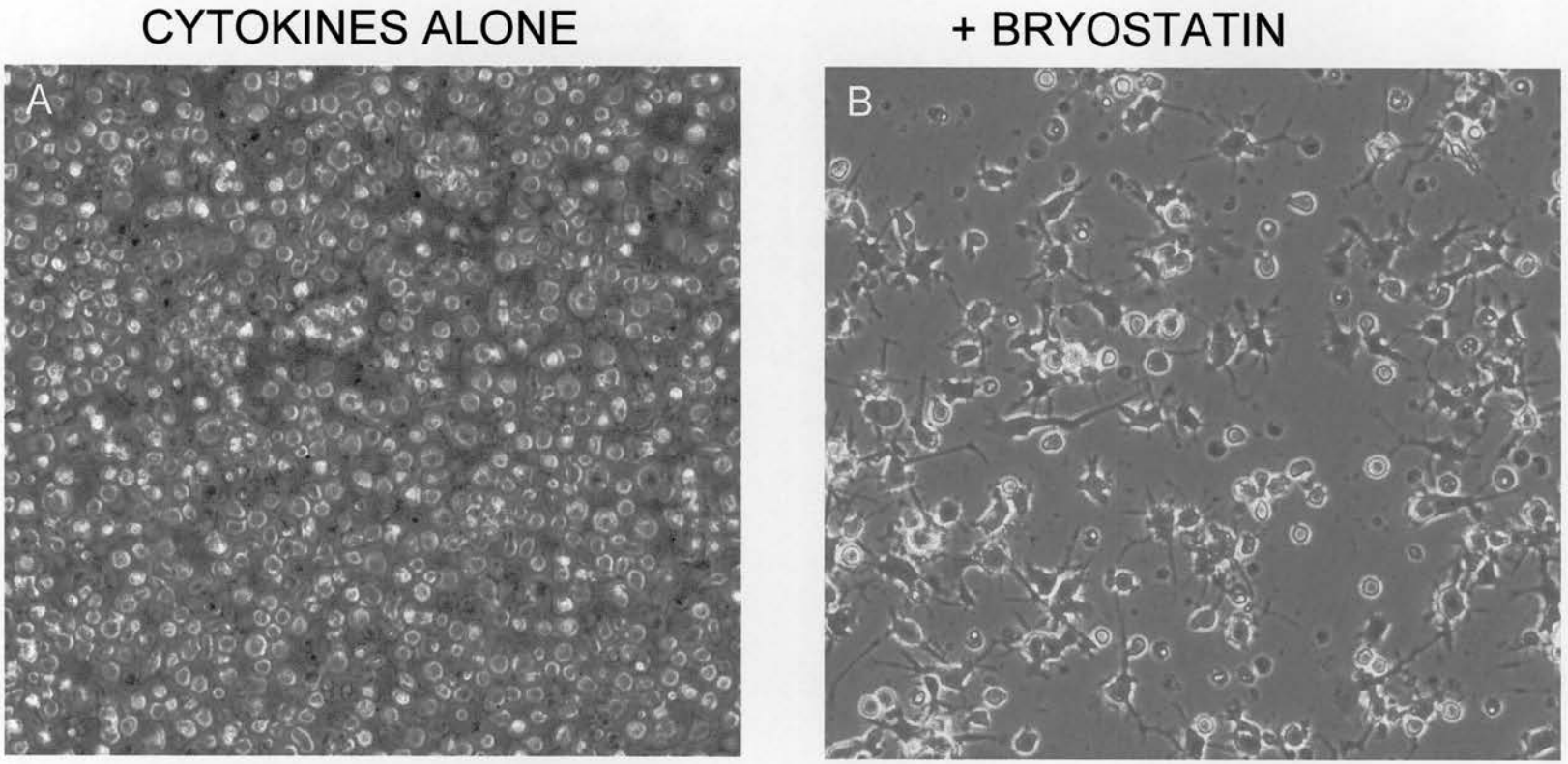


Fig 4.17 Morphological features of Bryostatin-1 treated leukaemia cells.
Phase contrast micrographs (x1000 magnification)
A. Leukaemia cells, from AML case UPN20, were cultured for 96 hours with GM-CSF, IL-4, and then a further 72 hours with GM-CSF and TNFα. At the end of the culture period the cells maintained an undifferentiated morphological appearance
B. Adding bryostatin-1 to GM-CSF and IL-4 for the first 96 hours of the culture period followed by 72 hours culture with GM-CSF and TNFα led to the acquisition of dendritic-cell like morphology by the leukaemia cells.

TSA and AZA were similarly ineffective in inducing differentiation in eight other differentiation resistant AML cases. When given in combination they failed to lead to induction in costimulatory expression above that achieved by culturing with cytokines alone. However BRYO again proved to be effective at overcoming differentiation resistance. Out of the 9 patients in whom BRYO was used in combination with cytokines to induce DLLC differentiation 6 showed evidence of differentiation based on upregulation of CD40, CD83, CD80 or CD86 expression. Figure 4.18 illustrates the immunophenotypic evidence for DLLC differentiation in three of these cases.

In addition to showing immunophenotypic and morphological evidence of DLLC differentiation BRYO treated leukaemia cells were also tested for their ability to induce allogeneic T lymphocyte proliferation in MLLR. Figure 4.19 shows the results in two AML cases in which allogeneic T lymphocytes were stimulated with leukaemia cells that had been cultured with BRYO in combination with cytokines or with cytokines alone. BRYO treatment of the leukaemia cells significantly enhanced the allostimulatory capacity of the leukaemia cells in comparison with those cultured with cytokines alone. In addition the amount of T cell proliferation induced by BRYO treated leukaemia cells, based on titriated thymidine uptake, was comparable in these two cases to that achieved with cytokine-induced DLLC from differentiation permissive AML cases.

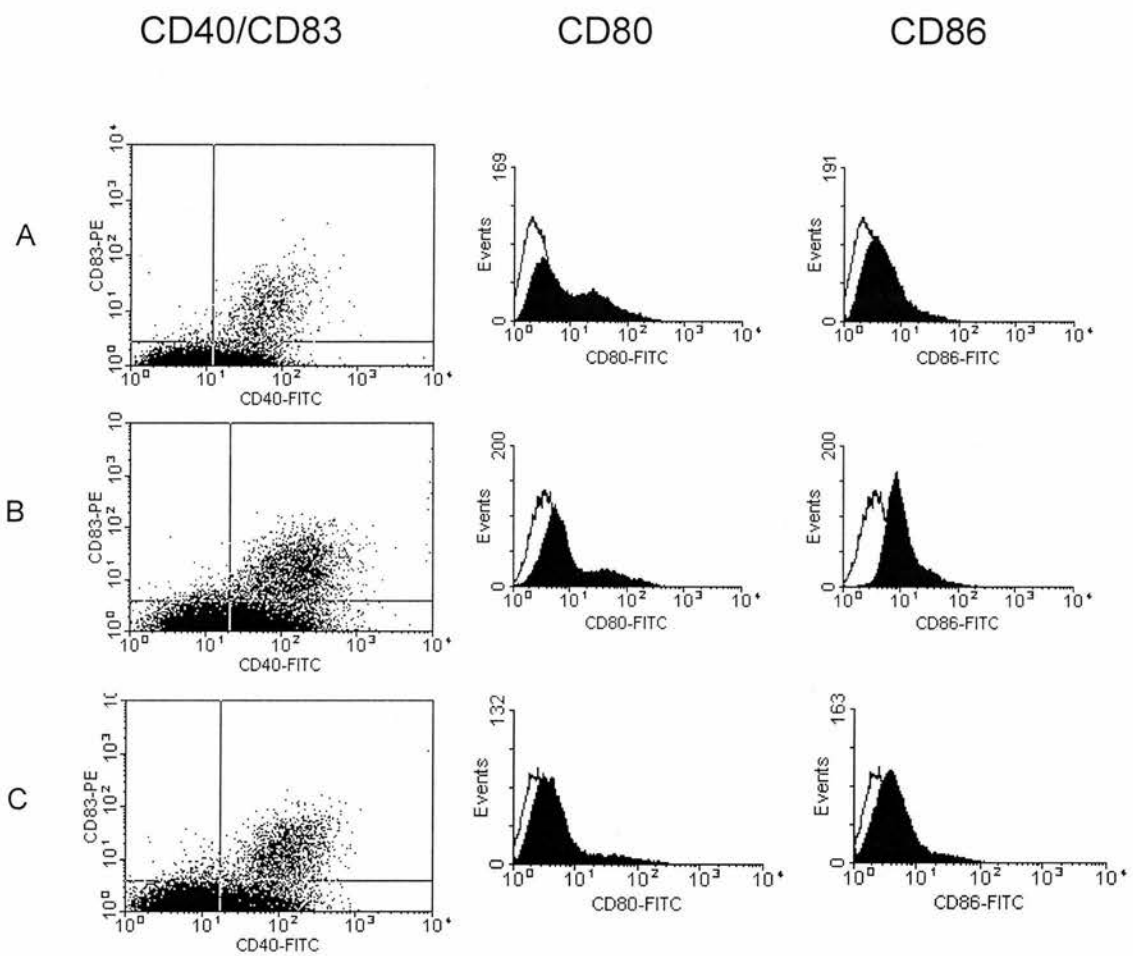


Fig 4.18 Immunophenotypic profile of Bryostatin-1 treated leukaemia cells.
The effects of combining BRYO with cytokines in overcoming the differentiation block in resistant AML cases. On the dot plot there is now a significant population of CD40/CD83 dual expressing cells. This accompanied by upregulation of CD80 and CD86 expression as shown on the histogram plots. Experiments were performed using leukaemia cells from AML cases A. UPN2 B. UPN5 and C. UPN19

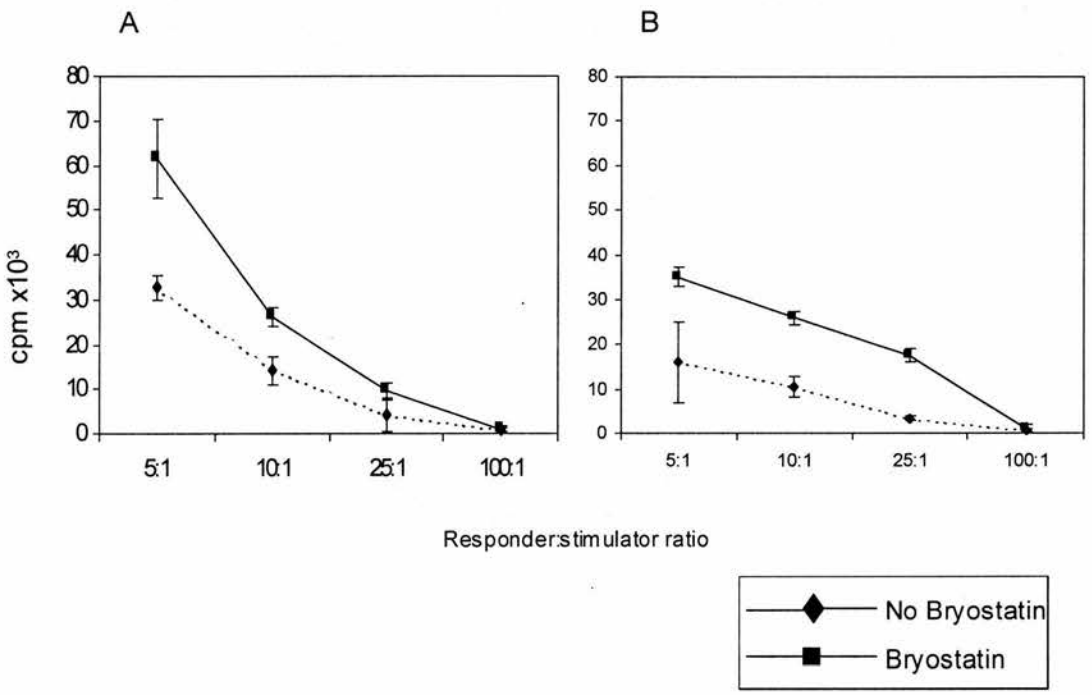


Fig 4.19 Allostimulatory properties of Bryostatin-1 treated leukaemia cells. Allogeneic MLLR. Responders: allogeneic lymphocytes. Stimulators: leukemic cells cultured with GM-CSF/IL4 with or without BRYO from AML cases A. UPN20 and B. UPN26 that had previously been shown to be resistant to DLLC differentiation.

4.2 Discussion

These results confirm the work of previous studies in showing that it is possible to induce the differentiation of primary leukaemia cells into DLLC by culture with certain cytokine combinations. It was shown that cytokine-induced DLLC were both morphologically and immunophenotypically reminiscent of MDDC, that they produced in a proportion of cases the immunostimulatory cytokine IL-12 and that they were potent stimulators of allogeneic T lymphocyte proliferation. Confirmation that the DLLC did in fact derive from the leukaemic clone was established in two AML cases by FISH analysis for a leukaemia-associated chromosomal rearrangement. It was not possible, however, to definitively establish that DLLC were capable of generating leukaemia reactive CTL by co-culture with autologous T lymphocytes. Although in two AML cases cytotoxicity against unmodified leukaemia targets was demonstrated, the assay system at that time had not been optimised and it is possible that the differences observed may have arisen by chance. Unfortunately although subsequent work led to the establishment of a more reliable and sensitive assay system, due to the small numbers of patients achieving remission, there was only one case where both leukaemia cells and remission PBMC were available with which to perform the assay. In this case no cytotoxicity against leukaemia targets by T lymphocytes cultured with DLLC could be detected. It remains, therefore, an importance priority to confirm the results of previous studies showing that leukaemia reactive CTL can be generated by DLLC.

Although the previous studies of DLLC differentiation had shown that in a significant proportion of AML cases the leukaemia cells fail to undergo cytokine-induced differentiation to DLLC these studies had not addressed the reasons for this.

One of the purposes of this study was to try to identify the factors important in determining differentiation potential and, in those cases found be resistant to DLLC differentiation, to try to find ways of overcoming this resistance. In order to achieve this it was necessary to establish criteria for successful DLLC differentiation. It was found that the appearance of a dual positive CD40/CD83 population was a useful marker in confirming that DLLC differentiation had taken place. In differentiation resistant cases, following attempted cytokine-induced differentiation, the CD40/CD83 positive population was <5% whereas for cases undergoing DLLC differentiation it was >5%. Expression of CD80 and/or CD86 invariably accompanied the presence of this CD40/CD83 population. Using these immunophenotypic criteria it was possible to divide AML cases into those that were differentiation permissive and those that were differentiation resistant. Examination of the clinical features of these two groups showed that there appeared to be a preponderance of poor risk karyotypic features amongst the differentiation resistant AML cases. The differentiation permissive AML cases possessed either standard risk or favourable karyotypic features. An association between differentiation potential and karyotype is plausible given that several lines of evidence point towards a common theme in the molecular pathogenesis of AML, namely that the end result of the majority of the commonest chromosomal translocations is the repression of transcription of genes involved in haematopoietic differentiation (Redner *et al*, 1999). The rationale for using the histone deacetylase inhibitor TSA and DNA demethylase AZA in an attempt to overcome differentiation resistance was based on evidence demonstrating that changes in histone acetylation and DNA methylation status are important in mediating transcriptional repression in AML. There may be a

variety of possible reasons why this approach was not successful. AML cases possessing complex karyotypic alterations will have multiple molecular rearrangements, many or all of which might contribute to the differentiation block. In addition the molecular consequences of these poor risk cytogenetic/molecular rearrangements may be to repress haematopoietic gene transcription by mechanisms unrelated to changes in histone acetylation or DNA methylation status. BRYO-1 promotes differentiation by interfering with protein kinase C activity (Steube & Drexler, 1993). It has been shown to be effective at inhibiting the clonogenicity of myeloid leukaemia cells (Matsui *et al*, 2000). The success of BRYO in allowing differentiation to DLLC in resistant AML cases might be a consequence of its inhibitory effects on cell cycle progression. Cell cycle arrest in these differentiation resistant cases may be sufficient to permit the cell to respond positively to differentiation signals induced by cytokines thereby allowing DLLC differentiation to proceed. Therefore by using agents such as BRYO, in combination with cytokines, it should be possible to increase the proportion of AML cases that are capable of undergoing DLLC differentiation. This is of particular significance because it would appear that the patients most likely to be excluded from receiving DLLC vaccination are those with karyotypically poor risk AML. However, it is these patients who are most in need of alternative therapeutic approaches, as their outlook with conventional combination chemotherapy regimens is extremely poor. Data from 1711 patients entered into the UK MRC X trial showed that 5 year survival for patients with karyotypically poor risk AML was only 17% (Wheatley *et al*, 1999).

Whereas FAB subtype was not considered to be an important factor in predicting DLLC differentiation potential it did appear to influence subsequent DLLC

maturation. Only AML cases of FAB subtypes M4/M5 produced appreciable levels of IL-12(p70) following maturation with CD40L. Whether this represents a true inability of DLLC generated from AML cases of FAB subtypes other than M4 and M5 to produce IL-12 or whether additional maturational stimuli are required to fully mature DLLC derived from these cases remains to be determined. This may be important if DLLC are to be used in an attempt to generate immunity to leukaemia *in vivo*. Data from studies examining maturation of monocyte derived DC suggests that IL-12 production by MDDC plays a crucial role in directing T cells towards a Th1 cytokine phenotype (Cella *et al*, 1996; Kalinski *et al*, 1999). Th1 type responses are believed to be more relevant in the development of effective anti tumour immunity (Terheyden *et al*, 2000). It will be important therefore to characterise the nature of any *in vivo* T cell responses following DLLC vaccination in order to determine whether these are dependent on the maturational status of the DLLC. These issues will need to be addressed by performing *in vivo* studies of DLLC vaccination.

Chapter 5

RESULTS

5.1 Differentiation induction in murine myeloid leukaemia cells

5.1.1 Passage of murine leukaemia cells

The initial passage was performed using an aliquot of murine AML cells derived from the spleen of a CBA mouse with radiation-induced leukaemia, which had been harvested and cryopreserved in 1995. 10 syngeneic CBA mice were irradiated with 200R and then injected intravenously with 10^6 murine AML cells. The outcome from these injections is summarised in Table 5.1. For passage 2 the source of the leukaemia cells was the spleen of a mouse that had developed frank leukaemia from passage 1. In the second passage a higher dose of leukaemia cells was injected and in addition the mice received a slightly higher conditioning dose of irradiation of 300R. The outcome from this passage is also summarised in Table 5.1. The mice injected in passage 2 had a 100% incidence of acute leukaemia and the natural history was of a faster rate of disease progression, necessitating earlier sacrifice of animals than was required in passage 1. Cells harvested from passage 2 mice had morphological features typical of AML. These features were identical whether the source of the leukaemia cells was spleen, peritoneal exudate or peripheral blood (Figure 5.1). Cytochemical staining with combined esterase demonstrated positivity

for both chloracetate and non-specific esterase thereby classifying the leukaemia as myelomonocytic (Figure 5.1).

Passage number	No. of mice injected	Radiation dose	Source of leukaemia cells	No. of cells injected	No. of mice developing AML	Survival from injection (days)
1	10	200R	Thawed aliquot of spleen cells (MC7.3 14.9.95)	10^6	4	106-117
2	10	300R	Fresh spleen cells from passage 1 mouse	$2x10^6$	10	49-60

Table 5.1 Outcome of injection of CBA mice with passage 1 and passage 2 murine leukaemia cells

5.1.2 Immunophenotypic profile of undifferentiated leukaemia cells

Murine leukaemia cells were stained with a more limited panel of mAb antibodies than was used in the human studies. This panel consisted of mAb directed against Gr-1 and CD11b, used for the purpose of confirming that the leukaemia cells were myeloid in origin, and CD40, CD80, CD86 and MHC class II, which were felt to be the most useful for establishing that DLLC differentiation had taken place.

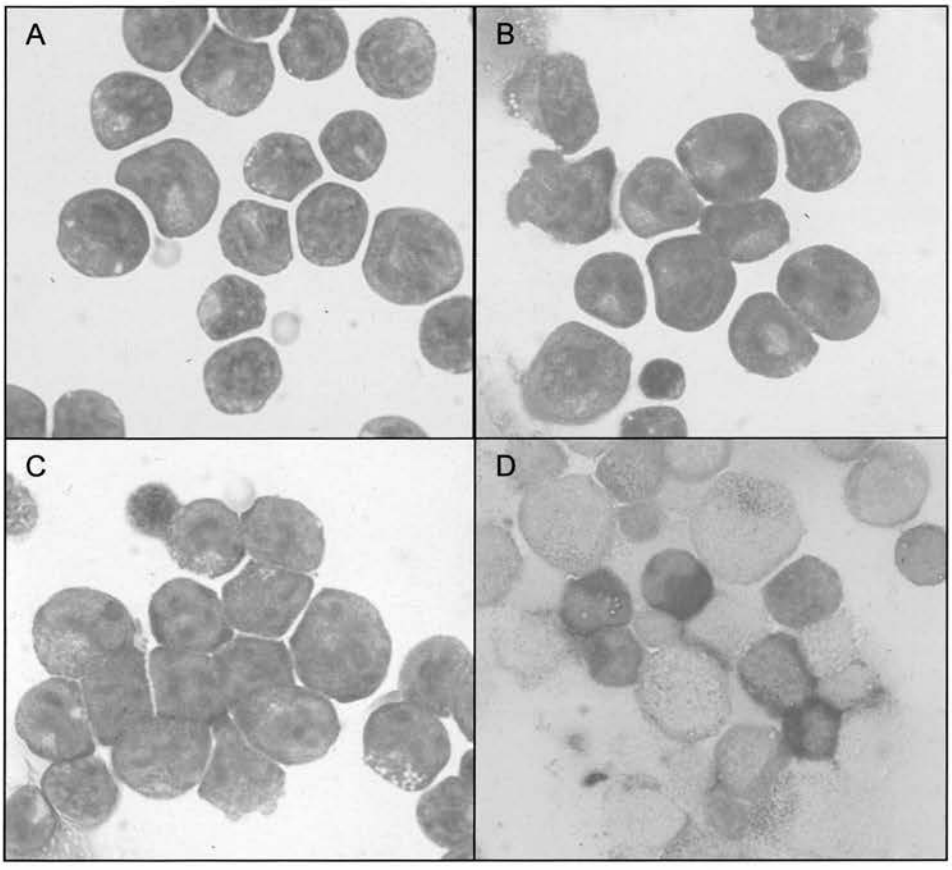


Figure 5.1 Morphological and cytochemical characteristics of murine AML. Cytospin slides made from mononuclear cell preparations of murine AML A. Peripheral blood B. Peritoneal exudate and C. Spleen cells stained with MGG and D. Spleen cells stained with dual esterase

The immunophenotypic profile of undifferentiated murine leukaemia cells is shown in Figure 5.2. The leukaemia cells show expression of Gr-1 and CD11b, which would be consistent with the morphological and cytochemical features that suggested a myelomonocytic leukaemia. MHC class II expression was negative (In 6 of the 42 human AML cases tested the leukaemia cells were HLA class II negative). The murine leukaemia cells showed no expression of costimulatory molecules CD40, CD80 and CD86.

5.1.3 Cytokine-induced differentiation

Murine leukaemia cells were cultured under similar conditions to human leukaemia cells. For the first 4 days cells were cultured in complete medium supplemented with murine GM-CSF and murine IL-4. The cells were then harvested and the medium replaced with complete medium supplemented by GM-CSF and murine TNFα. Morphologically cytokine cultured murine leukaemia cells showed none of the features that characterised human DLLC differentiation but instead maintained an undifferentiated appearance. In addition the murine leukaemia cells show very poor viability over the duration of the culture period. Immunophenotypic analysis of the cultured leukaemia cells confirmed the fact that they had failed to undergo DLLC differentiation based on their lack of expression of MHC class II and costimulatory molecules. Despite repeated experiments, using aliquots of murine leukaemia cells from both passages 1 and 2, cytokine-induced DLLC differentiation could not be achieved.

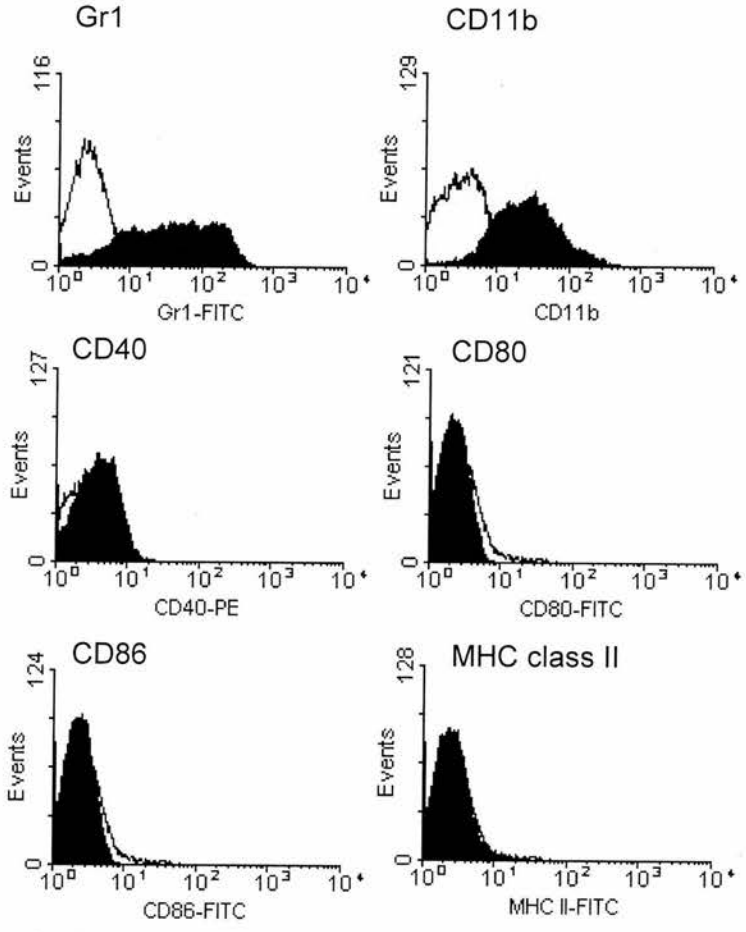


Fig 5.2 Immunophenotypic profile of undifferentiated murine AML cells

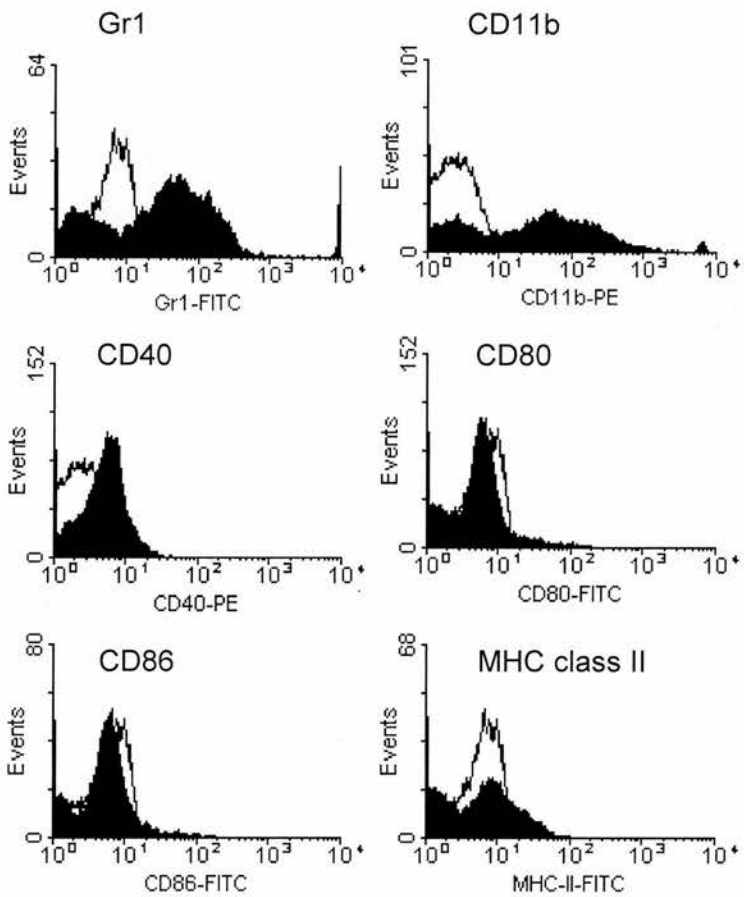


Fig 5.3 Immunophenotypic profile of murine leukaemia cells following culture with cytokines GM-CSF/IL-4 for 4 days and then GM-CSF/TNFα for 3 days.

5.1.4 Bryostatin in combination with cytokines

Because BRYO had proven to be an effective agent in overcoming DLLC differentiation resistance in human AML cases this approach was attempted with the murine leukaemia cells. Murine leukaemia cells were culture with GM-CSF and IL-4 in combination with BRYO 10nM for the first 4 days of the culture period. Then cells were harvested and the medium replaced with fresh complete medium supplemented by GM-CSF and TNFα. A similar morphological appearance of these BRYO treated cells to the human leukaemia cells was observed with the appearance of an adherent population of cells that had dendritic processes. Immunophenotypic analysis demonstrated that differentiation had taken place with the appearance of a population of cells that had expression of CD40, CD80, CD86 and MHC class II (Figure 5.4). However it is important to note that the viable cell population at the end of the culture period was only 20% (based on staining with PI). Experiments performed to confirm this finding were unsuccessful either because poor viability over the culture period prevented any meaningful evaluation or because, based on morphological and immunophenotypic appearances, DLLC differentiation did not take place.

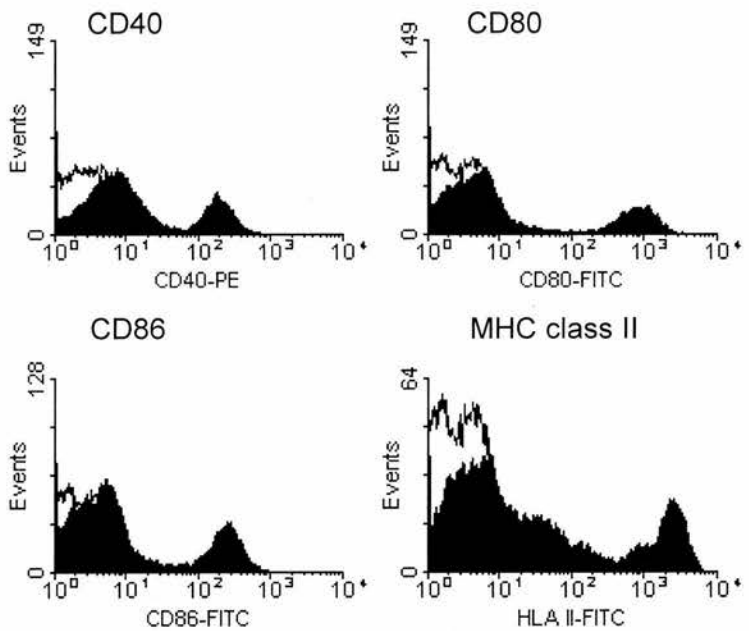


Figure 5.4 Upregulation of costimulatory molecules and MHC class II expression following culture with cytokines in combination with bryostatin.

5.2 Discussion

The murine leukaemia model used in this study in many ways represents a useful system for evaluating immunotherapy-based approaches for the treatment of AML (Rithidech *et al*, 1999). It is a radiation-induced leukaemia, in CBA mice, that morphologically and immunophenotypically resembles human AML (Major, 1979). Cytogenetic abnormalities are reported to parallel those found in human AML (Rithidech *et al*, 1999). The leukaemia can be maintained by passage through CBA mice, which means that immunotherapeutic strategies designed at stimulating host immunity against leukaemia could be tested for in a syngeneic setting. Unfortunately the murine leukaemia cells proved to be resistant to DLLC differentiation. As evidence from the human studies demonstrates that a significant proportion of AML cases are resistant to cytokine-induced differentiation to DLLC, the fact that the leukaemia cells from this murine AML model were resistant to differentiation was not wholly unanticipated. An additional factor that may have been important in leading to differentiation resistance is that the leukaemia cells had been passaged. Following each passage of the murine leukaemia cells through a host animal the AML behaves in a more aggressive manner and the time from injection to death becomes shorter (Personal Communication, E.Wright). It could be hypothesised that with each passage the leukaemia cells acquire new cytogenetic abnormalities culminating in a complex karyotypic picture, which based on the data from the human studies would decrease the chances of DLLC differentiation occurring. Mice in passage 2 certainly had a higher incidence and shorter natural history of their leukaemia than mice in passage 1, although there were other factors that could have contributed to this, such as the fact that in passage 1 the leukaemia

cells had been cryopreserved 6 years previously and that the number of leukaemia cells used for the injection and the dose of conditioning radiation used was higher in passage 2. A karyotype was not performed on the leukaemia cells from passage 1 and 2 in order to establish whether cytogenetic clonal evolution had taken place. Although attempts were made to overcome the differentiation resistance using BRYO this approach was only successful on one occasion. Because of the difficulties in trying to reproduce the results of this experiment and as the murine leukaemia cells showed such poor viability over the culture period it was felt to be logistically impossible to pursue the murine AML model as a means of evaluating the effectiveness of an *in vivo* DLLC vaccination strategy. There has been one previous study in which murine erythroleukaemia cells were induced to differentiate into DLLC by culture with GM-CSF, which suggests that DLLC differentiation is possible in at least some types of murine leukaemia (Cao *et al*, 1998). There may be other murine AML models, therefore, that could be used for the evaluation of a DLLC vaccination strategy. In the absence of these murine models, clinical trials would be necessary for the assessment of *in vivo* anti-leukaemia responses to DLLC vaccination.

Chapter 6

CONCLUDING REMARKS

The objective of this study was the development of a leukaemia cell vaccine by the modification of autologous leukaemia cells, which could be used for the immunotherapy of AML. At the beginning of this study it was thought that an immune gene transfer approach would prove to be the most suitable way to achieve this objective. At that time there were few published studies on gene transfer into primary AML blasts and none that had used adenoviral vectors for this purpose. It was therefore considered to be important to perform a comprehensive evaluation of gene transfer methods into myeloid leukaemia cell line and primary AML blasts for the purpose of selecting a gene transfer system that could be used for generating sufficient numbers of genetically modified leukaemia cells to allow further study of their immunostimulatory properties. Unfortunately it transpired that none of the gene transfer methods evaluated in this study were sufficiently efficient to be of use in a clinical setting. The most successful method, based on an adenoviral vector system, gave high levels of gene transfer in a minority of AML cases, but in a significant proportion gave only low levels of gene transfer. In addition these experiments were performed at high MOI with relatively small numbers of target cells. Scaling up these methods in order to generate clinically relevant numbers of genetically modified leukaemia cells would have been likely to have led to a fall in GTE, rather than the converse. Whilst this study was being performed data was published on novel vectors that appeared to show considerable promise for the transfer of genes to

primary AML blasts. A recombinant adenoviral vector was engineered with a modified fibre protein thereby allowing it to bind with much greater efficiency to haematopoietic cells (Wickham *et al*, 1996). This vector, when used for gene transfer to primary AML blasts, gave a mean GTE of 94.3% in 25 AML cases (Gonzalez *et al*, 1999). The second vector that shows promise in gene transfer to primary leukaemia cells is a lentiviral vector pseudotyped with VSV-G (Stripecke *et al*, 2000). Mean GTE of this vector in leukaemia cells from 5 AML cases was 77.6%. Based on the evidence from the preliminary studies with these vectors it appears that it may be possible to generate sufficient numbers of genetically modified leukaemia cells for clinical use as vaccines for the immunotherapy of AML.

There are, however other potential problems, with pursuing this form of immunotherapeutic strategy. Although the gene transfer of single immune genes, such as CD80, may be sufficient to restore the immunogenicity of leukaemia cells *in vitro*, in a clinical setting vaccines based on single immune gene transfer might prove to be ineffective at generating anti-leukaemia immunity because of their inability to overcome the effects of the immunosuppressive microenvironment. In order to enable the genetically modified leukaemia cells to favourably alter the leukaemia microenviroment it may be necessary to use vectors containing combinations of immune genes. For example a tumour cell vaccine based on leukaemia cells transduced with a bicistronic vector containing the cDNA for CD80 and the immunostimulatory cytokine GM-CSF, was evaluated in a murine leukaemia model and was found to be more effective at inducing leukaemia eradication than leukaemia cells transduced with CD80 only containing vector (Stripecke *et al*, 1999). Another potential concern with the use of viral based vectors is that immune

responses may be directed against viral proteins rather than the leukaemia-specific antigens (Gahery-Segard *et al*, 1998; Molinier-Frenkel *et al*, 2000). Although all non-essential genes, other than those necessary for transgene expression, are eliminated from viral based gene transfer vectors immune responses can still be generated against viral proteins (Gahery-Segard *et al*, 1998; Molinier-Frenkel *et al*, 2000). For the future development of a vaccination strategy based on immune gene transfer to leukaemia cells it will be necessary to have vectors that are highly efficient at gene transfer to primary AML blasts, do not elicit host immune responses against viral proteins and are capable of supporting transgene expression of combinations of immune genes.

The second approach that was investigated for the purposes of developing a leukaemia cell vaccine was the cytokine-induced differentiation of leukaemia cells to dendritic-like cells. One of the potential advantages of this approach over an immune gene transfer strategy is that following successful DLLC differentiation the leukaemia cells express a wide range of costimulatory molecules, have high levels of expression of ICAM-1 and HLA class I and II molecules and, in a proportion of cases, produce the immunostimulatory cytokine IL-12 (Boyer *et al*, 2000; Brouwer *et al*, 2000a; Brouwer *et al*, 2000b; Charbonnier *et al*, 1999; Choudhury *et al*, 1999; Cignetti *et al*, 1999; Costello *et al*, 2000; Kohler *et al*, 2000; Oehler *et al*, 2000; Robinson *et al*, 1998). Therefore DLLC may be better equipped to alter the microenvironment in such a way as to promote effective immune responses and, based on their ability to produced IL-12 (Charbonnier *et al*, 1999), skew T cell cytokine responses towards a Th1 phenotype (Kalinski *et al*, 1999a; Vieira *et al*, 2000). A potential concern with this approach is that leukaemia-specific antigens

may in fact be down regulated during DLLC differentiation. WT1, the product of the Wilms tumour gene is a leukaemia-specific antigen and therefore a potential target for CTL recognition (Gaiger *et al*, 2000; Gao *et al*, 2000; Inoue *et al*, 1997; Menssen *et al*, 1995; Ohminami *et al*, 2000). However the expression of WT1 has been shown to be down regulated during differentiation of CD34+ haematopoietic progenitors (Menssen *et al*, 1997). If the same pattern were to be observed during leukaemia cell differentiation then DLLC might be incapable of generating CTL specific for WT1, or for other leukaemia-specific antigens whose expression is dependent on differentiation status. Although FISH analysis does demonstrate that in selected cases the leukaemia associated chromosomal rearrangement is still carried by the DLLC (Boyer *et al*, 2000; Brouwer *et al*, 2000b; Choudury *et al*, 1999; Kohler *et al*, 2000; Oehler *et al*, 2000; Robinson *et al*, 1998) it cannot be assumed that the protein products of these rearrangements would act as antigenic determinants. Therefore in developing this strategy it will be necessary to have reliable and sensitive assays for measuring T cell immune responses directed against leukaemia cells, both *in vivo* and *ex vivo*. There are a number of potential ways of measuring T cell activity *in vivo*. These include measuring delayed type hypersensitivity responses to intradermal injections of leukaemia cells, measuring IFN-γ production by leukaemia specific CTL using the ELISPOT assay and by quantifying CTL specific for known leukaemia antigens by the use of fluorescence labelled HLA-peptide tetramers. At the present time it is not known which, if any, of the above assays are the most appropriate for detecting clinically relevant immune responses to leukaemia *in vivo*. Only by performing clinical trials of DLLC vaccination and correlating clinical

responses with the results obtained from the immunological assays will it be possible to establish the best methods for monitoring immune responses to leukaemia cells.

Any future clinical trials of DLLC vaccination will need to take into account a number of important considerations in study design. One of the most serious potential side effects of this type of strategy is that it could encourage the development of autoimmunity. Following DLLC differentiation leukaemia cells become potent APC and therefore, in addition to stimulating T cell responses to leukaemia-specific antigens, might conceivably stimulate immunity against self-antigens. Autoimmunity has been observed in a vaccination study using DC that had been pulsed with leukaemia-derived peptides. Vaccination of leukaemia bearing mice with pulsed DC, when given in combination with CD40L and IL2 transduced fibroblasts, led to the development of a severe systemic autoimmune disease resembling GVHD (Roskrow *et al*, 1999). Therefore in clinical trials of DLLC vaccination patients will need to be monitored closely for evidence of loss of tolerance to self-antigens. Another important consideration is the selection of patients most likely to benefit from the DLLC vaccination strategy. Based on the experience with DLI it is assumed that patients with a low leukaemia burden i.e. following prior chemotherapy induced remission are most likely to derive clinical benefit from immunotherapy type approaches. It is probably unlikely that vaccination with DLLC would be able to lead to the eradication of high leukaemia cell burdens. However as MRD is the major contributor to relapse and subsequent death in patients with AML eradication of MRD might well lead to improvements in leukaemia-free survival rates. Given that recruitment of patients with low leukaemia burden states seems most appropriate for the purposes of a clinical study it still

remains to be determined what would be the best vaccination regimen. Clinical studies will need to address the questions of DLLC dose, routes of vaccination and frequency and duration of vaccination in order to determine the most effective method for generating clinically relevant anti-leukaemia immunity.

Following on from our own *in vitro* studies in DLLC generation and characterisation a phase I/II clinical study of DLLC vaccination in AML has now been designed. A successful application to the Leukaemia Research Fund was made and the clinical study is to be jointly funded by the Scottish National Blood Transfusion Service and the Leukaemia Research Fund. A brief outline of the study is that four subcutaneous injections of autologous DLLC, in escalating doses, will be given to patients with AML who have undergone prior, successful, remission-induction chemotherapy. In addition to assessing the tolerability of DLLC vaccination other outcomes to be measured include the changes in immune function induced by vaccination and the durations of leukaemia-free and overall survival.

Appendix I

Optimisation of conditions for commercial transfection reagents

DNA(μG)	0	3	4	4	6	10
Transfast (μl)	12	9	12	24	18	30
Charge ratio	-	1:1	1:1	2:1	1:1	1:1

Table I.1 Optimisation of conditions for Transfast in the K562 cell line alone

DNA(μg)	0	1	2	3	4	4
Transfast (μl)	1.2	3	6	9	12	24
Charge ratio	-	1:1	1:1	1:1	1:1	2:1

Table I.2 Optimisation of conditions for Transfast in the four leukaemia cell lines

DNA (μg)	0	1	2	1	2	3
Fugene (μl)	3	3	3	6	6	6

Table I.3 Optimisation of conditions for Fugene in the four leukaemia cell lines

DNA(μg)	0	0.5	1	0.5	0.5
Enhancer (μl)	4	4	8	4	4
Effectene (μl)	5	5	10	12.5	25
Ratio	-	1:10	1:10	1:25	1:50

Table 1.4 Optimisation of conditions for Effectene in the four leukaemia cell lines

DNA (μg)	0	2	4
Superfect (μl)	8	8	16
Ratio	-	1:4	1:4

Table I.5 Optimisation of conditions for Superfect in the four leukaemia cell lines

Appendix II

Publications arising from this work

Roddie, P.H., Paterson, T. & Turner, M.L. (2000) Gene transfer to primary acute myeloid leukaemia blasts and myeloid leukaemia cell lines. *Cytokines.Cell Mol.Ther.*, **6,** 127-134.

Abbreviations

ABS	Human AB Serum
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
APC	Antigen Presenting Cells
APML	Acute Promyelocytic Leukaemia
ATRA	All-Trans Retinoic Acid
AZA	Azacytidine
BM	Bone Marrow
BRYO	Bryostatin-1
c.p.m.	Counts Per Minute
cDNA	Complementary Deoxyribonucleic Acid
CAR	Coxsackie virus and adenovirus receptor
CFU	Colony Forming Units
CI	Calcium Ionomycin
CML	Chronic Myeloid Leukaemia
CTL	Cytotoxic T Lymphocytes
DLI	Donor Lymphocyte Infusion
DLLC	Dendritic-like leukaemia cells
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
FAB	French-American-British
FITC	Fluorecein isothiocyanate
FACS	Fluorescence Activated Cell Sorting
FasL	Fas ligand
FCS	Foetal Calf Serum
FISH	Fluorescence In Situ Hybridisation
g	Relative Centrifugal Force
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GTE	Gene Transfer Efficiency

GTU	Gene Transfer Unit
GVHD	Graft Versus Host Disease
GVL	Graft Versus Leukaemia
hi	Heat Inactivated
HIV-1	Human Immunodeficiency Virus-1
HLA	Human Leucocyte Antigen
IFN	Interferon
IL	Interleukin
kb	Kilo-Bases
kDA	Kilo-Daltons
LDH	Lactate Dehydrogenase
LTR	Long Terminal Repeat
mAb	Monoclonal Antibody
MDDC	Monocyte-derived dendritic cells
MFI	Mean Fluorescence Intensity
MGG	May-Grünwald Giemsa
MHC	Major Histocompatibility Complex
MLLR	Mixed Leukaemia Lymphocyte Reaction
MLV-A	Murine Amphotrophic envelope protein
MOI	Multiplicity of Infection
Mo-MLV	Moloney-Murine Leukaemia Virus
MRD	Minimal Residual Disease
NK	Natural Killer
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFU	Plaque Forming Units
PHA	Phytohaemagglutinin
PI	Propidium iodide
PMA	Phorbol Myristate Acetate
PolyI:C	Polyriboinosinic polyribocytidylic acid

R	Rads
RD114	Feline Endogenous Retrovirus Envelope Protein
SCT	Stem Cell Transplantation
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor beta
Th	T helper type
TNF-α	Tumour Necrosis Factor alpha
TSA	Trichostatin-A
VSV-G	Vesicular Stomatitis virus-G envelope protein

List of Figures

List of Tables

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