DEVELOPMENT OF A DENDRITIC CELL BASED VACCINE FOR THE IMMUNOTHERAPY OF ACUTE MYELOID LEUKAEMIA

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Declaration

I declare that this thesis is my own work and has been planned, conducted and written by myself, unless stated otherwise in the acknowledgements. The entire project was undertaken whilst I was in post in the South East of Scotland and has not been submitted in candidature for any other degree, diploma or professional qualification.

Matthias Klammer

26th of June 2007

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Abstract

22 patients with de novo or relapsed Acute Myeloid Leukaemia (AML) were recruited into a Phase I/II clinical trial aimed at vaccinating with autologous Dendritic-like Leukemia Cells (DLLC) once in complete remission. At trial entry leukaemia cells were harvested and tested for their permissiveness to cytokine-induced dendritic cell differentiation. Study patients were then treated with induction chemotherapy. Five patients achieved both complete remission and had leukaemia cells that were permissive to differentiation, and were therefore eligible to proceed to vaccination. Four escalating doses of DLLC were administered weekly by subcutaneous injection. Levels of minimal residual disease were measured by Wilms tumour-1 gene expression. Development of anti-leukaemic T-cell responses was assessed by ELIPSOT analysis of gamma interferon secreting T-lymphocytes and by measurement of WT1 specific T-cells by HLA tetramer analysis. An increase in anti-leukaemic T-cell responses was demonstrated in four patients. Vaccination was generally well tolerated with the only significant side effect being the development of extensive eczema in one patient. Two patients relapsed during or shortly after the vaccination schedule. In the remaining three patients, one relapsed at 12 months with two in continued remission more than 12 months post vaccination.

In a parallel investigation, the potential of Tumour Cell / Dendritic Cell Fusion Hybrids to generate *in vitro* anti-leukaemic T-cell responses following co-culture with autologous remission lymphocytes was assessed in six patients with AML. Comparison was made to anti-leukaemic responses induced by mature Dendritic Cells (mDC) co-cultured with autologous, irradiated myeloid blasts. Fusion Hybrids

induced anti-leukaemic T-cell immune responses in three out of six patients. Tumour pulsed mDC induced T-cellular responses in two other patients. Only one of six patient's remission lymphocytes failed to develop leukaemia directed immune responses following stimulation with either construct. Anti-proliferative properties of Fusion Hybrids against allogeneic lymphocytes were observed in mixed lymphocyte-leukaemia reactions (MLLR) and were found not to be specific to the cell fusion partners and do not prevent the ability of AML-mDC heterokaryons to induce autologous anti-leukaemic cytotoxicity. In conclusion, Tumour Cell / Dendritic Cell Fusion Hybrids hold promise as a cellular vaccine for Acute Myeloid Leukaemia.

List of Abbreviations

AML	Acute Myeloid Leukaemia
APC	Antigen Presenting Cells
BRYO	Bryostatin
CI	Calcium Ionophore
CM	Complete Medium
CML	Chronic Myeloid Leukaemia
CTL	Cytotoxic T-Lymphocytes
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusion
FAB	French American British
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GMP	Good Manifacturing Practice
GVHD	Graft Versus Host Disease

GVL	Graft Versus Leukaemia
HLA	Human Leucocyte Antigen
HSCT	Haematopoietic Stem Cell Transplantation
IFN-γ	Interferon gamma
IL	Interleukin
KIR	Killer Immunoglobulin-like Receptor
LPS	Lipopolysaccharide
MDS	Myelodysplastic Syndrome
MGG	May-Grünwald Giemsa
MHC	Major Histocompatibility Complex
MLLR	Mixed Leukaemia Lymphocyte Reaction
MRD	Minimal Residual Disease
NK	Natural Killer
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin

PEG	Polyethylenglycol
Poly I:C	Polyriboinosinic polyribocytidylic acid
PR3	Proteinase 3
TAA	Tumour Associated Antigen
TGF-β	Transforming Growth Factor beta
Th	T-Helper Cell
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor alpha
TREG	Regulatory T-Cells
TRM	Transplantation Related Mortality
VEGF	Vacular Endothelial Growth Factor
WHO	World Health Organisation

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Chapter 1: Introduction

1.1 Current concepts of immune-mediated treatment modalities for Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is a biologically and clinically heterogeneous disease characterised by a clonal, neoplastic proliferation of haematopoietic progenitor cells. Immunological control of this disease, resulting in spontaneous remission, is exceedingly rare, but has been reported (Muller *et al*, 2004) after bacterial infections, suggesting that immune recognition of leukaemic cells is possible under certain circumstances. The discovery of tumour-related antigens, recognized by cytotoxic lymphocytes, has been a significant advance in understanding the role of an immune response seen in patients with leukaemia.

Intensive multi-agent chemotherapy, however, has been the backbone of AML treatment for decades. Complete remissions are achievable with this approach in 70-80% of patients below 60 years of age. However, disease relapse originating from minimal residual disease (MRD) (Yin & Tobal, 1999) results in an overall 5-year survival of less than 30%. Autologous transplantation in AML confers some degree of benefit in respect of improved disease-free survival, but is associated with a not insignificant risk of treatment related mortality (Burnett *et al*, 1998;Ravindranath *et al*, 1996). Superior long-term disease free survival of 50-60% can be achieved in patients that have undergone allogeneic haematopoietic stem cell transplantation (HSCT), and in recent years evidence has emerged that much of the therapeutic benefit of this procedure is derived from an immunologically mediated graft versus leukaemia (GvL) effect (Korngold *et al*, 1994;Falkenburg *et al*, 1997). This has led

to the development of reduced intensity, non-myeloablative transplant conditioning regimes, designed to allow donor engraftment rather than direct tumour cytotoxicity, and the use of donor-lymphocyte infusion (DLI) (Kolb *et al*, 1995) (Mackinnon *et al*, 1995;Helg *et al*, 1998;Collins, Jr. *et al*, 1997)as a means to treat relapse following allogeneic HSC transplantation, proving that immune surveillance of residual leukaemic blasts by donor T-lymphocytes is possible.

Recently, a number of specific peptides have been identified that may represent appropriate targets for immune responses in the context of allogeneic HSCT. The haematopoiesis –specific minor histocompatibility antigens HA-1 and HA-2 may act as malignancy-specific targets in the context of allogeneic HSCT (Mutis *et al*, 1999a;Mutis *et al*, 1999b;Marijt *et al*, 2003).

Newer evidence points towards the role of Natural Killer (NK) cells in controlling and eradicating leukaemia in a situation of haplotype mismatched transplantation. Mismatch for killer immunoglobulin-like (KIR) receptors between donor and recipient pairs in this particular subgroup is associated with activation of donor derived NK cells that contribute to immune control of MRD and lower relapse risk compared to KIR matched donor-recipient pairs (Ruggeri *et al*, 1999;Ruggeri *et al*, 2002;Farag *et al*, 2002).

These treatment modalities provide a model for the successful integration of immuno-therapeutic approaches to MRD in current clinical practice and have improved long-term clinical outcomes. However, there is a need for novel treatment strategies, in particular for older patients, because of the unacceptably high morbidity and mortality associated with HSC transplantation approaches. In clinical practice, the development of GvL is often associated with significant Graft versus Host

Disease (GVHD). Unfortunately, GVHD remains a major cause of transplantation-related mortality (TRM) and still limits the use of HSCT in older patients.

In recent years a better understanding of mechanisms involved in the immune escape of tumours together with the identification of tumour associated antigens (TAA) in AML, such as the Wilms tumour gene product WT1 (Mailander *et al*, 2004;Elisseeva *et al*, 2002;Oka *et al*, 2004), proteinase 3 (Gao *et al*, 2000) (Molldrem *et al*, 1996;Ohminami *et al*, 2000)and protein products of abnormal fusion genes, e.g. BCR-ABL in Chronic Myeloid Leukaemia (CML) and PML-RARα in AML (Clark *et al*, 2001) (Osman *et al*, 1999)have promoted new immunotherapeutic approaches to leukaemia outside the setting of HSC transplantation. Dendritic cells (DC), as professional antigen presenting cells (APC) able to elicit a cytotoxic response from naïve T-cells, have been at the centre of interest in developing a cellular vaccine for AML.

1.2 Dendritic cell development and T-cell interaction in vivo

In vivo DCs form a heterogeneous group of subsets, continually produced from myeloid and lymphoid haematopoietic precursors in the bone marrow, and widely distributed as immature DCs into lymphoid and non-lymphoid tissues (Banchereau & Steinman, 1998;Cella *et al*, 1997;Sousa *et al*, 1999;Moser & Murphy, 2000). Immature DCs, including epidermal Langerhans cells, splenic marginal zone DCs and interstitial DCs in non-lymphoid tissues, continually sample self-antigen to maintain T-cell tolerance. However, immature DC's can also take up foreign antigens. When triggered by pathogens, the pattern-recognition receptors expressed by immature DC's cause them to mature in the presence of inflammatory "danger

signals", such as Tumour Necrosis Factor (TNF), Interferon (IFN) α or bacterial lipopolysaccharide (LPS) (Matzinger, 1998). Mature DCs are able to initiate primary T-cell immune responses by activating naïve T-cells (Steinman, 1991;Banchereau & Steinman, 1998), because they express high amounts of cell surface major histocompatibility complexes (MHC) and co-stimulatory molecules (Banchereau & Steinman, 1998;Cella *et al*, 1997;Sousa *et al*, 1999;Moser & Murphy, 2000), as well as adhesion molecules and chemokine receptors such as CCR-7 and 8(Qu *et al*, 2004), enabling them to migrate to the secondary lymphoid tissues where they present antigen to T-cells. The APC are thought to take up exogenous antigens, including apoptotic tumour cells and present these to lymphocytes via a classical MHC class II pathway, but "cross-presentation" of these antigens through MHC class I molecules has also been demonstrated. Such cross-presentation has been associated with the stimulation of CD4+ T-helper cells and CD8+ cytotoxic T-cells (CTL), together providing the best conditions for cell-mediated cytotoxicity. T-cell activation requires two different signals (Fig.1.1). The first signal is provided by the interaction of the MHC molecule-peptide with the corresponding T-cell receptor. The second signal consists of the binding of co-stimulatory molecules on the APC with their respective ligands on the T-cell. CD40 and B7 on the APC interact with CD40 ligand and CD28 on the T-cell to mediate T-cell activation.

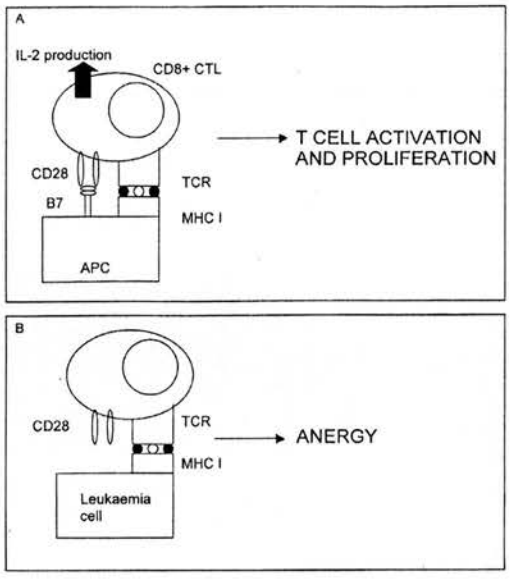


Figure 1.1: Mechanisms of T-cell activation
A : Recognition of MHC/peptide complexes on the surface of an antigen presenting cell by a T-cell receptor in the presence of a second co-stimulatory signal mediated by B7 leads to T-cell activation and proliferation
B : Absence of B7 expression by leukaemia cells results in absent co-stimulatory signal following T-cell receptor binding of MHC/peptide complex with consequent T-cell anergy

The remarkable functional heterogeneity of DC subsets rests on their maturational state, the microenvironment to which the DCs are exposed in various tissues in vivo and a variety of other factors, such as the strength of the T-cell receptor-MHC class II interaction and antigen density, which are also known to influence the ability of DC to induce a) an Interleukin (IL)-12 driven type 1 T-helper cell (Th1) response, promoting T-cellular cytotoxicity, b) a Th2 T-cell response, polarizing towards antibody-mediated immunity, or c) to induce anergy of a T-cell following a tolerizing interaction with a DC. Any immuno-therapeutic approach utilising DC will have to consider whether DC function, in their interaction with other components of the immune system (T-cells, NK-cells), may be altered or defective in patients with AML, favouring tumour escape from immunological surveillance as previously

demonstrated in animal models of malignancy and in cancer patients (Chaux *et al*, 1996;Chaux *et al*, 1997;Nestle *et al*, 1997;Thurnher *et al*, 1996).

1.3 Dendritic cells in leukaemia: part of the problem or part of the solution?

The role of DCs in cancer has been described in numerous studies in various types of tumours (Ambe *et al*, 1989;Fox *et al*, 1989;Schroder *et al*, 1988), postulating an association of good clinical prognosis with presence of DCs in tumour tissues. However, functional defects, mainly due to lack of expression of co-stimulatory molecules in tumour associated DCs, have also been identified in more recent animal model studies and also in cancer patients (Chaux *et al*, 1996;Chaux *et al*, 1997;Nestle *et al*, 1997;Thurnher *et al*, 1996). In leukaemia, similar to other malignancies, release of immunosuppressive cytokines, such as Interleukin (IL)-10 (Gastl *et al*, 1993;Maeda & Shiraishi, 1996), transforming growth factor beta (TGF-β) (Nagy & Vanky, 1998;Espinoza-Delgado *et al*, 1994;Luscher *et al*, 1994), vascular endothelial growth factor (VEGF), other known soluble factors, such as soluble IL-2 receptor (Nakase *et al*, 1992), soluble tumour necrosis factor receptor (Vinante *et al*, 1998) and soluble Fas ligand (Tanaka *et al*, 1996), as well as other, yet unspecified factors (Buggins *et al*, 1999)have been implicated in creating an immunosuppressive microenvironment that may not favour the induction of an effective immune response. Recent investigations (Ma *et al*, 2004) (Mohty *et al*, 2001)have shone more light on the ontogeny of both myeloid and plasmacytoid DC in the peripheral blood of patients with AML and Myelodysplastic Syndrome (MDS). While in MDS both DC subsets are present, albeit in lower numbers compared with those in normal blood, both populations are demonstrably derived from the malignant clone (Ma *et*

al, 2004). Likewise, in the peripheral blood of AML patients both subsets have been identified (Mohty *et al*, 2001), however, the identification of subsets by immunophenotypic characteristics may be complicated by the fact that DCs and leukaemic blasts share many markers, hence leukaemic blasts can be misidentified as DC on the basis of abberant expression of DC associated markers on the surface of leukaemic blasts that are not DC.

In vitro, cytokine driven differentiation of AML blasts to a dendritic-like phenotype (AML-DC) can be achieved in a proportion of patients (Robinson *et al*, 1998;Panoskaltsis *et al*, 2002;Harrison *et al*, 2001;Choudhury *et al*, 1999;Roddie *et al*, 2002). As these AML-DCs arise from the leukaemic clone, they offer potential as autologous cellular vaccines, expressing both leukaemic antigens and also DC-characteristic, co-stimulatory molecules that are mostly lacking in native leukaemic blasts. Co-culture experiments of AML-DC with autologous T-cells have induced anti-leukaemic cytotoxicity (Charbonnier *et al*, 1999). However, as DC populations originating from leukaemic blasts *in vivo* are part of the malignancy and may potentially also produce "tolerogenic" signals, the question arises if AML-DC *in vivo* might be implicated in the disease process and, if so, would their use in therapy be advisable? The fact that the malignant cells are DC precursors and therefore components of the immune interaction one attempts to study complicates the characterisation of immune responses to AML. Studies of anti-leukaemic T-cell responses in leukaemic patients in remission (Panoskaltsis *et al*, 2002) (Molldrem *et al*, 2000)and those with fulminant disease (Molldrem *et al*, 2003) (Elisseeva *et al*, 2002)have been undertaken. Cytotoxic T-lymphocytes (CTL) directed against tumour-associated antigens, such as WT1 and proteinase 3 have been demonstrated

at some point of the disease process. Such observations have led some investigators to the suggestion that immune responses to tumour might be actively inhibited in advancing disease (Staveley-O'Carroll *et al*, 1998;Takasugi *et al*, 1977), while others have postulated that in patients with malignancy immune responses to tumour associated antigens (TAA) are only initiated in disseminated, overwhelming disease and are only detectable at low levels in a situation of low tumour burden (Mintz *et al*, 2003;Valmori *et al*, 2000). In either case, immune control of the tumour ultimately would be ineffective, but the exact immunosuppressive mechanisms remain unclear (Panoskaltsis, 2005).

1.4 Cellular vaccine design strategies in AML

Any successful cellular vaccine needs to overcome the reduced immunogenicity of leukaemic cells. This requires the generation of vaccine cells in large numbers to express leukaemia-specific antigens in the context of adaequate co-stimulation. Two principal strategies have been pursued in DC based leukaemia vaccine generation.

1) Improving the antigen-presenting capacity of leukaemia cells themselves: This can be achieved either by :

a) transfer of genes that encode the necessary co-stimulatory molecules into leukaemic blasts (Rousseau *et al*, 2006);(Chan *et al*, 2005;Nakazaki *et al*, 1998;Stripecke *et al*, 2000;Dilloo *et al*, 1996;Hirst *et al*, 1997;Boyer *et al*, 1997;Mutis *et al*, 1998;Saudemont *et al*, 2002), or

b) cytokine-driven leukaemic blast maturation into dendritic-like leukaemic cells (AML-DC) (Buhmann *et al*, 1999;Cardoso *et al*, 1997;Charbonnier *et al*, 1999;Choudhury *et al*, 1997;Choudhury *et al*, 1999;Eibl *et al*, 1997;Kohler *et al*, 2000;Mohty *et al*, 2002;Robinson *et al*, 1998).

2) Utilising the superior T-cell stimulatory capacity of non-leukaemic DCs: either

a) by adoptive immunotherapy, using ex vivo generated CTL specific for TAAs, or

b) through vaccination protocols with professional APCs expressing leukaemic antigens (Fig.1.2).

To deliver leukaemia-associated antigens, DCs can be generated ex-vivo from either monocytes (Thurner *et al*, 1999) (Bender *et al*, 1996)or CD34+ haematopoietic progenitor cells (Ferlazzo *et al*, 2000;Ye *et al*, 1996;Fujii *et al*, 1999). They can be pulsed as immature DCs *ex vivo* with tumour antigens, matured with cytokines, and then injected as cellular vaccines. Reported approaches for this purpose include the following:

(a) Loading DC with leukaemic blast lysates (Lee *et al*, 2004;Schui *et al*, 2002)

(b) Loading DC with irradiated or apoptotic blasts (Spisek *et al*, 2002;Fujii *et al*, 1999)

(c) Loading DC with synthetic peptides from tumour-associated antigens, such as WT1 in AML, PR1 in AML/CML (Mailander *et al*, 2004;Elisseeva *et al*, 2002;Oka *et al*, 2004;Gao *et al*, 2000;Molldrem *et al*, 1996;Ohminami *et al*, 2000;Menssen *et al*, 1995), or

(d) Loading DC with tumour-specific antigens derived from protein products of abnormal fusion genes, such as BCR-ABL in CML, PML-RARα and DEK-CAN in AML (Clark *et al*, 2001;Osman *et al*, 1999;Ohminami *et al*, 1999)

(e) Loading DC with tumour derived RNA or DNA (Zeis *et al*, 2003)

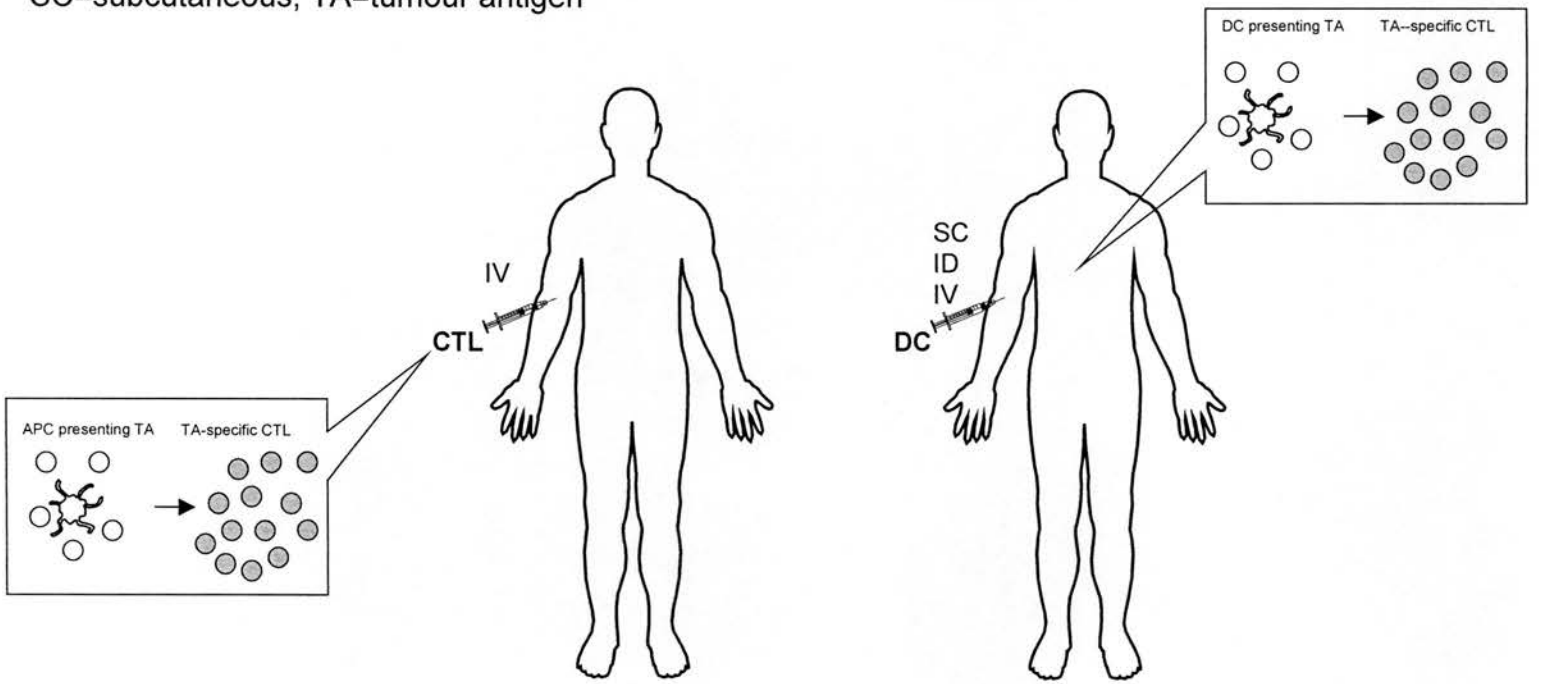
(f) Fusing DC with leukaemic blasts, generating AML-DC Hybridomas (Galea-Lauri *et al*, 2002)

Fig.1.2: Two strategies to generate anti-tumour immunity

A: Cytotoxic T-Lymphocytes are generated ex vivo by co-culture of autologous or allogeneic T-Cells with antigen presenting cells expressing a defined tumour antigen. Tumour-antigen specific T-lymphocytes are then injected into the patient.

B: Dendritic cells are generated from the patient and are engineered to express a defined tumour antigen. The Dendritic cells are then injected into the patient (either by subcutaneous, intradermal or intravenous route) and stimulate the in vivo development of anti-tumour directed specific immununity.

APC= antigen presenting cell, CTL=cytotoxic T-lymphocyte, DC=dendritic cell, ID=intradermal, IV=intravenous, SC=subcutaneous, TA=tumour antigen



A. Ex vivo generation of tumour specific CTL

B. Dendritic cell vaccination

1.5 Adoptive immunotherapy with *ex-vivo* generated CTL

This approach has been exploited in several clinical and pre-clinical studies. Mutis *et al* (Mutis *et al*, 1999b) have built on the recognition of minor histocompatibility antigen (mHags) mismatch as important factors in the development of GvHD and GvL in allogeneic HSC transplantation. Some mHags show ubiquitous tissue distribution, others, such as the mHags HA-1 and HA-2 are only expressed in haematopoietic cells (de Bueger *et al*, 1992). These antigens are therefore attractive TAAs for immunotherapy, as they should direct an allogeneic cytotoxic response selectively against the patient's haematopoietic cells, whether neoplastic or not. Donor-derived DCs from an HA-1 or HA-2 mismatched donor are pulsed with HA-1 or HA-2 peptides corresponding to the recipient's mHag type. These DCs are co-cultured, *ex vivo*, with donor lymphocytes to generate CTL specific for these peptides. Subsequently the patients undergo an allogeneic HSC transplantation from their HLA-compatible, but HA-1 or HA-2 mismatched donor. After complete engraftment of donor derived haematopoiesis the *ex vivo* generated mHag-specific CTLs are infused into the patient to antileukaemic cytotoxic effect, without causing GvHD. This approach is limited to a small number of patients that are undergoing an allogeneic HLA-matched HSC transplantation from a donor with HA-1 or HA-2 disparity. More widely applicable strategies have used the characterisation of TAAs demonstrated to induce CTL responses specific to tumour cells in haematological malignancies. The transcription factor WT1, which is widely expressed on the leukaemic cells from patients with AML and CML has been shown to be a potential target for *in vitro* CTL generation. Using APCs pulsed with HLA-A2 restricted WT1 peptides, CTL were generated from peripheral blood mononuclear cells that showed

exquisite specificity of action against CD34+ leukaemia cells, but spared normal CD34+ progenitors (Gao *et al*, 2000). Some of the limiting factors to successful translation of this type of *ex-vivo* CTL generation into clinical practice are the very prolonged period of culture necessary to obtain clinically relevant numbers of CTL, with all the attendant difficulties that this entails, and the limitations set by HLA class-restricted DC presentation of tumour peptides that may not always be available for every HLA type.

1.6 AML-DC as cellular vaccine

A strategy explored by several investigators over the last decade (Buhmann *et al*, 1999) (Cardoso *et al*, 1997;Charbonnier *et al*, 1999;Choudhury *et al*, 1997;Choudhury *et al*, 1999;Eibl *et al*, 1997;Kohler *et al*, 2000;Mohty *et al*, 2002;Robinson *et al*, 1998)is the differentiation and maturation of leukaemic blasts into dendritic-like cells (AML-DC). It followed the emerging knowledge of DC development from myeloid progenitors. A variety of different cytokine cocktails including GM-CSF, IL-4, IL13, TNF-α, FLT3 ligand and immunomodulators, such as soluble CD40 ligand have been proposed to induce dendritic-like differentiation and maturation. The "essential cocktail", identified for generating DC from blood monocytes, consisting of GM-CSF and IL4(Sallusto & Lanzavecchia, 1994), has been complemented by CD40 ligand or TNF-α to induce differentiation in 18/19 primary AML samples in a seminal study by Choudhury *et al* (Choudhury *et al*, 1999). The resulting AML-DC showed the characteristic morphology, immunophenotypic properties and T-cell stimulatory properties of mature DC. Importantly, co-culture of autologous T-lymphocytes with the AML-DC induced

cytotoxic responses directed against the native leukaemic blasts, but not against normal autologous cells. Several other studies have confirmed these findings in a proportion of AML blasts investigated (Brouwer *et al*, 2000;Harrison *et al*, 2001). There are to date no published clinical vaccination studies utilising AML-DC as cellular vaccines in AML. We have therefore initiated a Phase I/II clinical vaccination study using autologous dendritic-like leukaemic cells (AML-DC) in adult patients with AML in remission, following multi-agent chemotherapy, which is the subject of this thesis, in an attempt to evaluate the clinical feasibility of this approach and to demonstrate anti-leukaemic immune responses following AML-DC vaccination.

1.7 DC-based vaccination

Clinical phase I/II study protocols utilising tumour-antigen loaded DC have in principle documented the feasibility of this approach and demonstrated variable degrees of immunological and clinical *in vivo* responses in patients with melanoma, prostate and ovarian cancer (Nestle *et al*, 1998;Brossart *et al*, 2000;Banchereau *et al*, 2001;Marchand *et al*, 1999;Murphy *et al*, 1996;Hernando *et al*, 2002). Partial regressions of solid tumours have been demonstrated in a proportion of patients in these studies, with occasional complete responses demonstrated. These early reports are encouraging, but difficult to compare and reproduce, due to methodological differences in all aspects of study design.

Few clinical vaccination trials in leukaemia are published. T-cellular cytotoxic responses to TAA derived peptides have been studied in most depth. Molldrem *et al* (Molldrem *et al*, 1996) have demonstrated that HLA-A2 restricted CTL, raised

against PR1 peptide, a human leukaemia-associated antigen derived from proteinase3, an aberrantly expressed protein in myeloid leukaemia cells, preferentially killed leukaemic over normal haematopoietic precursors (79% cytotoxicity against CML cells, 54% lysis of AML blasts). A later study (Molldrem *et al*, 2000) investigated the correlation between the presence of PR1 specific T-cells, detected by PR1/HLA-A2 tetrameric complexes in the peripheral blood of leukaemic patients following treatment with Interferon alpha and allogeneic bone marrow transplantation, giving first time evidence of the role of T-cell immunity in clearing malignant leukaemic cells. A subsequent clinical vaccination study with PR1 peptide and adjuvant GM-CSF in 35 patients with AML, CML and MDS elicited both immunologically demonstrable (60% of patients) and clinical responses in patients with refractory and relapsed myeloid leukaemia (Qazilbash *et al*, 2004).

In AML there are to date no published clinical trials exploiting DC based vaccination strategies as an adjuvant therapeutic approach for Minimal Residual Disease following multi-agent intensive chemotherapy, where, in our opinion, the chances of immunological control of disease recurrence appear most promising. The only clinical DC vaccination study, undertaken by Lee *et al* (Lee *et al*, 2004) utilised blast cell lysate-pulsed DC in two patients in relapse following autologous HSC transplantation. In this setting, immunological responses to vaccination are difficult to differentiate from those directed against advancing leukaemic tumour burden, but evidence of delayed hypersensitivity and increased mixed lymphocytes responses (MLR) were attributed to the subcutaneous vaccination protocol.

A larger number of pre-clinical studies have been conducted, exploiting various DC-loading protocols.

Schui *et al (Schui et al, 2002)* pulsed DC with tumour lysates and have induced autologous anti-leukaemic cytotoxicity in co-cultured T-cells in 5 out of 25 patients only, while in other patients an inhibitory effect was seen. Spisek *et al* (Spisek *et al*, 2002) demonstrated the ability of non-leukaemic DCs loaded with late apoptotic leukaemic blasts to induce *in vitro* leukaemia-specific T-cellular cytotoxicity in 3 patients; direct comparison to the cytotoxicity induced by AML-DC was only possible in one patient and found to be similar. An earlier study by Fuji *et al* showed the induction of anti-leukaemic CTL's by CD34+ derived DC-clusters pulsed with autologous, irradiated blasts (Fujii *et al*, 1999).

In AML and ALL, HLA-A2-binding peptides derived from the Wilms tumour gene-encoded transcription factor WT-1 (Inoue *et al*, 1997) stimulate a specific anti-leukaemic T-cell response (Ohminami *et al*, 2000;Gao *et al*, 2000;Scheibenbogen *et al*, 2002). Similarly, DCs loaded with MUC1 protein, an epithelial mucin that is overexpressed in AML and multiple myeloma, facilitate the generation of anti-tumour T-cell responses *in vitro*.

In a subgroup of leukaemia patients, chromosomal translocations are demonstrable in the leukaemic clone, resulting in gene rearrangements and giving rise to fusion gene protein products, such as BCR-ABL in Chronic Myeloid Leukaemia (CML) and PML-RARα in AML (Clark *et al*, 2001;Osman *et al*, 1999). These proteins are potential targets of an immune response. They are attractive for immunotherapy, because they represent true tumour-specific antigens. Furthermore, as some of these fusion proteins are essential for the malignant transformation of the clone, tumour escape through antigen loss in response to selective immunological pressure is unlikely. However, in spite of these attractive features, the potentially immunogenic

site of a fusion protein is only located in the aminoacid sequences immediately flanking the fusion site. Cleaving by proteases is necessary to arrive at candidate antigenic peptides. Those peptides will have to fit the binding motif of a given patient's HLA molecules in order to elicit cytotoxic T-cell responses. This HLA restriction makes it unlikely to find a universal antigenic peptide sequence derived from a fusion protein that would be useful in the majority of patients presenting with a given chromosomal translocation.

Fusion Hybrids of tumour cells and monocyte-derived DC have been investigated as possible cellular vaccine constructs in a variety of malignancies (Gong *et al*, 2000;Vasir *et al*, 2005;Raje *et al*, 2004;Avigan *et al*, 2004). In principle, remission monocytes can be matured into DCs and then fused to autologous tumour cells, using either Polyethyleneglycol (PEG) as the fusing agent or electro-poration protocols. The resulting constructs express the full repertoire of tumour antigens on their surface in conjunction with DC-derived co-stimulatory molecules. There is no need to establish the HLA type of the patient, in contrast to approaches using pulsing of DC's with specific peptides or antigens, no prior identification of TAAs expressed by the individual tumour cells is required, and the fusion technology using PEG is well established and simple, making this approach more easily applicable. It also avoids the technical difficulties and safety concerns that surround the use of viral vectors to generate genetically modified cells for vaccination. The use of Fusion Hybrids of remission Monocyte-derived DC with autologous AML blasts has so far not been reported and pre-clinical experimental experience is limited to a single report (Galea-Lauri *et al*, 2002), prompting their further exploration as potential cellular vaccine constructs in this thesis.

1.8 Monitoring responses to DC vaccination in AML

The use of objective criteria to describe clinical responses, as codified by the World Health Organisation and in solid tumour response evaluation schemes (RECIST) (Therasse *et al*, 2000) would advance the field of DC immunotherapy. Many early studies are compromised by a lack of standardisation of clinical responses and no demonstration of specific anti-neoplastic immune responses.

In a proportion of myeloid leukaemias recurrent chromosomal abnormalities (translocations and inversions) can be detected. Those can be used to quantify the residual tumour burden using highly sensitive, quantitative Reverse Transcriptase (RT)-PCR assays. In cases without demonstrable cytogenetic defects, stably expressed TAAs, such as WT-1 (Inoue *et al*, 1997), have been used to quantify MRD from blood and bone marrow. These assays represent minimally invasive, sensitive and validated tools, applicable to the monitoring of treatment responses post chemotherapy and HSCT. They can be utilised to objectively quantify treatment responses to DC-immunotherapy in a similar way.

Any future attempt to increase the effectiveness of vaccination will also depend on the ability to measure the development of anti-leukaemic T-cell immune responses with a high degree of sensitivity and specificity. In recent years, it has become possible to visualise antigen-specific T-cells under flow cytometry by using soluble, fluorescently labelled multimeric HLA-peptide complexes (Altman *et al*, 1996) that bind stably, specifically and avidly to antigen-specific cells. We proposed to combine tetramer assays for WT1-recognising T-cells with highly sensitive assays of T-cellular IFN-γ responses to autologous AML blasts (ELISPOT) together with RT-

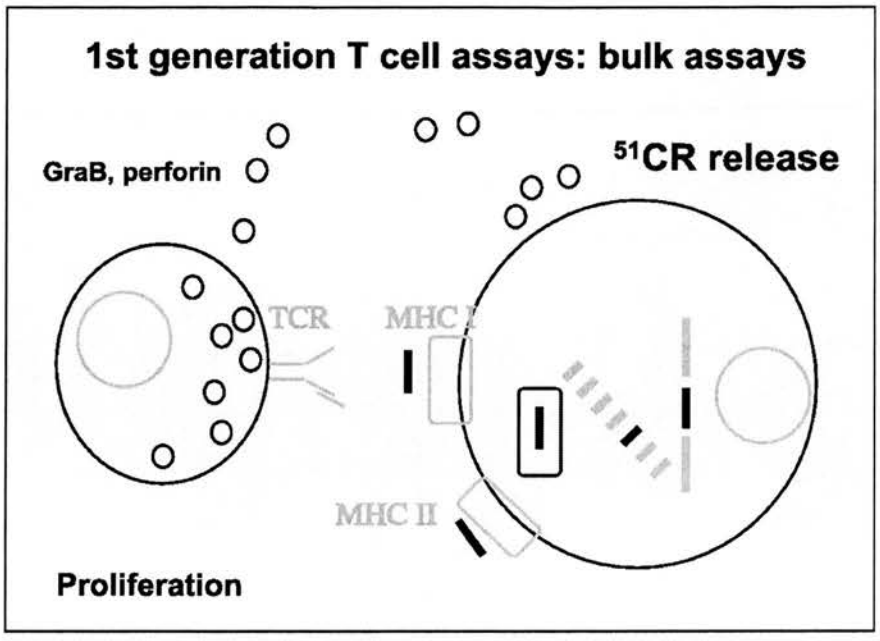


Fig.1.3(a): T-cell assays: 1st generation bulk assays measuring either overall T-cell proliferation after exposure to target (standard readout: [^{3}H]thymidine uptake) or lysis of target cells after exposure to an effector population (standard readout: ^{51} Cr release from target cells into the supernatant).

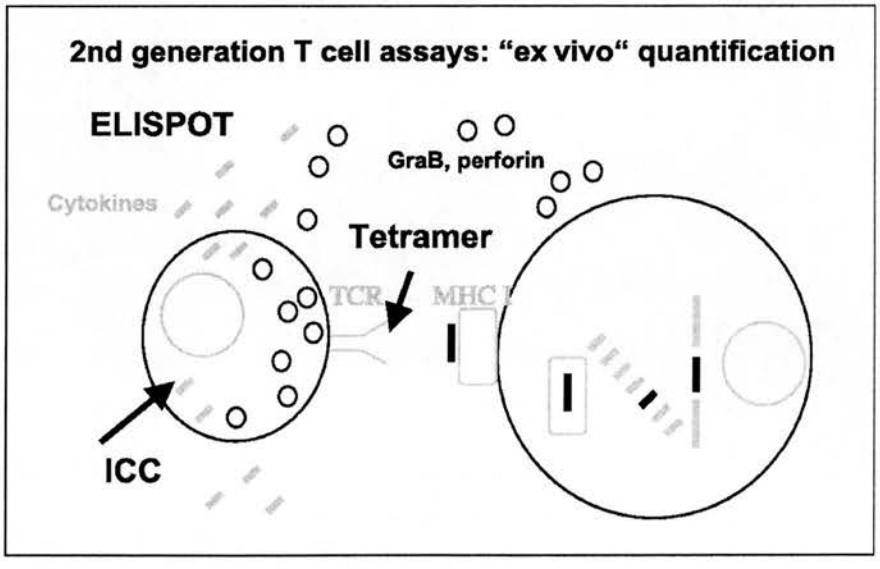


Fig.1.3(b): T-cell assays: 2nd generation: using single cell-based analysis of T-cell properties. T-cell receptor analysis using major histocompatibility multimers or assesment of cytokine production capacity of T-cells after exposure to antigen (readouts: intracellular cytokine accumulation in T-cells by flow cytometry assays, or secretion of cytokine captured by antibodies bound to cell surface or plate (ELISPOT assay). GraB= Granzyme B; ICC=intracellular cytokine; TCR=T-cell receptor (modified from Keilholz *et al*, Clin.Canc.Res. 2006)

PCR for WT-1 assays of tumour burden in order to monitor the impact of AML-DC vaccination in the clinical study undertaken for this thesis.

Regulatory T-cells (T_{reg}) have been investigated in many recent studies because of their apparent immuno-modulatory role in infection, auto-immunity and inhibitory effect on anti-cancer immune responses (Casares *et al*, 2003a;Mesel-Lemoine *et al*, 2005;Morse *et al*, 2002;Wolf *et al*, 2003a). Conversely, evidence has emerged that effective anti-cancer immunity can be restored by suppression of T_{reg} numbers (Ghiringhelli *et al*, 2004;Morse *et al*, 2002). Correlating the number of T_{reg} cells before, during and following DC vaccination with the variation of measurable anti-leukaemic cytotoxicity and MRD burden may provide insight into the role of these cells in dampening anti-leukaemic immune responses.

1.9 Considerations for delivering a DC-based vaccine for AML

Many facets of DC-based vaccines in AML remain unknown, as the clinical application of this approach is in its infancy. From experiences with earlier DC vaccination studies in solid tumour malignancies it may be deducted that the state of minimal residual disease (MRD), achieved following conventional chemotherapy or HSCT, is a desirable setting for the trial of leukaemia cell vaccines. Patients with MRD have a low tumour burden. DC-induced CTL responses are likely to be overwhelmed by rapidly advancing leukaemia (Haining *et al*, 2005) and, importantly, immunological control of MRD has been demonstrated to be associated with superior long-term survival and lower relapse rates originating from residual leukaemia in allogeneic HSCT. Pre-clinical studies have emphasized the importance of

vaccinating with fully mature DCs in order to avoid tolerogenic responses associated with immature or semi-mature forms. Optimal dose, scheduling and route of delivery remain controversial. DCs are frequently administered subcutanuously, intradermally or by direct injection into lymphatics / draining lymph nodes. Recent studies suggest that immature DCs migrate less than mature DC's. De Vries *et al* (De Vries et al, 2003) demonstrated that in general, less than 5% of intradermally administered mature DCs reach the draining lymph node. From this data it can be speculated that increased migration would increase the efficacy of DC vaccines. Attempts to increase migration include the concomitant use of inflammatory cytokines to condition tissues (MartIn-Fontecha *et al*, 2003). Matrix metalloproteinases and Toll-like receptor (TLR) ligands might also enhance DC migration (Ratzinger *et al*, 2002).

Any *ex-vivo* manipulation of human cells must be undertaken conforming to good manufacturing practice (GMP) standards laid down in North America and the European Union. Facilities complying with these standards are regulated in the US by the Center for Biologics Evaluation and Research (CBER). In the UK the Department of Health, through the Medicines and Healthcare Products Regulatory Authority, had established an inspectorate whose remit included cellular therapy. Investigators have to ensure that clinical vaccination studies using cellular products are compliant with standards that are already established in the commercial pharmaceutical industry. This includes cytokines used in the process that must be of clinical grade standard and cell culture media, which should only contain human serum protein, or ideally be serum free. The use of fetal calf serum (FCS) is no longer deemed appropriate.

For any vaccination approach that uses leukaemia derived DCs, prevention of re-engraftment of viable, potentially proliferating cells is of importance. We established that 25 Gy of γ-irradiation from a Caesium 137 source, routinely used for irradiation of blood products, completely abolished the proliferative potential of AML blasts in tissue culture and irradiated all AML-DC vaccine with this dose prior to inoculation (Appendix VIII).

1.10 Objectives and scope of the clinical and pre-clinical studies undertaken for this thesis

The work outlined in this thesis encompassed a multi-centre clinical stage I/II study of vaccination with Dendritic-like Leukaemia Cells (DLLC) generated from primary acute myeloid leukaemia blasts as immuno-therapy for Acute Myeloid Leukaemia. The main objectives of this clinical trial were to establish the feasibility, safety and tolerability of this approach. Clinical endpoints included the assessment of cellular immune responses, impact of the vaccination on Minimal Residual Disease and evaluation of remission duration, leukaemia free survival and overall survival.

In an attempt to develop the next generation of cellular AML vaccines, a pre-clinical assessment of alternative DC-based constructs was undertaken in parallel to the clinical vaccination trial. The approaches explored include the addition of the differentiation agents Calcium Ionophore and Bryostatin-1 in an attempt to overcome blast resistance to cytokine induced DLLC maturation. The ability of Tumour Cell / Dendritic Cell Fusion Hybrids to generate *in vitro* anti-leukaemic T-cell responses following co-culture with autologous remission lymphocytes was investigated in six

AML patients. Comparison was made to anti-leukaemic responses induced by mature dendritic cells (mDC) loaded with autologous, irradiated myeloid blast tumour bodies *in vitro*.

Chapter 2: Materials and Methods

2.1 Study Protocol: A Clinical Phase I/II Study of Vaccination with Dendritic-like Leukaemia Cells Generated From Primary Acute Myeloid Blasts As Immunotherapy For Acute Myeloid Leukaemia

2.1.1 Study Objectives

2.1.1.1 Primary Endpoint

- To establish the feasibility of generating clinically-relevant numbers of DLLC from primary AML blasts *ex vivo* and the safety/tolerability of subcutaneous injection

2.1.1.2 Secondary Clinical Endpoints

- To assess the immune function in AML
- To assess cellular immune responses to autologous leukaemia prior to and following DLLC vaccination
- To gain evidence of reduction in minimal residual disease (MRD) level by real time quantitative RT-PCR in patients using suitable molecular markers
- To assess for inversion in remission durations for relapsed patients
- To ascertain the duration of leukaemia free survival
- To ascertain the duration of overall survival

2.1.2 BACKGROUND

2.1.2.1 Generation of Dendritic-Like Leukaemia Cells from Patients with AML

Peripheral blood or bone marrow samples were taken from patients with newly presenting or relapsed AML. Mononuclear cells were prepared over Ficoll-Hypaque, washed in RPMI medium, resuspended in RPMI with 5% AB serum, 20ng/ml IL-4 and 100ng/ml GM-CSF and transferred to plastic tissue culture flasks. After 4 days culture medium and cytokines were replaced with fresh RPMI supplemented with GM-CSF 100ng/ml and TNFα 25ng/ml and cells cultured for a further 4-7 days. Using this regimen, based on our own experience and on data from previously published studies, between 50-70% of AML cases would be expected to show evidence of dendritic cell-like differentiation based on morphology, expression of costimulatory molecules CD40, B7.1, B7.2, and the expression of the dendritic cell-associated marker CD83 (Appendix V). The remaining AML cases that fail to show differentiation, in our pre-clinical experience, often had associated poor risk karyotypic features.

2.1.2.2 Pre-clinical Functional Analysis of DLLC

The immunostimulatory properties of DLLC have been tested in pre-clinical studies preceeding the clinical trial. Results of these MLLR and CTL assays are attached in Appendix VI.

2.1.3 PATIENT SELECTION AND ENROLMENT

We proposed to recruit adult patients to a phase I/II clinical trial of vaccination with autologous dendritic-like leukaemic cells:

2.1.3.1 Inclusion Criteria

- Adult patients with newly presenting AML or with relapsed disease
- The predicted survival should exceed 3 months
- Patients must be fit enough to undergo leucapheresis or to have 100mL of blood removed by iso-volaemic venesection or a 10mL bone marrow aspirate
- Patients must give written informed consent

2.1.3.2 Exclusion Criteria (Initial Enrolment)

- Patients with acute promyelocytic leukaemia and a 15-17 translocation
- Patients, whose predicted survival is less than 3 months
- Patients unfit for leucapheresis or to have 100mL of blood removed by isovolaemic venesection or a 10mL bone marrow aspirate
- Patients who decline informed consent
- Patients who fail virological screening

2.1.3.3 Exclusion Criteria (Vaccination Programme)

- Patients who, following chemotherapy, fail to go into complete remission (as defined by a cellular marrow showing <5% blasts)
- Patients who fail to achieve good haematological recovery (as defined by haemoglobin >120g/L, neutrophils >$1x10^9$/L and platelets >$100x10^9$/L)
- Patients whose AML blasts do not mature into DLLC on pre-screen

- Those who decline consent

Patients who enter complete remission but whose AML blasts do not mature into DLLC on pre-screen were offered continued monitoring of immune function and minimal residual disease.

2.1.4 DESIGN OF CLINICAL TRIAL

2.1.4.1 Harvesting of Leukaemia Cells

At trial entry patients underwent a single leucapheresis, or 100mL isovolaemic venesection or 10mL bone marrow aspirate depending on the peripheral blood blast count as shown in Table 2.1.

Table 2.1: Summary of leukaemia harvest methods depending on peripheral blast count

Peripheral blood blast count ($x10^9$/l)	Method of leukaemic cell harvest
≥10	100mL peripheral blood
≥1<10	Leucapheresis
<1	10mL bone marrow aspirate

- Patients with peripheral blast counts ≥10 x 10^9/L underwent isovolaemic venesection of 100mL of peripheral blood - volume replacement was with normal saline.
- Patients with peripheral blast counts of ≥1 but <10 x 10^9/L underwent a leucapheresis procedure performed. Patients were evaluated prior to the

procedure by a blood transfusion physician to ensure they were fit enough to undergo the procedure.

- Patients with low peripheral blast counts (<1 x 10^9/L) underwent a 10mL marrow aspiration. This was performed under local anaesthesia, and sedation with Midazolam was offered.

All patients underwent routine virological testing, in line with the UK requirements for the handling of autologous donations. In addition, 50mL of peripheral blood were used for assessment of baseline immune function including peripheral blood count, flow cytometry and proliferative responses to mitogens and recall antigens.

2.1.4.2 Pre-Screen and Cryopreservation of the Leukaemia Cell Harvest

The leukaemia cell harvest was transferred to the Scottish National Blood Transfusion Service Tissue Bank at Liberton, Edinburh, where it could be handled in Good Manufacturing Practice (GMP) grade sterile clean room facilities. The majority of the harvested cells were cryo-preserved using standard techniques. A portion of the harvest was used for a pre-screen. The purpose of the pre-screen was to establish whether it was possible to induce differentiation of the patient's leukaemia cells into DLLC. Only patients whose leukaemic cells pass the pre-screen were deemed eligible for the vaccination programme.

2.1.4.3 Induction and Documentation of Remission

Patients received intensive chemotherapy. The number of courses given and regimens used were at the discretion of the treating consultant. Patients achieving complete remission (a cellular marrow showing <5% blasts) then underwent a

marrow aspirate following haematological recovery (Hb >120g/L, neutrophils $>1x10^9$/L and platelets $>100x10^9$/L) from the last cycle of chemotherapy and those confirmed to be in continuing complete remission were eligible to receive the vaccination therapy (Figure 2.1). DLLC vaccination was performed at least 2 weeks from the time of haematological recovery.

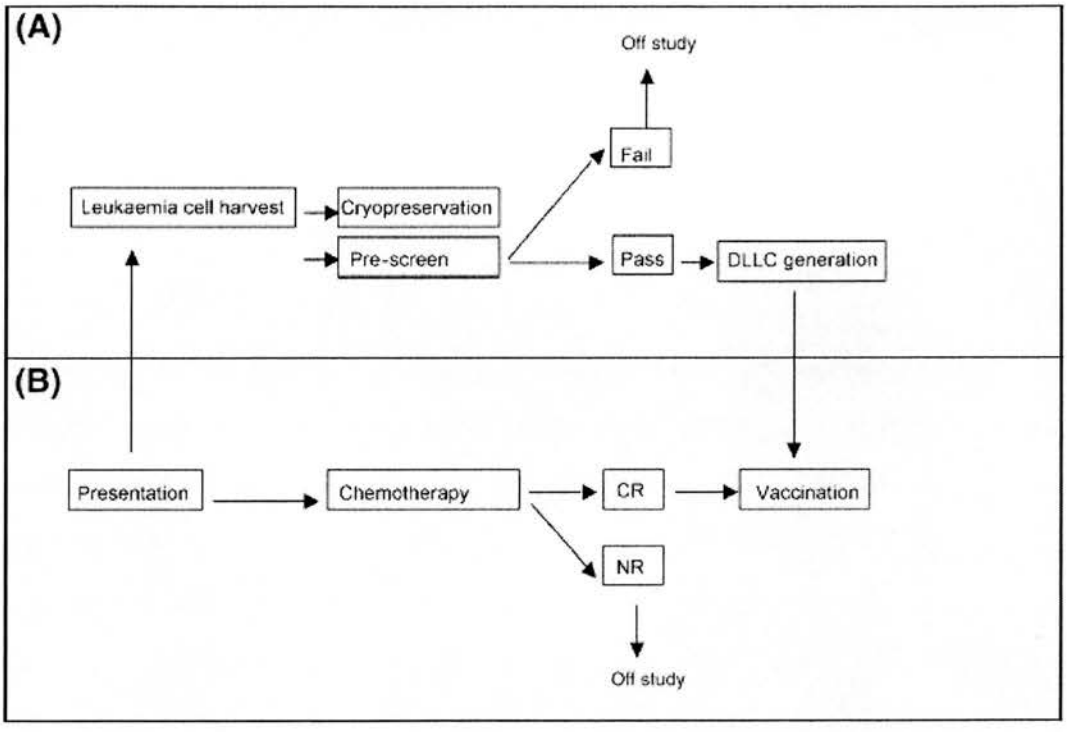


Fig.2.1: Clinical trial outline: The top half of the diagram outlines the steps required to fulfil the laboratory requirements for DLLC vaccination whilst the bottom half represents the clinical requirements. CR=complete remission NR=no remission.

2.1.4.4 Generation of Clinical Grade DLLC

Generation of DLLC for clinical use was performed once the patient had been confirmed to be in remission, based on bone marrow examination. Initiation of culture was timed so that DLLC for vaccination were available 2 weeks following the marrow examination. Cell manipulation, culture and storage was undertaken within the SNBTS Tissue Bank facility at Ellen's Glen Road, Edinburgh, according to a standard operating procedure (Appendix VII).

For patients in Edinburgh, cultured DLLC were washed and re-suspended in a small volume (1mL) of RPMI with 5% AB serum. The DLLC were then subjected to 25Gy γ-irradiation and transferred to the administering hospital in a capped syringe enclosed in a protective sterile container. DLLC for subsequent doses were cryo-preserved at the Tissue Bank, and thawed, washed, re-suspended and irradiated prior to transfer to the administering hospital as above.

For patients in other centres, all doses of DLLC were transferred post thawing, washing, re-suspension and irradiation, undertaken at the Scottish National Blood Transfusion Service tissue bank facility.

Irradiation was undertaken at 25-50Gy from an MCA accredited caesium 137 source in accordance with national guidelines and local Standard Operating Procedures. This irradiation dose has been validated to eliminate the proliferative potential of any residual AML blasts in the inoculum (Appendix VIII).

2.1.4.5 Re-administration and Dosage/Scheduling

DLLC were administered in 1mL of carrier medium by a subcutaneous injection under the direct supervision of an experienced physician in an appropriate clinical facility where resuscitation and supportive care facilities were available. An intra-dermal test dose of $1x10^6$ irradiated DLLC was administered prior to the vaccination protocol and after completion of the DLLC vaccination schedule. Patients were maintained under close observation in the ward for 4 hours following vaccination. Patients received 4 escalating doses of DLLC at weekly intervals of $0.125x10^6$/kg, $0.25x10^6$/kg, $0.5x10^6$/kg and $1x10^6$/kg.

Expected Toxicity

Our previous experience from using autologous dendritic cells pulsed with MUC-1 peptide in patients with breast cancer has failed to show any significant systemic toxicity (Appendix IX). Inflammation and ulceration at the local site of injection has been described in other clinical studies of dendritic cell vaccination. In this study the sites of inoculation were rotated.

2.1.4.6 TREATMENT MONITORING

a) Data Collection

Case Report Forms were provided for each patient. Each page was to be completed in blue or black ink. Any errors were crossed out with a single line and the correct value entered alongside. The change was to be be dated and initialled by the person

making the correction with the original entry still being legible. Snopake, Liquid Paper etc. was not be used.

b) Patient Details

- Name, date of birth
- Height and weight
- French-American-British Classification (FAB) AML subtype
- Newly presenting or relapsed disease
- Cytogenetic and Fluorescent in situ Hybridisation (FISH) analysis
- Chemotherapy given to achieve remission
- Concomitant disease
- Drugs taken in the 7 days prior to DLLC infusion
- Karnofsky score

c) Pre-Treatment Assessment (at presentation)

- Clinical evaluation
- Full Blood Count (FBC), renal function, biochemistry and liver function tests
- Flow-cytometric evaluation of T-and B lymphocytes, CD4:CD8 ratios.
- Immunoglobulin electrophoresis, complement, C-reactive protein (CRP)
- Bone marrow examination
- Human Leucocyte Antigen (HLA) type
- Virology testing (antibodies to HIV, HBV, HCV and for VDRL evaluation)

d) Pre-Vaccination Assessment

- Clinical evaluation
- FBC, renal function, biochemistry and liver function tests
- Bone marrow examination including evidence of MRD (Appendix XI)
- Immune function monitoring (Appendix X)
- Skin test for delayed type hypersensitivity (DTH) response to autologous irradiated leukaemia cells

e) Immediate Post-Vaccination Assessment

Monitoring of vital signs (pulse, BP, temperature) ¼ hourly for first hour, hourly for the next 4 hours. Patients were monitored for adverse events.

An enquiry was made about post treatment morbidity

Clinical and laboratory parameters as outlined in the Trial Schedule (Table 2.2) were evaluated at 14, 28, 42 and 84 days following the first DLLC vaccination. Patients were monitored for adverse events.

A patient diary was kept

f) Monitoring of disease activity

Disease activity was monitored on a weekly basis by FBC measurement and examination of a blood film. Bone Marrow examination, including examination for evidence of MRD, was to be performed at 3 monthly intervals or if there was a suspicion of relapse based on FBC findings.

2.1.4.7 STATISTICAL DESIGN

Descriptive statistical analysis were performed where appropriate

2.1.4.8 TRIAL TERMINATIONS

The study was to be terminated if

- Recruitment was too slow to allow accrual of an adequate number of patients in the anticipated length of time,
- Evidence was gained that patients were being exposed to an unacceptable risk
- For any reason it proved not possible to continue to produce the trial material.

2.1.4.9 PATIENT WITHDRAWAL

Patients were able to withdraw from the study at any time without giving a reason. Patients experiencing any kind of adverse reaction were able to be withdrawn by the investigator at his/her discretion.

2.1.4.10 INFORMED CONSENT

Fully informed written consent was obtained from the patients with the option that they may refuse to take part or withdraw from the study at any time without giving a reason and without affecting their treatment in any way. Copies of the Information for Patients and Patient Consent Form are attached as Appendices I & II. Patient confidentiality was strictly maintained. Patients' names appear only on internal documentation and in any external report or publication relating to the study patient anonymity was ensured. Data relating to the study was stored in compliance with Good Clinical Practice and the Data Protection Act 1998.

With the patient's consent their general practitioner was informed of their participation in the study. A letter for this purpose is attached as Appendix IV.

2.1.4.11 ETHICAL CONSIDERATIONS

The protocol has received approval by the Multi-Centre Research Ethics Committee and the Local Research Ethics Committee prior to entry of any patients into the study The trial was performed in accordance with the requirements of the ICH Tripartite Guideline for Good Clinical Practice, which is consistent with the principles laid down in the Declaration of Helsinki as adopted by the 18th World Medical Assembly, Helsinki, Finland, 1964 and amended by The 29th World Medical Assembly, Tokyo, Japan, October 1975, the 35th World Medical Assembly, Venice, Italy, October 1983, the 41st World Medical Assembly, Hong Kong, September 1989, the 48th World Medical Assembly, Republic of South Africa, October 1996 and the 52nd World Medical Assembly, Edinburgh, Scotland, October 2000.

Table 2.2: Outline of trial monitoring schedule

Time in days in relation to first DLLC vaccination										
	At study entry	Pre Vacc	D+0 **DLLC injection #1**	D+7 **DLLC injection #2**	D+14 **DLLC injection #3**	D+21 **DLLC injection #4**	D+ 28	D+ 42	D+ 84	Notes
History	X									
Examination	X	X	X	X	X	X	X	X	X	
Observation			X	X	X	X				
Weight	X	X								
Height	X									
Karnofsky score	X	X	X	X	X	X	X	X	X	
Adverse events			X	X	X	X	X	X	X	C.T.C grading
HLA type	X									
Virology/VDRL	X									HIV/HBV/HCV/VDRL
ECG	X	X								
Urinalysis	X	X								
Drugs in the last 7 days	X	X	X	X	X	X	X	X	X	Patient diary
BM aspirate	X	X			X		X		X	Then at 3 monthly intervals
FBC	X	X	X	X	X	X	X	X	X	
Flow cytometry	X									T&B lymphocytes, CD4:CD8
U&Es/LFTs	X	X	X	X	X	X	X	X	X	
Immunoglobulins	X	X	X				X		X	
Complement	X	X	X				X		X	
CRP	X	X	X				X	X	X	
ANF	X	X					X		X	
Skin testing		X					X			10^6 γ-irradiated autologous leukaemia cells
Immune function monitoring		X			X		X	X	X	50mL peripheral blood
MRD monitoring		X			X		X		X	3 monthly after D+84
Injection site			X	X	X	X	X			

2.2 Laboratory Materials and Methods

2.2.1 Cells and culture conditions

2.2.1.1 Harvesting of primary leukaemic blasts

Leukaemic blasts were harvested as per study protocol either from peripheral blood, bone marrow or a leukapheresis procedure using a COBE spectra apheresis machine (Gambro BCT, Lakewood, CO, USA) was performed. 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 of peripheral blood and bone marrow were taken into 50 ml polypropylene tubes containing 100 iu/ml preservative-free heparin (Leo, Risborough, UK). Leukapheresis harvests were sent for further processing in the original apheresis bag. Cell manipulation, culture and storage was undertaken within the SNBTS Tissue Bank facility at Ellen's Glen Road, Edinburgh, according to a standard operating procedure in Good Manufacturing Practice (GMP) grade sterile clean room facilities. (Appendix VII). An aliquot of the leukaemic blast sample was transferred to the John Hughes Bennett Laboratory, Edinburgh to pre-screen the differentiation potential of the blasts into DLLC under identical conditions to those at the Tissue Bank facility. Blast samples were diluted at 1:1 ratio with RPMI 1640 culture medium (Sigma-Aldrich, Gillingham, UK). The blast suspension was layered at volume ratio 3:1 in 50 ml polypropylene conical tubes onto Ficoll (Histopaque

1077, Sigma Diagnostics, St.Louis, MO) for density-gradient centrifugation. Cells were centriguged for 25 minutes at 400g and the mononuclear cell layer aspirated from the Ficoll / Serum interface using sterile pipettes. The mononuclear cell layer containing enriched leukaemic blasts was transferred to 15 ml conical tubes and re-suspended with 10 ml RPMI 1640 culture medium and centrifuged at 400 g for 5 minutes. After decanting of the supernatent the cell pellet was re-suspended by flicking of the tube and subsequently re-suspended to 15 ml medium for a second centrifugation at 400 g for 5 minutes. Finally, blast samples were re-suspended to 10 ml using RPMI 1640.

2.2.1.2 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/ml x10.

2.2.1.3 Cryopreservation

Cells were aliquoted not exceeding $1x10^8$/ 500µl culture medium in cryovials (cryotube™, Nunc, Denmark). An equal volume of freezing medium consisting of 90% fetal calf serum (FCS) and 10% tissue culture grade DMSO (Sigma-Aldrich, Gillingham, UK) was added drop-wise and samples placed in sequence into a -20^0C freezer for 2 hours, followed by -70^0C freezing overnight and finally transfer to a -140^0C freezer for storage.

2.2.1.4 Thawing of cryopreserved cells

Cryopreserved cells were thawed rapidly at 37^0C in a water bath. The cell suspension was diluted by initial drop-wise addition of RPMI supplemented with 5% heat-inactivated human AB serum and then by accelerating suspension up to 20 mls followed by centrifugation at 400g for 5 minutes. The resulting supernatant was then discarded and the pellet was re-suspended in medium supplemented by 5% human AB serum for cell culture.

2.2.1.5 Cell culture medium and culture conditions

All cell culture was performed using Complete Medium (CM), RPMI 1640 (Sigma-Aldrich, UK) supplemented with 5% heat inactivated human AB serum, 100 U/ml Penicillin/Streptomycin (Sigma-Aldrich, UK) and 2mM/l L-glutamine (Sigma-Aldrich, UK), and the medium was supplemented with cytokines and maturational agents as directed by the work undertaken. Plastic-ware used for tissue culture (25- and 75cm^2 culture flasks and 6-well plates) was resourced from Corning, NY, USA. All cultures were incubated at 37^0C in a humidified incubator (Heraeus) containing 5% CO_2.

2.2.2 Generation of Dendritic-Cell vaccine constructs

2.2.2.1 Generation of Dendritic-Like Leukaemic Cells (DLLC) from AML blasts

Complete medium as described above was used. Cell density was adjusted to 1x 10^6/ml and GMCSF 100ng/ml (Sandoz, Surrey, UK) and IL-4 20ng/ml (R+D Systems, Abingdon, UK) were added. After 3 days of culture at 37^oC / 5% CO_2

fresh GMCSF (100 ng/ml) and Tumour Necrosis Factor-α (TNF-α) 25ng/ml (Peprotech, Rocky Hill, NJ, USA) was added. At day 6 of culture further maturational agents Polyriboinosinic polyribocytidylic acid (Poly I: C) at 12.5 mcg/l (Sigma-Aldrich, UK) and Interferon-gamma at 30ng/ml (Boehringer, Ingelheim, Germany) were added and cells were harvested at day 7 of culture for immuno-phenotypic characterisation by flow-cytometry and microscopic analysis of cytospin preparations.

In addition to the outlined standard cytokine combination, various concentrations of IL4, GMCSF with or without the addition of the Bryostatin-1, 10-20 ng/ml, as well as blast culture in medium supplemented with calcium ionophore A23187 100 ng/ml (Sigma-Aldrich) were explored in an attempt to overcome differentiation resistance of AML blasts to a dendritic-like phenotype.

2.2.2.2 Determination of successful DLLC differentiation of leukaemic blasts

Confirmation of successful DLLC differentiation was determined by flow-cytometric analysis using a FACSCalibur flow-cytometer (Becton Dickinson, San Jose, CA, USA) following staining of the cells with the following monoclonal antibodies: CD40, CD80, CD86, CD83, CD11c (all Caltag, Burlingame, CA, USA). DLLC differentiation was deemed to have occurred, if more than 20% of cells expressed two or more of the above markers. This phenotype was based on previous studies that demonstrated that this correlated with the acquisition of functional dendritic cell characterisitics, such as increased allo-stimulatory capacity and production of IL-12(Roddie *et al*, 2002). The acquisition of a characteristic Dendritic Cell

morphological phenotype was confirmed by light microscopy of cytospin preparations of AML blasts before and after attempted cytokine maturation. These assessments were performed each time in pilot screening cell cultures and also in subsequent bulk cultures for vaccine generation.

2.2.2.3 Generation of mature DCs from Peripheral Blood Monocytes of patients in complete morphological remission or from healthy donors.

Mature DCs (mDC) were generated from the adherent fraction of PBMCs collected after the resolution of cytopenias following combination intravenous chemotherapy or from healthy donors. PBMCs were suspended at 10^7/ml in Complete Medium (CM), RPMI 1640 (Sigma-Aldrich, UK) supplemented with 10% FCS, 100 U/ml Penicillin/Streptomycin (Sigma-Aldrich, UK) and 2mM/l L-glutamine (Sigma-Aldrich,UK), and incubated for 2 hours at 37°C / 5% CO_2 in 75cm^2 culture flasks (Corning, NY,USA). Non-adherent lymphocytes were subsequently removed and cryo-preserved. Adherent monocytes were re-suspended with CM in the presence of granulocyte-macrophage colony stimulating factor (GMCSF; Sandoz, Surrey, UK) at 100ng/ml and IL-4 (R+D Systems, Abingdon, UK) at 15ng/ml for 5 days. DCs were then matured for further 2 days by the addition of tumour necrosis factor-alpha (TNF-α; Peprotech, Rocky Hill, NJ) at 25ng/ml, Poly I:C (Sigma-Aldrich, UK) at 12.5 mcg/l and Interferon-gamma (IFN-γ; Boehringer, Ingelheim, Germany) at 30ng/ml.

2.2.2.4 Generation of AML-mDC Fusion Hybrids

Thawed AML blasts and fresh mDC were mixed at a ratio of 1:1, not exceeding 5 x 10^6 each in 37°C CM and centrifuged for 5 min at 400g to pellet. After removal of all supernatant, cells were fused by drop-wise addition of 500µl of 50% Polyethylene Glycol (PEG)/10% DMSO (Sigma) over 1 min in a 37°C water-bath . The reaction was stopped after a further minute by adding 1ml of CM and further dilution to 15 ml with 37°C heated CM prior to use as stimulators in autologous cytotoxicity assays and allogeneic mixed lymphocyte leukaemia reactions (MLLR).

2.2.3 Morphological and flow-cytrometric assessment of cells

2.2.3.1 Cytospin preparations

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). Digital photographs of cytospin preparations of native AML blasts at diagnosis prior to cell culture and post attempted cytokine differentiation into DLLC were taken using a Leica Microscope (Leitz, Wetzlar, Germany) and Zeiss Axiovision Digital Image Archive System (Jena, Germany).

2.2.3.2 Assessment of AML-mDC fusion efficiency

A series of experiments was conducted to establish the efficiency of PEG induced AML-DC hybrid formation. Prior to fusion the AML blasts were labelled with the red fluorescent linker dye PKH 26 (Sigma) according to manufacturer's instructions. Cells were pelleted by centrifugation and all supernatant removed. 0.5ml of manufacturer's diluent and 0.5ml of 0.004 mMol PKH26 was added and cells left to stain for 3 minutes. The reaction was then stopped with 1ml FCS and cells were left for a further 1min incubation prior to re-suspension with 10 mls of PBS and centrifugation at 1200rpm for 5 minutes. Cells were then re-suspended and transferred to a fresh centrifugation tube and washed and pelleted by centrifugation three times, as outlined above. Final re-suspension was in 1ml PBS.

DCs were labelled using the green Cell Tracker CMFDA. Cells were pelleted by centrifugation and all supernatant removed from the pellet. 1ml of 1micromolar CMFDA was added in a 37°C water-bath and left to stain for 15 minutes. The stained cells were then re-suspended with PBS up to 10 mls and centrifuged at 12000 rpm for 5 minutes. The supernatant was again discarded and further fresh 10 ml PBS was added, followed by a post-stain incubation period of 30 minutes in a 37°C water-bath. After a further 5-minute centrifugation the pelleted cells were finally re-suspended in 1 ml PBS.

Un-fused and fused cells were analysed by flow-cytometry. Cells presenting both membrane dyes after fusion, therefore fluorescing in both the FL1 and FL2 channels, were scored as fusion hybrids. In addition, fluorescent microscopy using motorised micro-focussing through the generated heterokaryons was undertaken to visualise double fluorescent membrane of AML-DC Fusion Hybrids. Separate images of

cytospin preparations were created using FITC, Texas Red and DAPI excitation filters in a Zeiss Axioplan fluorescence microscope with motorised focus and images were subsequently combined. Chroma filter sets, Vector shield mountant and a Princeton Instruments Micromax digital camera were used.

2.2.3.3 Immunophenotypic characterisation of myeloid cells

Myeloid cells underwent immunophenotypic analysis using a panel of mAb directed against co-stimulatory molecules, HLA molecules and dendritic cell-associated markers, prior to and following attempted dendritic cell differentiation (Table 2.3).

Table 2.3: Panel of immuno-phenotypic markers used to characterize leukaemia cells and DLLC

CD marker or name	Brief description of function
CD11c	Type I transmembrane protein-high level expression on most human DC's
CD14	Expressed on Monocytes and Macrophages- binds bacterial lipopolysacharide
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
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
HLA I	MHC molecule involved in antigen presentation to CD8+ T lymphocytes
HLA II	MHC molecule involved in antigen presentation to CD4+ T lymphocytes

Cells were harvested and pelleted by centrifugation at 400g for 5 minutes. A viable cell count was performed and $2x10^5$ cells were taken and added to 12x75mm plastic tubes for staining with murine monoclonal antibodies (mab) pairs, either fluorescein

isothiocyanate (FITC) conjugated or phycoerythrin (PE)-conjugated: CD14/CD11c, CD40/CD83, CD86/CD80, HLA Class I/HLA Class II (all Caltag, Burlingame, CA, USA). 20µl was used of each antibody. Phosphate buffered saline (PBS) with 0.1% sodium azide was added to give a final volume of 100µl. Tubes were then incubated for 20 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 PBS/0.1% sodium azide was added. After 5 minutes incubation analysis was performed on a FACSCalibur flow-cytometer (Becton Dickinson, San Jose, CA, USA). Appropriate isotype controls for the monoclonal antibodies were used in all immunophenotypic stains. Non-viable cells were identified by Propidium Iodide (PI) uptake of cells in a separate sample tube and viable cells were defined with a forward scatter (FS)/PI gate. Data analysis was performed using WinMDI software (Scripps, La Jolla, CA, USA).

2.2.3.4 Characterisation of lymphoid responder cells

Cells were harvested and pelleted by centrifugation at 400g for 5 minutes. A viable cell count was performed and $2x10^5$ cells were taken and added to 12x75mm plastic tubes for staining with murine monoclonal antibodies (mab) pairs, either fluorescein isothiocyanate (FITC) conjugated or phycoerythrin (PE)-conjugated (all Caltag, Burlingame, CA, USA). The T-cells were characterised by estimating numbers and proportions of CD3 positive cells, CD4:CD8 ratio, HLA Class I and II expression, regulatory T-cell profile (CD4/CD25 expression) and activation status (CD25/CD45RO expression). B-cell numbers and proportions were determined by their expression of CD19. Flow-cytometry protocols and data analysis were performed according to methods outlined above.

2.2.4 Assessment of immuno-stimulatory properties of vaccine constructs

2.2.4.1 Stimulation of leukaemia-specific lymphocytes by co-culture with autologous DLLC, AML, Fusion Hybrids of AML with mature DC, or mature DC co-cultured with irradiated AML blasts

The non-adherent fraction of fresh or thawed remission blood samples provided the source of autologous responder cells. Unmodified AML blasts, DLLC, AML–mature DC (mDC) Fusion Hybrids and mDC mixed 1:1 with irradiated (26Gy) AML blasts were used as autologous stimulators. PBMC cultured in the absence of stimulators or primed by un-pulsed mDCs and AML-AML fusions served as negative controls, as these are not expected to stimulate leukaemia directed cytotoxicity.

All stimulators received 26Gy of irradiation from a Cs137source. Co-culture of 10^7 responder cells with 10^6 irradiated stimulators was prepared in 12-well plates at a cell density of 10^6/ml effectors. CM was supplemented with IL-2 5ng/ml and IL-7 5ng/ml (Peprotech, Rocky Hill, NJ). For subsequent Chromium^{51} cytotoxicity assays, responder cells were re-stimulated at day 7 and day 14 using the same stimulators that had been added at the beginning of co-culture. CM and cytokines were half exchanged weekly. Effectors were harvested on day 21 for subsequent Chromium^{51}cytotoxicity assays against native, autologous AML blasts.

2.2.4.2 Allogeneic Mixed Leukaemia-Lymphocyte Reactions (MLLR)

Responder T-cells for allogeneic MLLR were obtained from the non-adherent lymphocytes of healthy, unrelated donors, whose PBMC had been separated by

gradient density centrifugation followed by 2hrs plastic adherence. Adherent donor monocytes were matured in a 7-day culture into mature DC (mDC) as described above and subsequently fused to patient AML blasts to generate AML-DC fusion hybrid stimulators. Un-fused AML blasts and mDC as well as AML co-cultured with mDCs served as stimulator controls. AML-AML or mDC-mDC fusions and titrating concentrations of polyethyleneglycol (PEG) were also assessed regarding their ability to stimulate the proliferation of lymphocytes allogeneic to the AML blasts used. Responder cells were plated at 1 x 10^5/well in CM in U-bottomed 96 well plates. Stimulators were added at graded Responder/ Stimulator ratios of 5:1, 10:1, 25:1 and 100:1. The proliferation of lymphocytes was determined by the uptake of [3H]-thymidine (ICN Biochemicals, Basingstoke, UK), added for the final 18 hours of a 5 day co-culture at 37°C / 5% CO2. Results were expressed as mean counts per minute ± standard deviation in triplicates.

2.2.4.3 Cr^{51}-release cytotoxicity assays

Following re-stimulation co-culture with a variety of autologous stimulators the resulting leukaemia directed *in vitro* cytotoxicity was measured in a 4 hr ^{51}Cr-release cytotoxicity assay in patients, where uptake of the isotope by their native AML blasts was demonstrable. 1 x 10^6 AML blasts were incubated for 1 hr at 37°C / 5% CO_2 with 100μCi ^{51}Cr (ICN Biochemicals, Basingstoke, UK), washed three times with CM and 1 x 10^4 labelled targets were subsequently co-cultured with autologously activated lymphocytes in 200μl CM in 96 well U-bottomed plates at Effector:Target ratios of 50:1, 25:1, 12.5:1 and 6.25:1. After 4 hours 25μl of cell free supernatent were collected and mixed with 150μl of scintillant (Ecolite+, ICN Biochemicals, Basingstoke,UK). Scintillation was analysed using a liquid scintillation and

luminescence counter (1450 microbeta trilux, Wallac, Finland). Specific lysis was calculated using the following formula: [(cpm experimental release – cpm spontaneous release) / (cpm maximal release – cpm spontaneous release)] x 100. Spontaneous ^{51}Cr release was determined from wells containing target cells and medium only. Maximum ^{51}Cr release was gained by lysing target cells with 100 µl of 2% Triton x 100 / sample well. Spontaneous release was less than 15% of maximum release. All assays were performed in triplicates and expressed as mean counts per minute ± standard deviation.

2.2.4.4 Measurement of IFN-γ secreting CTL by enzyme linked immunospot essay (ELISPOT).

Peripheral blood mononuclear cells (PBMNC) were used as a source of T-cells for the purposes of the assay. 10^7 cells were co-cultured with 10^6 irradiated autologous stimulators: AML, DLLC, Fusion hybrids of AML with mature DC, or mature DC co-cultured with irradiated AML blasts in complete medium (CM), supplemented with IL-2 5ng/ml and IL-7 5 ng/ml (both Peprotech). Cells were harvested on day 7 for subsequent enzyme-linked immunospot essay (ELISPOT). Sterile Multiscreen-IP plates (PVDF membranes, Millipore, UK) were coated overnight at 4°C with 50µl / well anti-human IFN-γ antibody (Bender MedSystems, Vienna, Austria) diluted to 10µg/ml in carbonate buffer. Coated plates were washed 4 times with PBS / 0.05% Tween 20 and blocked with CM for 2 hours at 37°C / 5% CO_2. Effector cells (100µl) were added to triplicate wells at graded concentrations, followed by 1 x 10^4 target cells/well (100µl) to Effector:Target ratios of 20:1, 10:1, 5:1 and 2.5:1. After co-culture for 36 hours at 37°C / 5% CO_2, cells were removed from the assay and biotinylated, anti-IFN-γ avidin conjugated alkaline phosphatase and BCIP NBT

substrate (Sigma Aldrich, St Louis,MO) were used for the calorimetric reaction. Spots were quantitated using an automated ELISPOT reader (AID Systems, Strassberg, Germany).

2.2.4.5 Wilms Tumour 1 (WT1) HLA-A2 tetramer-positive CTL analysis

Numbers of WT1 specific CTL were measured in the peripheral blood of unique patient number (UPN) 18, who was HLA-A*201 positive. WT1-HLA-A2 tetramer (Beckman Coulter, Fullerton, CA, USA) was custom-made for the WT126 peptide (sequence RMFPNAPYL) (Gao *et al*, 2000). PE-conjugated WT1/HLA-A2 tetramer was incubated with $1x10^6$ PB MNCs at final concentration of 50μg/ml in phosphate buffered saline at 4^0C. After washing, cells were incubated with FITC-labelled CD8 mab (Caltag, Burlinghame, CA, USA) for 30 mins at 4^0C. Cells were then washed and then analysed on a FACSCalibur Flow-Cytometer.

2.2.5 Measurement of Minimal Residual Disease (MRD)

Monitoring for MRD was performed by real-time quantitative PCR (RT-PCR) for WT1 gene expression prior to and then at time-points 14, 28 and 84 days following vaccination. In four patients WT1 expression was measured in BM with the remaining patient having PB WT1 expression measured due to practical considerations. Total RNA was extracted from mononuclear cells using TRI-Reagent (Sigma-Aldrich, UK). Reverse transcription was carried out using random hexamers (GE Healthcare, Amersham UK) and Superscript II (Invitrogen, Paisley UK). Relative quantitation of WT1 expression was performed by realtime PCR using the ΔΔCt method on an ABI 7900 HT thermal cycler. cABL was used as the

endogenous control. The WT1 primers and Taqman probe were as previously described(Ogawa *et al*, 2003). The c*ABL* primers and probe were:

ABL-146F; GATACGAAGGGAGGGTGTACCA

ABL-240R; CTCGGCCAGGGTGTTGAA and

ABL-183T; 5'-FAM-TGCTTCTGATGGCAAGCTCTACGTCTCCT-TAMRA-3'.

Fifty cycles of PCR were performed. Absolute quantitation of WT1 transcript was performed using the Profile*Quant* (Ipsogen, Marseille, France).

2.2.6 Measurement of T regulatory cell numbers

At the same time points as measurement of antileukaemic CTL responses were measured, T regulatory (Treg) cell numbers were assessed. 2 x 10^5 peripheral blood mononuclear cells were stained with the following combinations of monoclonal antibodies; CD3-FITC alone, CD4-FITC + CD25-PE and CD8-FITC + CD25-PE (all Caltag, Buringame, CA, USA) and cells analysed by flow-cytometry on a FACSCalibur flow-cytometer. Treg were gated on the basis of CD4 positivity and bright expression of CD25.

2.2.7 Statistical methods

Statistical analysis of Cr^{51} cytotoxicity, ELISPOT and [3H]-thymidine uptake assay results was performed using Student's t-test.

Chapter 3: Clinical Results

3.1 Results of the clinical DLLC vaccination study

3.1.1 Feasibility and safety

Patients were recruited from several clinical centres throughout the United Kingdom during a 2-year period from November 2001 and DLLC vaccination schedules for 5 eligible patients were completed by October 2004. There were no protocol violations throughout the study duration. The clinical details of the 22 patients recruited into the study are summarised in Table 3.1.

Only five patients were eligible to enter the vaccination program. Failure to proceed to vaccination was due to one or more of the following reasons; induction death or failure to achieve complete remission (27%), insufficient cell numbers in the initial leukemia cell harvest (22%, including 5 of 6 marrow harvests) and failure of leukemia cells to undergo DLLC differentiation (68%). Vaccination was generally well tolerated. Only one patient, UPN 20, developed any side effects likely to be attributable to the vaccination. At day 14 post vaccination she had an extensive recurrence of eczema. In addition her anti-nuclear factor (ANF) which was positive at a titre of 1/40 pre vaccination, increased to a titre of 1/160 (nucleolar pattern) at day 84 and remained at this level following the end of the vaccination course. Two of the patients, UPN12 and UPN14, had clinical relapse of their leukaemia shortly after

Table 3.1: Clinical details of patients recruited into the DLLC vaccination study.
Patient characteristics; AML presentation status, WHO subtype and karyotype; peripheral blood blast cell count and type of leukemia cell harvest; permissiveness of leukaemia cells to DLLC differentiation, outcome of induction chemotherapy and eligibility for DLLC vaccination

Patient identifier	Age (years)	Sex	Presentation status	WHO type	Cytogenetics	PB blast cell count ($\times 10^9$/l)	Type of harvest	DLLC differentiation	Treatment outcome	Eligible for vaccination
UPN1	67	M	*De novo*	M4	46, XY	5·7	PB	N	CR	N
UPN2	70	M	Relapse	M1	Complex	14·2	PB	N	ID	N
UPN3	64	F	*De novo*	M1	46, XX	<1	BM	N	CR	N
UPN4	65	M	tMDS	M2	Complex	17	PB	Y	RD	N
UPN5	62	M	*De novo*	M5	46, XY	md	PB	N	RD	N
UPN6	61	M	tMDS	M4	47, XY, +8	26·8	PB	Y	RD	N
UPN7	67	F	Relapse	M1	46, XY	3·98	BM	N	CR	N
UPN8	74	F	*De novo*	M1	46, XX	2·9	BM	N	CR	N
UPN9	52	F	Relapse	M2	46, XX	<1	BM	N	CR	N
UPN10	62	M	*De novo*	M0	46, XY	<1	BM	N	CR	N
UPN11	62	M	Relapse	AML with mld	46, XY	3·6	PB	N	ID	N
UPN12	60	M	Relapse	M0	46, XY	3·1	PB	Y	CR	Y
UPN13	63	M	*De novo*	AML with mld	46, XY	<1	BM	N	CR	N
UPN14	61	F	*De novo*	M5	46, XX	50	PB	Y	CR	Y
UPN15	69	M	*De novo*	M5	del 9	<1	BM	N	RD	N
UPN16	40	M	*De novo*	M5	46, XY	26	PB	Y	CR	Y
UPN17	30	F	*De novo*	M4	46, X	13·8	PB	N	CR	N
UPN18	67	F	*De novo*	M1	46, XX	36·8	PB	Y	CR	Y
UPN19	65	M	*De novo*	AML with mld	47, XY, +8	1·5	LP	N	CR	N
UPN20	43	F	*De novo*	M5	46, XY	37	PB	Y	CR	Y
UPN21	70	M	Relapse	M1	46, XY, add(8)	5·8	LP	N	CR	N
UPN22	42	M	*De novo*	AML with mld	46, XX	7	PB	N	CR	N

the start of vaccination. The clinical details of the five vaccinated patients are summarised in Table 3.2.

Table 3.2: Clinical details of the five patients who underwent the DLLC vaccination program. Patient and AML characteristics; type of induction chemotherapy; disease status at time of vaccination; current status; duration of leukemia free survival and overall survival from start of DLLC vaccination

UPN12	60	M	M0	46,XY	FLAGx1	CR3	†	0	28
UPN14	62	F	M5	46,XX	FLAGx2/idACx2	CR1	†	2	3
UPN16	40	M	M5	46,XY	DAx2/hdACx2	CR1	CR1	14*	14*
UPN18	67	F	M1	46,XX	DAx2/MidAC+ICE	CR1	†	12	13
UPN20	43	F	M5	46,XY	DAx2/MACE+MidAC	CR1	CR1	11*	11*

CR=complete remission † =dead
FLAG=fludarabine, cytarabine and G-CSF idAC=intermediate dose cytarabine DA=daunorubicin and cytarabine hdAC=high dose cytarabine
MidAC=mitoxantrone and cytarabine ICE=idarubicin, cytarabine and etoposide MACE=amsacrine, cytarabine and etoposide
LFS=leukaemia free survival from start of vaccination OS=overall survival from start of vaccination
*alive and remains in CR1

3.1.2 Cytokine induced DLLC maturation of AML blasts

The optimal cytokine cocktail to achieve DLLC maturation from AML blasts of different subtypes of myeloid leukaemia has been subject of much scientific debate. In this study an approach of minimal interference during the seven day cytokine incubation, adding further cytokines to medium at day 3 (GMCSF and TNF-α) and day 6 (Poly I:C and Interferon-γ) achieved best DLLC maturation results in most permissive blasts, compared to centrifuging and re-suspending maturing AML blasts in fresh cytokine enriched medium at these time-points. Previous investigators have reported a low likelihood of patients with complex karyotypic abnormalities to undergo blast differentiation to DLLC (Roddie *et al*, 2002) and this study reproduces these findings. Out of seven patients whose blasts were permissive to cytokine induced DLLC differentiation, only one had a complex karyotypic abnormality

(UPN4), a further patient was found to have a trisomy of Chromosome 8 (UPN6). These two patients showed a myelodysplastic phenotype and did not enter remission of their leukaemia with induction chemotherapy, and therefore were not eligible for subsequent DLLC vaccination. All five patients receiving the DLLC vaccination showed a normal male or female karyotype. Three of these patients had a monocytic subtype of AML (WHO: AML, not otherwise classified, FAB M5).

Details of the immuno-phenotypic profiles of AML blasts prior to and following cytokine incubation are shown in Table 3.3. Marked apoptosis (>75%) in response to cytokine culture was observed in several maturation resistant cases (UPN5, UPN11), limiting the immuno-phenotypic workup of these patients blasts.

Most of the cases permissive to cytokine maturation to DLLC did show some expression of one or several co-stimulatory markers of their blasts in the native state, but up-regulation in more than 20% of blasts together with the development of the characteristic DC-morphology, showing loosely adherent clumps of large cells with round or bean-shaped nuclei, a relatively low nuclear-cytoplasmatic ratio and characteristic dendritic processes on microscopic examination of MGG stains of cytospin preparations was only seen after 7 days culture with the cytokine maturation cocktail. Morphological changes of native AML blasts following DLLC maturation for UPN12, UPN14, UPN16 and UPN18 are shown in Fig.3.1.

	Native AML blasts							Post cytokine maturation						
							Apoptosis							Apoptosis
A)	*UPN*	*CD40*	*CD83*	*CD86*	*CD80*	*CD14*	*CD11c*	*PI*	*CD40*	*CD83*	*CD86*	*CD80*	*CD14*	*CD11c*	*PI*
	UPN4	(-)	(-)	++	(-)	nd	nd	(-)	++	++++	++	++	nd	nd	++
	UPN6	(-)	(-)	+	+	++	++	(-)	(-)	(-)	++	++	+	++	+++
	UPN12	(-)	(-)	+	++	(-)	+	(-)	++	+	+	+	(-)	++	++
	UPN14	(-)	+	++	++	++	++	(-)	+	+	+++	++	(-)	+++	++
	UPN16	(-)	+++	+++	+++	+++	+++	+	+	(-)	++	+	(-)	+++	+
	UPN18	(-)	++	(-)	++	++	+++	(-)	+++	++	+++	++	(-)	++++	++
	UPN20	(-)	+++	+++	+++	++	+++	(-)	(-)	(-)	+++	(-)	++	++++	++

B)	*UPN*	*CD40*	*CD83*	*CD86*	*CD80*	*CD14*	*CD11c*	*PI*	*CD40*	*CD83*	*CD86*	*CD80*	*CD14*	*CD11c*	*PI*
	UPN1	(-)	+	++	++	nd	nd	+	(-)	(-)	(-)	(-)	nd	nd	++
	UPN2	(-)	++	(-)	++	nd	nd	+	(-)	(-)	(-)	(-)	nd	nd	+++
	UPN3	(-)	+	(-)	(-)	nd	nd	(-)	(-)	(-)	(-)	++	nd	nd	++
	UPN5	(-)	+	(-)	++	(-)	++++	++	(-)	(-)	+	+	(-)	+	++++
	UPN7	(-)	+	(-)	+	(-)	++	(-)	(-)	(-)	+	++	(-)	++	++
	UPN8	(-)	(-)	(-)	(-)	(-)	(-)	+	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	UPN9	(-)	(-)	nd	nd	(-)	++	(-)	(-)	(-)	(-)	+	(-)	++	(-)
	UPN10	(-)	(-)	(-)	+++	(-)	+	(-)	(-)	(-)	(-)	(-)	(-)	(-)	++
	UPN11	(-)	(-)	(-)	++	(-)	+	nd	(-)	(-)	(-)	+	nd	nd	++++
	UPN13	(-)	(-)	(-)	++	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	+
	UPN15	(-)	++	(-)	++	+++	+++	(-)	(-)	(-)	(-)	(-)	++	+++	++
	UPN17	(-)	++	++	++	++	+++	(-)	(-)	(-)	(-)	(-)	(-)	+	+
	UPN19	(-)	++	(-)	+++	(-)	(-)	(-)	(-)	(-)	(-)	+	(-)	++	+++
	UPN21	(-)	(-)	++	(-)	(-)	+	(-)	(-)	(-)	+++	+	(-)	+	(-)
	UPN22	(-)	(-)	(-)	(-)	(-)	+	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Table 3.3: Immuno-phenotypic profile of AML blasts permissive (A) and resistant (B) to DLLC maturation prior to and following cytokine incubation

Scoring for immunophenotype: <10%(-), 10-19% **+**, 20-49% **++**, 50-75% **+++**, >75% **++++**

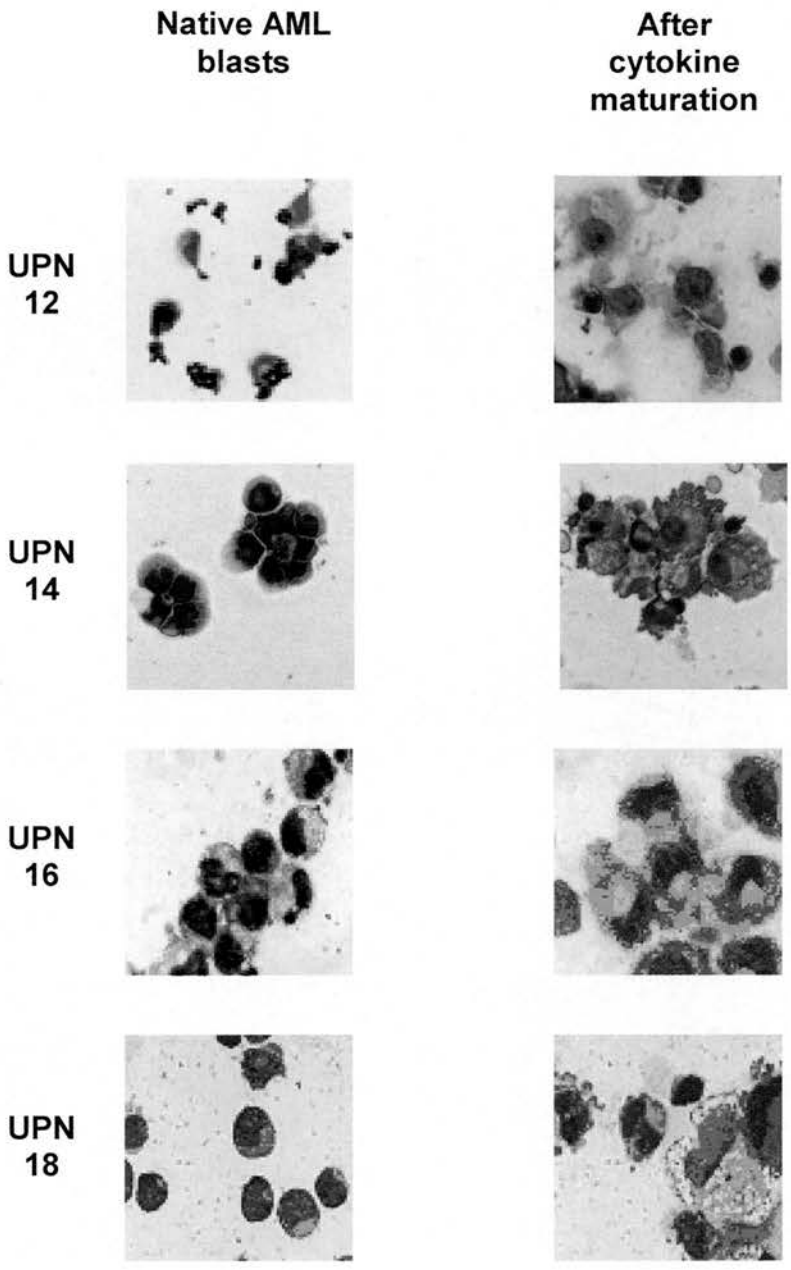


Fig. 3.1: AML blasts acquire Dendritic Cell-like morphology following cytokine culture

Shown are microscopic images of cytospin preparations of native AML blasts prior to and after successful cytokine-induced DLLC maturation for UPN 12, UPN 14, UPN 16 and UPN 18

The acquisition of a characteristic dendritic-like immuno-phenotype was accompanied by a marked increase in forward- and side scatter compared to native AML blasts on flow-cytometric analysis. This feature helped to differentiate those patients, whose blasts showed strong DLLC maturation from those with a weak immuno-phenotype. Fig. 3.3 shows the characteristic features of a differentiation permissive case in comparison to blasts from a patient resistant to cytokine induced blast maturation. A further helpful characteristic accompanying successful cytokine induced DLLC maturation was the observation of increased expression of CD11c compared to native AML blasts, which was, in cases with a monocytic or myelo-monocytic AML phenotype, associated with decreased expression of the monocytic marker CD14.

3.1.3 Use of additional differentiation agents

Previous studies have reported that AML resistance to cytokine induced maturation using GM-CSF, IL-4 and TNF-α can be overcome with the addition of Bryostatin-1, a differentiation agent that exerts its action by interfering with protein kinase C activity (Roddie *et al*, 2002).

In parallel to the standard cytokine maturation cocktail, additional experiments were undertaken to investigate the effect of adding Bryostatin at the beginning of the culture period to the combination of GM-CSF and Il-4, then adding further maturational agents TNF-α, Interferon-γ and Poly I:C at day 3 and 6 respectively of the culture period, as in the standard maturation protocol used in the clinical trial.

Calcium Ionophore has, likewise, been reported to induce dendritic like maturation in leukaemic blasts in the literature(Waclavicek *et al*, 2001;Kharfan-Dabaja *et al*, 2005). Calcium Ionophore was used as sole differentiation agent (UPN6, UPN8-UPN15) and in addition to the cytokine maturation cocktail (UPN16, UPN18-22) in parallel cultures to the standard pilot maturation assays to assess the potential of this molecule in overcoming differentiation resistance in the study patients AML blasts (Fig.3.2). No Bryostatin-1 or Calcium-Ionophore-treated blasts were used to vaccinate patients in the clinical trial.

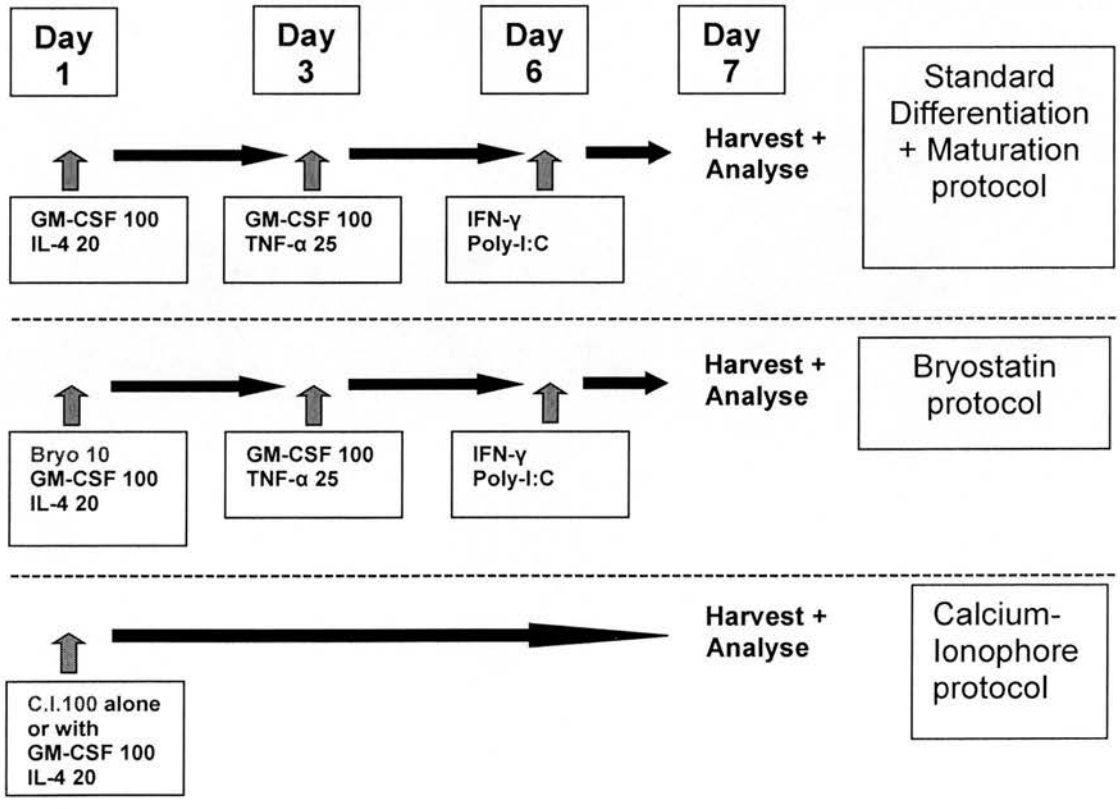


Fig.3.2: Schedules of Differentiation / Maturation Culture

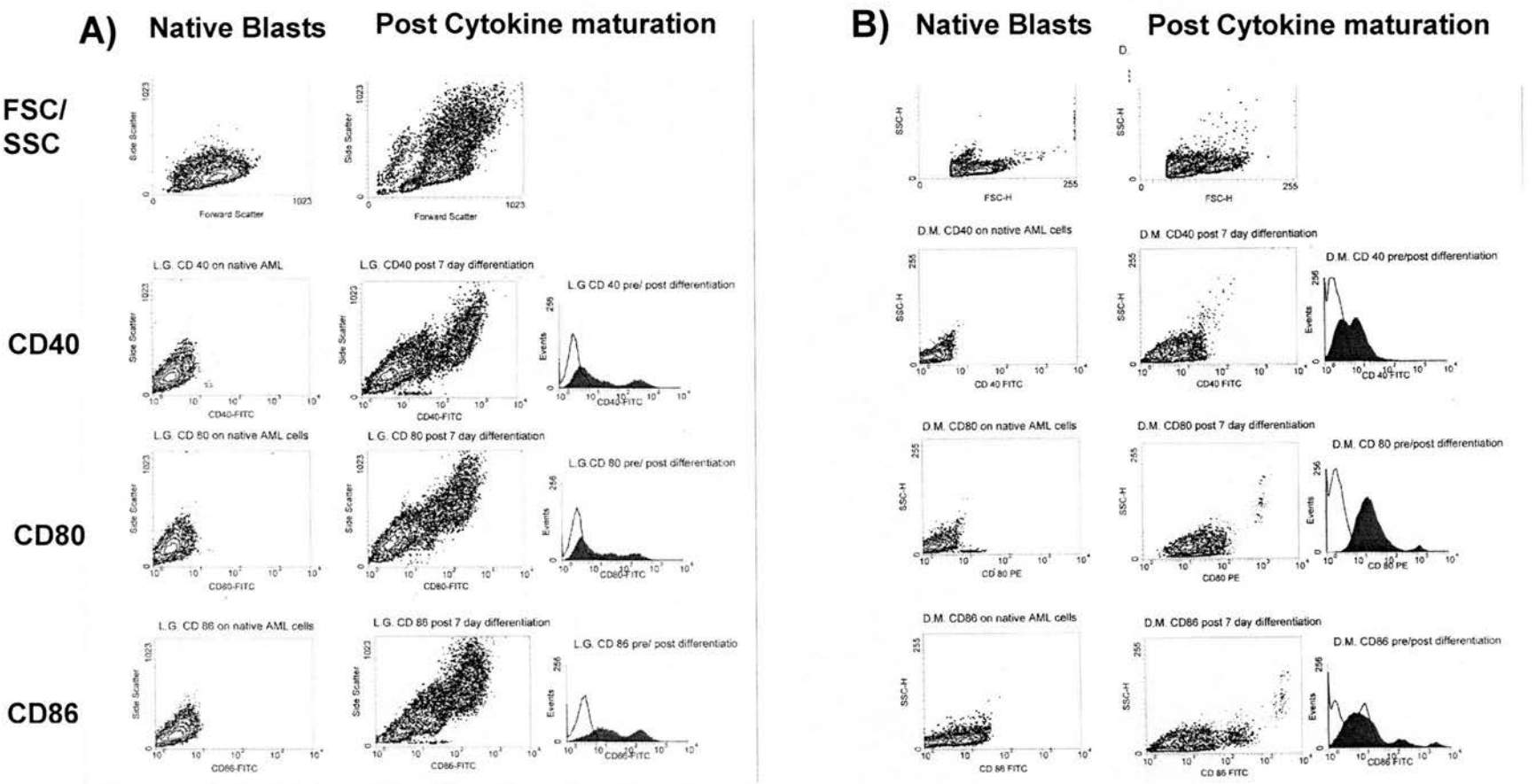


Fig.3.3: Examples of strong (A) and weak (B) cytokine induced AML maturation to DLLC.
Permissive blast (patient A) show marked increase in forward and side scatter in flow-cytometric analysis as they undergo DLLC differentiation, associated with expression of co-stimulatory molecules. In contrast, blasts from patient B (UPN1) do not undergo the characteristic morphological change and exhibit up-regulation of only selective co-stimulatory molecules (CD80).

3.1.3.1 Bryostatin-1

Bryostatin-1 was initially used in a concentration of 20 ng/ml, added at day 1 of culture to the cytokine maturation cocktail (UPN3-UPN5). In view of marked apoptosis observed, later experiments were conducted using a reduced dose of 10 ng/ml. Morphologically the addition of Bryostatin-1 to the maturation cocktail provoked the development of a plastic adherent phenotype with cytoplasmatic processes resembling those of dendritic cells in some AML blast samples (differentiation resistant or permissive cases). However, in flow-cytometric analysis, consistent up-regulation of co-stimulatory molecules as a consequence of Bryostatin-1 addition was not observed and no differentiation resistant cases were rendered permissive by the addition of Bryostatin-1. In two cases (UPN6, UPN14) that showed cytokine induced DLLC differentiation, the resulting immuno-phenotype was accentuated by Bryostatin-1 in further increasing expression of CD11c and reducing expression of the monocytic marker CD14 in these AML cases with monocytic- and myelomonocytic morphology respectively. A similar effect on CD11c was observed on a differentiation resistant case (UPN9, FAB type M2), but co-stimulatory markers were not up-regulated by Bryostatin-1 (Fig.3.4). In summary, the addition of Bryostatin-1 did not lead to DLLC differentiation in this cohort of patients. It did,however significantly increase apoptosis in the seven-day cytokine culture (Fig. 3.5), further limiting its usefulness.

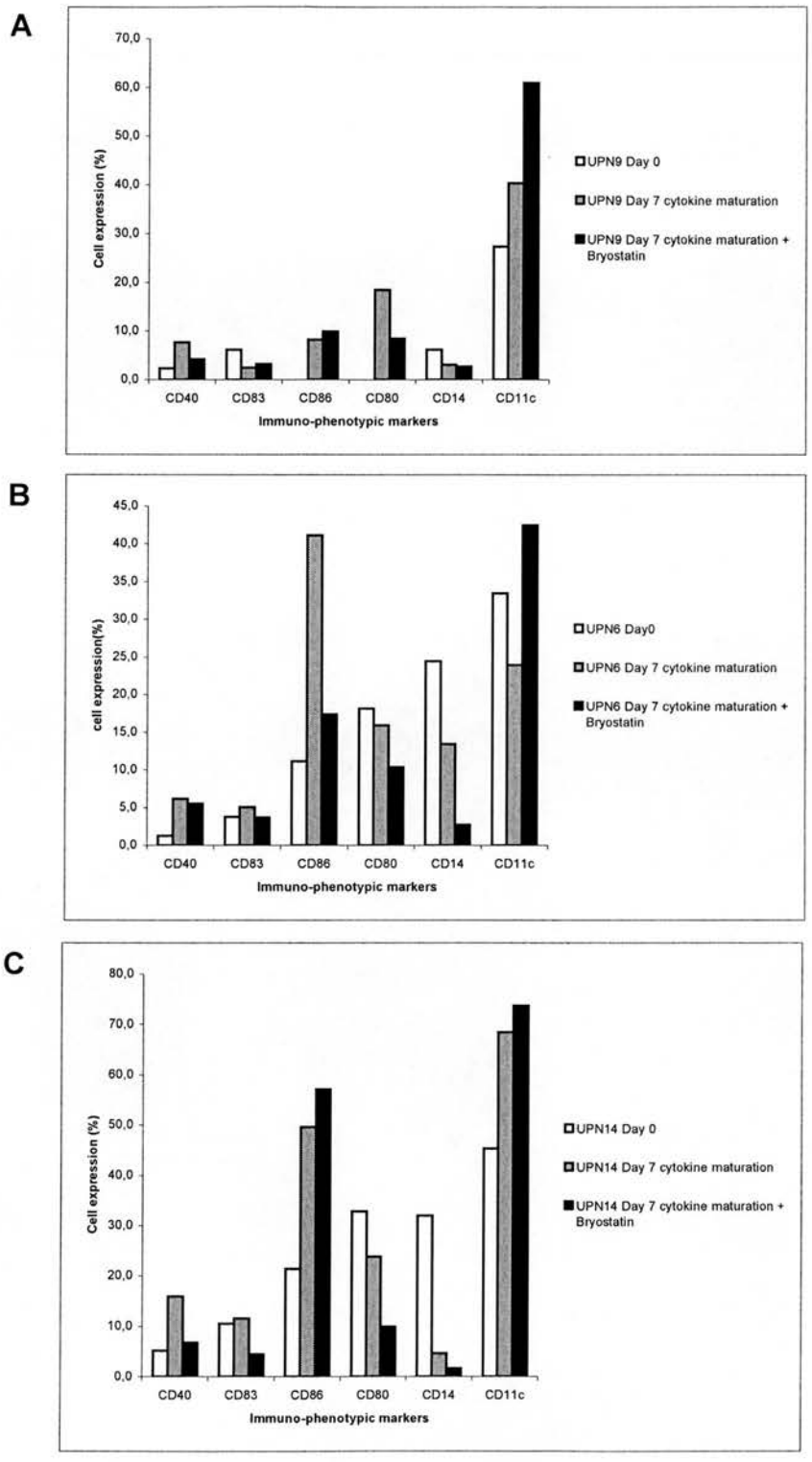


Fig.3.4: Results of Bryostatin addition to cytokine maturation cocktail. The addition of Bryostatin to the cytokine maturation cocktail enhanced the loss of CD14 and promoted gain of CD11c in AML blasts resistant **(A)** and permissive **(B, C)** to DLLC differentiation. Bryostatin did not, however, consistently up-regulate co-stimulatory markers and did not overcome maturation resistance in this cohort of patients.

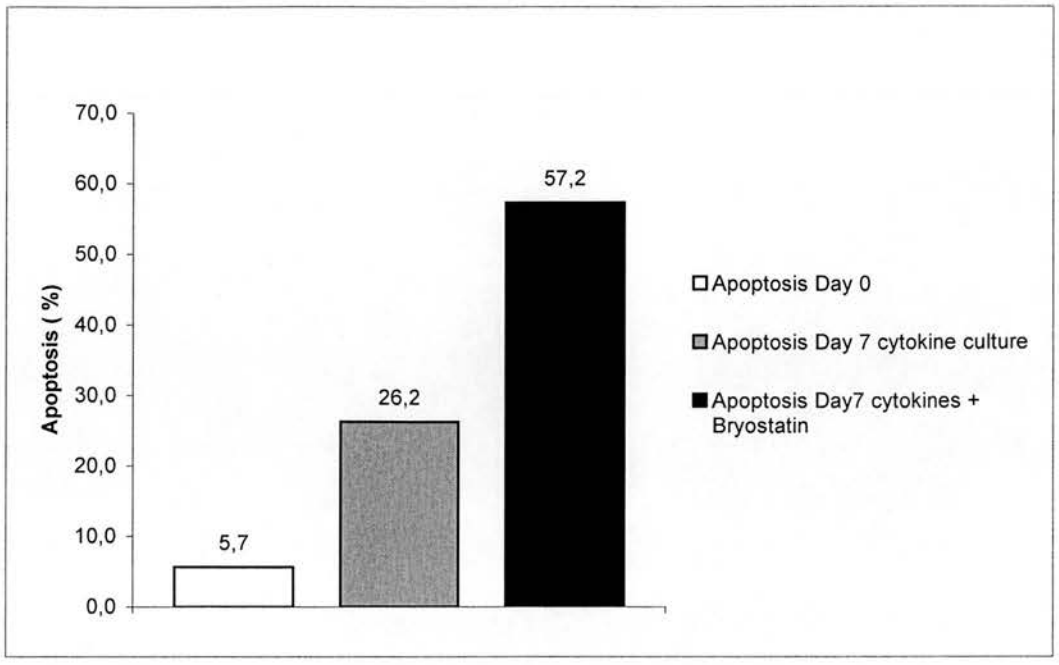


Fig.3.5: Addition of Bryostatin-1 to cytokine culture increased levels of apoptosis.
Mean of apoptosis is determined by flow-cytometric analysis of PI uptake and is shown for the AML blasts of study subjects prior to and after 7 days of cytokine culture with or without the addition of Bryostatin-1.

3.1.3.2 Calcium Ionophore

Calcium Ionophore was used as described in previous studies(Waclavicek *et al*, 2001) as sole agent added at Day 1 of 7 Day culture in 9 AML samples (UPN6, UPN8-UPN15). No morphological change indicative of DLLC conversion and no increase in expression of co-stimulatory markers was seen after the 7 day culture period. In subsequent experiments Calcium Ionophore was added to the cytokine cocktail of GM-CSF and IL-4 at Day 1 of culture (UPN16, UPN18-UPN22). No other maturational agents were added during the culture period. Again, no evidence of DLLC conversion was seen in cytokine resistant cases. 3 cases with AML blasts permissive to cytokine maturation were compared to results achieved with GM-

CSF/IL4/ Calcium-Ionophore culture (UPN 16, UPN18, UPN20). Significant differences in expression of co-stimulatory molecules were only seen in UPN 20, where Calcium-Ionophore maturation resulted in higher expression of CD80 and CD83 compared to the standard maturational agents (Fig 3.6).

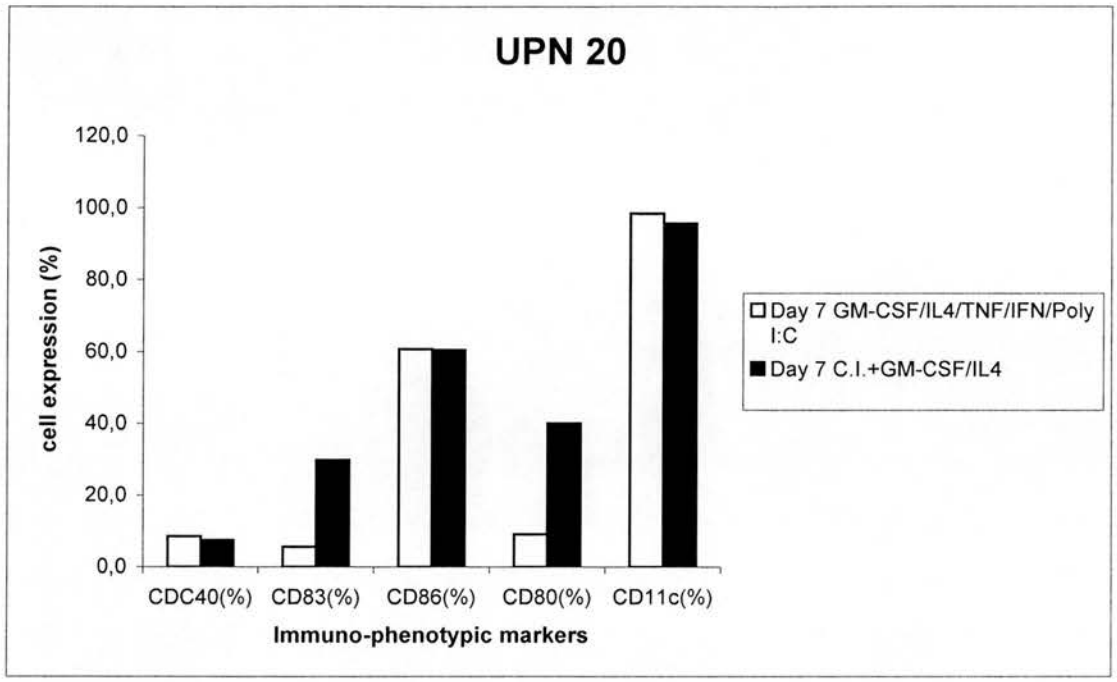


Fig 3.6: Calcium-Ionophore addition to GM-CSF/ IL-4 increased expression of CD80 and CD83 compared to maturation with TNF-α, Interferon-γ and Poly I:C (UPN 20)

3.1.4 Immune responses to DLLC vaccination

3.1.4.1 Delayed Type Hypersensitivity (DTH)

This was assessed by an intra-dermal test dose of 1x 10^6 irradiated autologous DLLC at the beginning and the end of the vaccination course. No significant erythema or swelling was observed in any of the five vaccinated subjects. The injection site was

scored by the patients themselves and recorded 48 hours following test dose injection in their patient diary. Likewise, none of the trial subjects experienced significant erythema, swelling or tenderness around the subcutaneous sites of vaccination.

3.1.4.2 Chromium^{51} - release cytotoxicity assay results (UPN 14)

A Chromium^{51} - release cytotoxicity assay was used to assess the development of anti-leukaemic cytotoxicity in response to DLLC in the first patient to be vaccinated (UPN12), but was subsequently replaced by enzyme- linked immunospot (ELISPOT) assay of Interferon-γ secreting CTL. Problems associated with using a Chromium^{51}-based assay centre around the lack of sensitivity, partly due to unreliable isotope uptake by AML blasts in some cases and high spontaneous release in others, leading to subsequent poor labelling of target cells and poor signal-to-background ratio, respectively. UPN12 experienced a relapse of his leukaemia during the vaccination program and we could not show a measurable increase in anti-leukaemic cytotoxicity responses using CR^{51}-CTL assaying.

3.1.4.3 Measurement of Interferon-γ secreting CTL by ELISPOT assay

For the estimation of anti-tumour specific immune responses following DLLC vaccination in patients UPN14, UPN16, UPN18 and UPN20, ELISPOT technology was utilised, as it provided the required assay sensitivity and reproducibility. Interferon-γ release of mononuclear effector cells was measured in response to native, autologous blasts and DLLC prior to the vaccination course and at set timepoints during and following the vaccination course. For UPN 14 an additional correlation between the release of Interferon-γ and Granzyme B, a cytotoxic T-

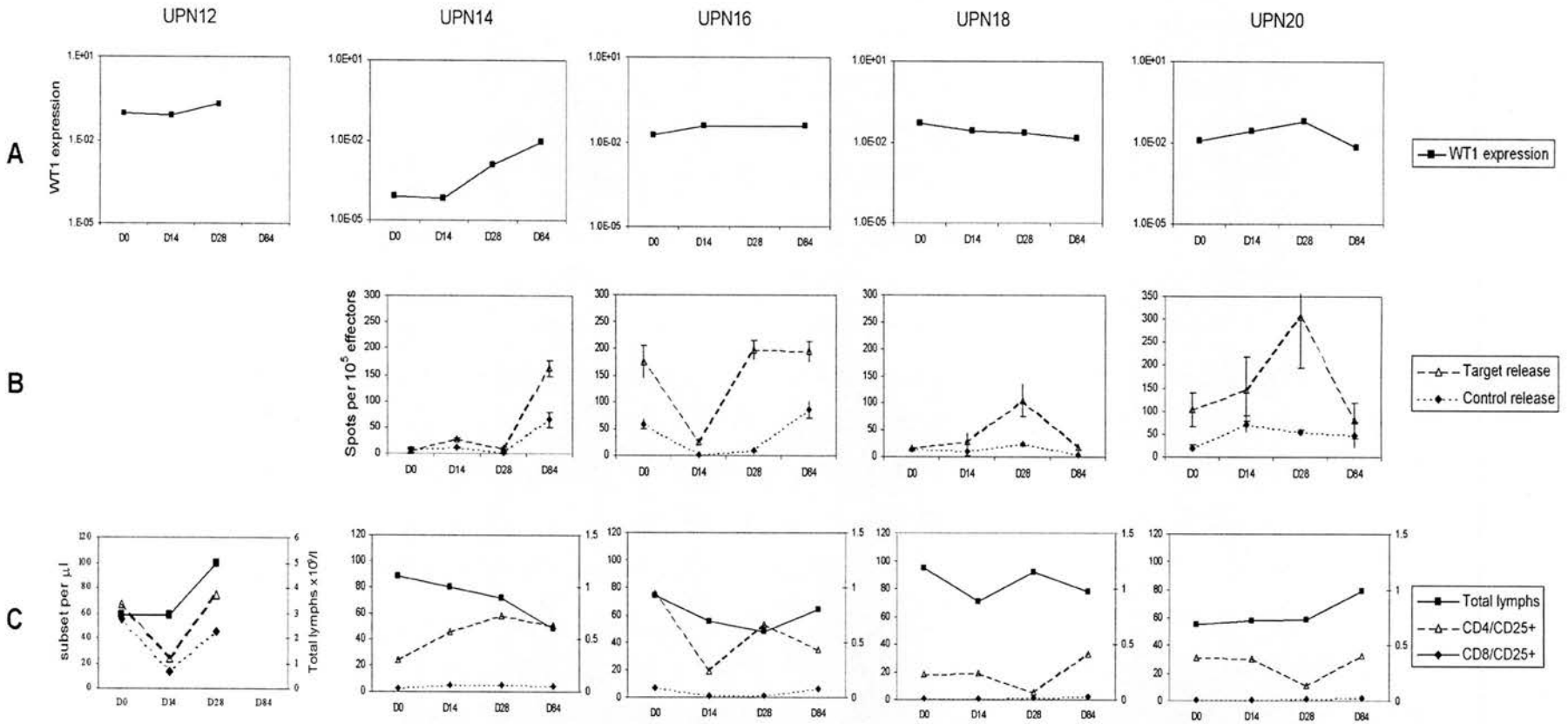


Figure 3.7: Results of monitoring for minimal residual disease and immunological responses in the five patients who underwent the DLLC vaccination program

A. Levels of minimal residual disease as assessed by RT-PCR for WT1 gene expression (reported as expression relative to K562 in logarithmic scale) **B.** Frequencies of gamma interferon secreting CTL measured by ELISPOT analysis. (UPN12 did not have this analysis performed) **C.**Frequencies of T-reg numbers. Measurement of T-reg numbers was assessed by flow cytometric analysis with T-reg gated on the basis of lymphocytes showing CD4/CD25 dual positivity. Total lymphocyte and CD8/CD25 dual positive cell numbers are provided for comparison.

cellular effector molcule was possible for two time-points during DLLC vaccination in parallel ELISPOT secretion assays.

Measurable increases in the frequency of anti-leukaemic CTL were demonstrable in all four patients at some timepoints during or following the vaccination course, when compared to pre-vaccination levels. The secretion of Interferon-γ correlated well with that of Granzyme B in response to autologous AML and DLLC at two time-points, Day 28 and Day 49 during vaccination of UPN 14, further validating the use of Interferon-γ secretion in the assessment of anti-tumour immune responses (Fig 3.8).

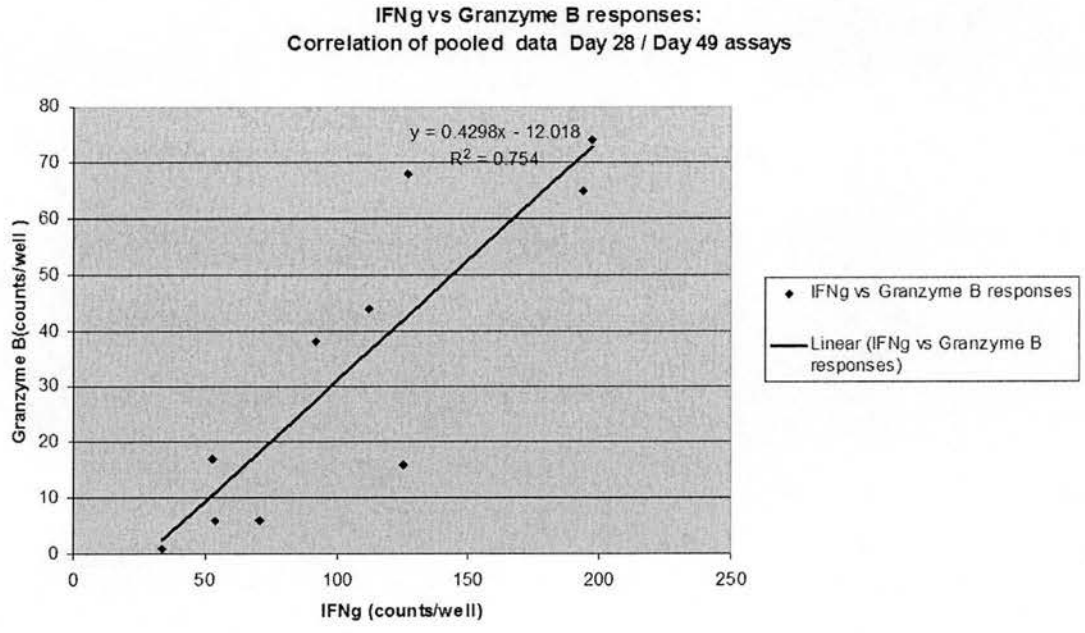


Fig 3.8: Correlation of Interferon- γ and Granzyme B-release

Parallel ELISPOT assays of Interferon- γ and Granzyme B release by T-cells in response to autologous AML and DLLC were conducted. Results of corresponding assay wells show good correlation.

3.1.4.4 Analysis of WT1/HLA-A2 tetramer positive CTL

It has been demonstrated that WT1 can serve as a target for CTL and that these WT1 specific CTL have particular activity against leukaemic blasts(Gao *et al*, 2000) The presence of WT1 specific CTL was measured using fluorochrome labelled HLA tetramer bound to a HLA-A*201 restricted 9mer WT1 peptide. Only one of the five vaccination patients was HLA-A*201 positive and therefore suitable for study. In this patient (UPN18) at the start of vaccination there was a very low frequency of WT1 specific CTL. Following vaccination there was a progressive rise in WT1 specific CTL in the PB with a maximum value of 0.35% of total T cell numbers on day 84 (Fig. 3.9).

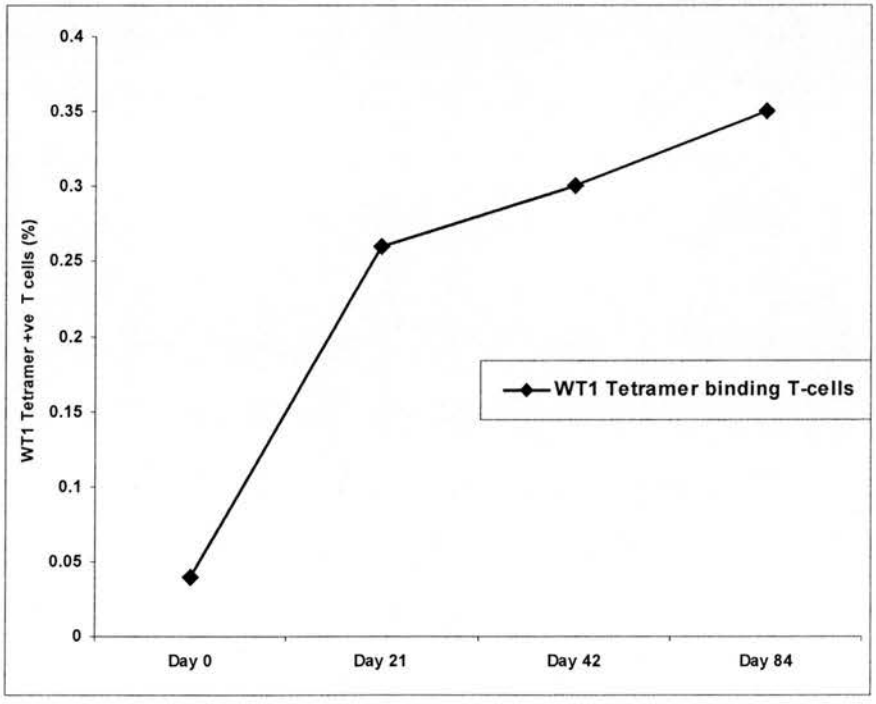


Fig.3.9: T-cells specifically recognising the WT1 leukaemic antigen increase following DLLC vaccination.
Peripheral blood CD8+ve T-cells of patient UPN 18 with WT1-expressing AML; Binding of WT1 HLA-Tetramer is expressed as percentage of all lymphocytes in a flow-cytometric assay.

3.1.4.4 Regulatory T-cell numbers

Frequencies of regulatory T-cells (Treg) were monitored though the vaccination programme. There was no consistent pattern either in the Treg frequency pre-vaccination or in the change in this frequency following vaccination between the five patients (Figure 3.7.C). In particular initial Treg frequencies did not appear to be appreciably different between the two patients who had received fludarabine based induction regimes (UPN12 and UPN14) compared to those that has received an anthracycline based regime (UPN16, UPN18 and UPN20). On selected time-points there did appear to be a relationship between the level of anti-leukemia CTL and Treg frequency in two of the patients. For cases UPN18 and UPN20 the peak in anti-leukemic CTL occurring at day 28 was associated with a dip in the Treg frequency. The subsequent fall in CTL numbers in these two patients at day 84 was accompanied by a rise in Treg numbers.

3.2 Discussion of the clinical study

This study has demonstrated that vaccination of patients with AML in CR with autologous DLLC is feasible. It was possible to generate clinically relevant numbers of DLLC in a GMP grade facility. In those cases, where the blast number in the peripheral blood exceeded 10^9/l, sufficient malignant cells for the planned vaccination course could mostly be gained from a peripheral blood aliquot of up to 100 ml. Bone marrow harvests for cases with a lower peripheral blood blast count, however, failed to yield sufficient cell numbers in five out of six cases and should be replaced by a leucapheresis procedure in cytopenic patients and those with blast counts below 10^9/l.

It remains impossible to predict, which patient's blasts might be permissive or resistant to cytokine-induced DLLC differentiation. Morphological categorisation according to FAB subtypes of AML did not co-segregate with successful DLLC differentiation. Marked cytokine-induced apoptosis in culture or failure to differentiate was seen in all morphological AML variants. The observed differentiation resistance in those patients with complex karyotypic abnormalities will be an obstacle to the application of this vaccine-generation stategy in many elderly AML patients and those with an antecedent myelodysplastic presentation. Further individualisation of cytokine concentrations and incubation periods may improve the results of DLLC differentiation in selected cases, an approach taken by previous investigators (Harrison *et al*, 2001). These procedural variations would, however, further complicate the standardisation and comparability of results achieved by this immuno-therapeutic intervention. The study did not show a clear benefit of adding Bryostatin-1 to the cytokine cocktail, nor could DLLC maturation be achieved by the substitution of TNF-α, IFN-γ and Poly I:C by Calcium-Ionophore. Vaccination was generally safe with only one of five patients developing side effects potentially attributable to the vaccination program. Interestingly in this patient the side effect was eczema that might have been as a consequence of the induction of autoimmunity through presentation of self-antigens by the DLLC. This same patient also developed an increasing ANF titre supporting this hypothesis. In terms of specific anti-leukaemic CTL activity four patients developed increased numbers of leukaemia specific gamma-interferon secreting CTL following vaccination. In the one HLA-A*201 positive patient this was also accompanied by an increase in WT1 specific CTL numbers.

However, despite the development of these anti-leukaemia immune responses, evidence of clinical benefit has not been established. Indeed, in one patient, UPN 14, leukaemic relapse developed despite an increase in anti-leukaemia CTL activity. There could be several reasons, why this increase in anti-leukaemic activity failed to prevent relapse in this patient. It is known that regulatory T cells inhibit anti-cancer immune responses (Casares *et al*, 2003b;Wolf *et al*, 2003b;Morse *et al*, 2002;Mesel-Lemoine *et al*, 2005;Brouwer *et al*, 2000) The rise in Treg numbers seen in this patient may have abrogated the anti-leukemic response to a degree that relapse could take place. Evidence suggest that suppression of Treg numbers can restore effective anticancer immunity (Morse *et al*, 2002;Ghiringhelli *et al*, 2004). It has been suggested that fludarabine may be a useful drug in terms of creating a favourable background for anti-tumor vaccination due to its activity against Treg. (Beyer *et al*, 2005). However, in our study we did not see any appreciable difference in pre-vaccination Treg frequency, or in the development of anti-leukemic activity, between those patients receiving prior fludarabine therapy compared with those receiving an anthracycline based regime. It may be therefore that type of the AML induction regime used prior to vaccination is not of fundamental importance in determining the probability of developing an anti-leukemic immune response following vaccination. Interestingly, the cytotoxic agent Cyclophosphamide has been shown to suppress Treg activity, allowing immunotherapeutic approaches for established malignancies (Ghiringhelli *et al*, 2004). An early vaccination time-point following high dose cytotoxic chemotherapy may be also of value in avoiding Treg mediated suppression of anti-tumour directed cytotoxicity. Although Treg may have a role in suppression of anti-leukaemia immunity, a more likely explanation for a lack of clinical benefit is

that DLLC vaccination is not sufficiently effective to generate a clinically relevant level of anti-leukemia immune response. If this is the case then it will be necessary to augment the vaccination method in order to generate clinically relevant immunity. Possible ways of achieving this are by improving the potency of the dendritic cell based vaccine through use of agents that promote dendritic cell maturation and/or polarisation or by supporting any anti-leukemia T cell responses by systemic administration of cytokines such as IL-12 (Napolitani *et al*, 2005;Portielje *et al*, 2005). It might then be possible to convert subclinical immune responses into ones that are clinical effective. However, as DC populations originating from leukaemic blasts *in vivo* are part of the malignancy and may potentially also produce "tolerogenic" signals, in particular if a fully mature DLLC phenotype can't be achieved, the question arises if AML-DC *in vivo* might be implicated in the disease process and, if so, whether their use in therapy would be advisable? The fact that the malignant cells are DC precursors and therefore components of the immune interaction one attempts to study complicates the characterisation of immune responses to AML. Could DCs derived from malignant precursors, one of the unique features of AML, be part of the pathology of AML? If AML-DC respond to stimuli in their environment with a capacity similar to that of normal marrow precursor derived DC, then the pathology in AML may not be at the level of the DC itself, but with the responder T- and NK cells. In this case, the use of AML-DC in therapy of AML may be of benefit. In cases, where AML-DCs differ from normal DCs in their responses to stimuli, AML pathology may in part be attributed to the AML-DC itself and their use in therapy of the disease may be less effective. Studies of anti-leukaemic T-cell responses in leukaemic patients in remission (Panoskaltsis *et al*,

2002;Molldrem *et al*, 2000)and those with fulminant disease (Molldrem *et al*, 2003) (Elisseeva *et al*, 2002)have been undertaken. Cytotoxic T-lymphocytes (CTL) directed against tumour-associated antigens, such as WT1 and Proteinase 3 have been demonstrated at some point of the disease process. Such observations have led some investigators to the suggestion that immune responses to tumour might be actively inhibited in advancing disease (Staveley-O'Carroll *et al*, 1998;Takasugi *et al*, 1977), while others have postulated that in patients with malignancy immune responses to tumour associated antigens (TAA) are only initiated in disseminated, overwhelming disease and are only detectable at low levels in a situation of low tumour burden (Mintz *et al*, 2003;Valmori *et al*, 2000). In either case, immune control of the tumour ultimately would be ineffective, but the exact immunosuppressive mechanisms remain unclear (Panoskaltsis, 2005). Semi-mature DC may trigger regulatory T-cells (T_{Reg}) like IL-10 producing CD4+ CD25+ cells, capable of silencing the immune response, whereas specific manipulation of malignant cells into APCs with a mature phenotype may trigger the immune system to suppress or eradicate leukaemia.

Any future attempts to increase the effectiveness of vaccination will depend upon the ability to measure to a high degree of sensitivity the development of anti-leukaemic T cell immune responses and the impact of vaccination on the levels of leukaemia burden. This study has demonstrated that by using techniques such as ELISPOT and HLA tetramer analysis in combination with RT-PCR for WT1 to measure MRD it is possible to sensitively monitor the impact of DLLC vaccination. This means that as dendritic cell based vaccination approaches are refined their impact on improving immunological and clinical effectiveness of the vaccine can be carefully followed.

Whilst this study has demonstrated that vaccination with DLLC is feasible, it is not an approach that would be broadly applicable. This is because the number of patients who were eligible to proceed to vaccination in comparison to those recruited into the study was only in the order of 25%. This was not unanticipated given the stringent requirements that had to be met in order to proceed to vaccination. The reason for these requirements was that it is believed that for cellular vaccination strategies to be effective they are best performed in a state of MRD. Conversely it is unlikely that any vaccination approach would be successful where the patient has active and uncontrolled leukemia (Haining *et al*, 2005). Given that CR is possible in >50% of patients suitable for intensive chemotherapy, this in itself would not necessarily prove a barrier to more widespread the adoption of this type of treatment approach. Unfortunately the additional requirement that the patient's leukemia cells need to be permissive to DLLC differentiation does restrict this approach to a minority of patients. It has been shown previously that in only around 25-40% of AML cases are leukemia cells are permissive to DLLC differentiation (Brouwer *et al*, 2000;Cignetti *et al*;Robinson *et al*, 1998). Unless methods for improving the proportion of AML cases that are permissive to DLLC differentiation can be found, different approaches to vaccination will need to be tried. Other methods, such as loading monocyte-derived dendritic cell with autologous leukaemia antigens or utilising fusion hybrids of tumour cells with autologous monocyte-derived DC may yield a potentially more broadly applicable cell based vaccine and are explored in the further laboratory studies undertaken for this thesis.

Chapter 4: Results of laboratory studies- Fusion Hybrids of Dendritic Cells and autologous Myeloid Blasts as possible cellular vaccine for AML

4.1 Overview of experimental strategy

Dendritic–like leukaemic cells (DLLC) generated from primary AML blasts by cytokine induced differentiation offer potential as autologous cellular vaccines as they acquire co-stimulatory and adhesion molecules and thereby stimulate cytotoxic T-lymphocyte (CTL) responses to leukaemic tumour antigens (Choudhury *et al*, 1997;Choudhury *et al*, 1999;Charbonnier *et al*, 1999;Choudhury *et al*, 1999;Charbonnier *et al*, 1999). In a significant number of cases, however, AML blasts prove resistant to cytokine driven differentiation into DLLC (Brouwer *et al*, 2000;Choudhury *et al*, 1999;Harrison *et al*, 2001;Robinson *et al*, 1998), therefore limiting this approach, in the experience of our clinical trial using DLLC as cellular vaccine constructs, to about 30% of cases.

To overcome these limitations, an alternative approach to generate potent anti-leukaemic cytotoxic responses was investigated by fusing autologous AML blasts to mature dendritic cells derived from peripheral blood monocytes of 6 AML patients in complete morphological remission (CR), thus generating heterokaryons that express leukaemic antigens derived from the blast fusion partner as well as co-stimulatory and adhesion molecules of mature dendritic cells. The aim of this strategy was to explore the potential of these AML-Dendritic Cell Fusion Hybrids to induce *in vitro*

T-cellular responses against the patient's unmodified leukaemic cells. Where possible, the potency of these constructs was compared to that of mature DCs co-cultured with irradiated autologous leukaemic blasts, previously shown to generate leukaemia specific T-cellular cytotoxicity (Galea-Lauri *et al*, 2002;Spisek *et al*, 2002).

Samples from 6 patients (median age: 54 years, range: 24-70 years) were used in this study. Table 4.1 shows relevant clinical and laboratory characteristics of these patients, in whom DLLC maturation was either not achievable (n=5), or DLLC proved ineffective in stimulating anti-leukaemic CTL (UPN3).

Table 4.1: Patient characteristics

Patient	Sex/Age	Sample origin	WHO/FAB classification	Karyotype	DLLC maturation
UPN1	M / 66	PB[a]	M4	46,XY	No
UPN2	M / 48	PB	AML with trilineage dysplasia / M5a	46,XY	No
UPN3	F / 61	PB	M5a	46,XX	Yes
UPN4	F / 24	PB	M5a	46,XX	No
UPN5	M / 39	PB	M4	46,XX	No
UPN6	F / 70	PB	M5	46,XY	No

[a] PB, peripheral blood

a) Leukaemic Blasts were taken with informed consent at the time of diagnosis. A highly purified population of leukaemic blasts (> 95%) was generated through Ficoll

density-gradient centrifugation and cells were immediately cryopreserved in 90% fetal calf serum/ 10% DMSO.

b) Remission samples were obtained after CR was confirmed (defined as less than 5% of blasts in the bone marrow). Ficoll density-gradient centrifugation was used to separate monocytes for generation of mature Dendritic Cells (mDC) and isolate responder T-cells for subsequent incubation with various autologous stimulators.

c) Generation of mature DCs from Peripheral Blood Monocytes of Patients in Complete Morphological Remission. Mature DCs were generated from the adherent fraction of PBMCs collected after the resolution of cytopenias following combination intravenous chemotherapy using a cytokine cocktail containing GMCSF at 100ng/ml and IL-4 at 15ng/ml for 5 days. DCs were then matured for further 2 days by the addition of TNF-α at 25ng/ml, Poly I:C at 12.5 mcg/l and Interferon-gamma at 30ng/ml. Mature dendritic cells were then used fresh for the generation of fusion hybrids with thawed autologous AML blasts and also co-incubated with irradiated AML blasts to generate an alternative vaccine construct.

d) Generation of Dendritic Like Leukaemic Cells (DLLC) from AML blasts. Maturation to DLLC was attempted in all cases to compare the immuno-stimulatory capacities of these constructs to that of AML-DC fusion hybrids and DCs loaded with AML tumour bodies in co-culture with autologous remission T-cells. Cytokine concentrations and culture conditions were identical to those used in the DLLC clinical vaccine trial.

e) Generation of AML-mDC fusion hybrids. Thawed AML blasts and fresh mDC were mixed at a ratio of 1:1, pelleted by centrifugation and fused by drop-wise addition of 50% Polyethylene Glycol (PEG)/10% DMSO. They were then used as

stimulators in autologous CTL assays and allogeneic mixed lymphocyte leukaemia reactions (MLLR) in comparison to other vaccine constructs and naïve AML blasts.

f) Stimulation of leukaemia-specific lymphocytes by co-culture with autologous AML-mDC fusion hybrids, mDc cocultured with irradiated AML and DLLC. The non-adherent fraction of fresh or thawed remission blood samples provided the source of autologous responder cells. Unmodified AML blasts, AML–mDC Fusion Hybrids and mDC mixed 1:1 with irradiated (26Gy) AML blasts were used as autologous stimulators. Dendritic-like differentiation was achieved from the leukaemic blasts of one of six patients. DLLC were generated as stimulators to autologous responder T-cells in this case. PBMC cultured in the absence of stimulators or primed by un-pulsed mDCs and AML-AML fusions served as negative controls, as these are not expected to stimulate leukaemia directed cytotoxicity. Co-culture of 10^7 responder cells with 10^6 irradiated stimulators was prepared in 12-well plates at a cell density of 10^6/ml effectors. CM was supplemented with IL-2 5ng/ml and IL-7 5ng/ml. Responder cells were re-stimulated at day 7 and day 14 for ^{51}Cr-release cytotoxicity assays, using the same stimulators that had been added at the beginning of co-culture. CM and cytokines were half exchanged weekly. Effectors were harvested on day 7 (ELISPOT assay) or on day 21 for subsequent ^{51}Cr-release cytotoxicity assays against native, autologous AML blasts.

g) Allogeneic Mixed Leukaemia-Lymphocyte Reactions (MLLR). Responder cells for allogeneic MLLR were obtained from the non-adherent lymphocytes of healthy, unrelated donors, whose PBMC had been separated by gradient density centrifugation followed by 2hrs plastic adherence. Adherent donor monocytes were

matured in a 7-day culture into mDC as described above and subsequently fused to patient AML blasts to generate AML-DC Fusion Hybrid stimulators. Un-fused AML blasts and mDC as well as AML co-cultured with mDCs served as stimulator controls. AML-AML or mDC-mDC fusions and titrating concentrations of PEG were also assessed regarding their ability to stimulate the proliferation of lymphocytes allogeneic to the AML blasts used.

h) ^{51}Cr-release cytotoxicity assay and Interferon-γ ELISPOT Immuno- assay.

Following re-stimulation co-culture with a variety of autologous stimulators the resulting leukaemia directed *in vitro* cytotoxicity was measured in a 4 hr ^{51}Cr-release cytotoxicity assay in 2 patients (UPN1, UPN2), where uptake of the isotope by their native AML blasts was demonstrable. Interferon-γ (IFN-γ) ELISPOT assays were used in 4 patients (UPN3-UPN6) to assess *in vitro* T-cell responses to autologous AML blasts.

4.2 Flow-Cytometry and Fluorescence Microscopy confirm AML-mDC fusion

Before testing the immune-stimulatory function of AML-mDC Fusion Hybrids a series of experiments was conducted to assess the efficiency of fusion using PEG / DMSO.

Flow-cytometric analysis following membrane staining of the fusion partners with different fluorescent dyes (CMFDA for DC's and PKH 26 for AML blasts respectively) allowed identification of fusion hybrids as double stained cells. Fusion efficiency was between 20% and 30% (mean 26%). Fig.4.1 shows a representative result.

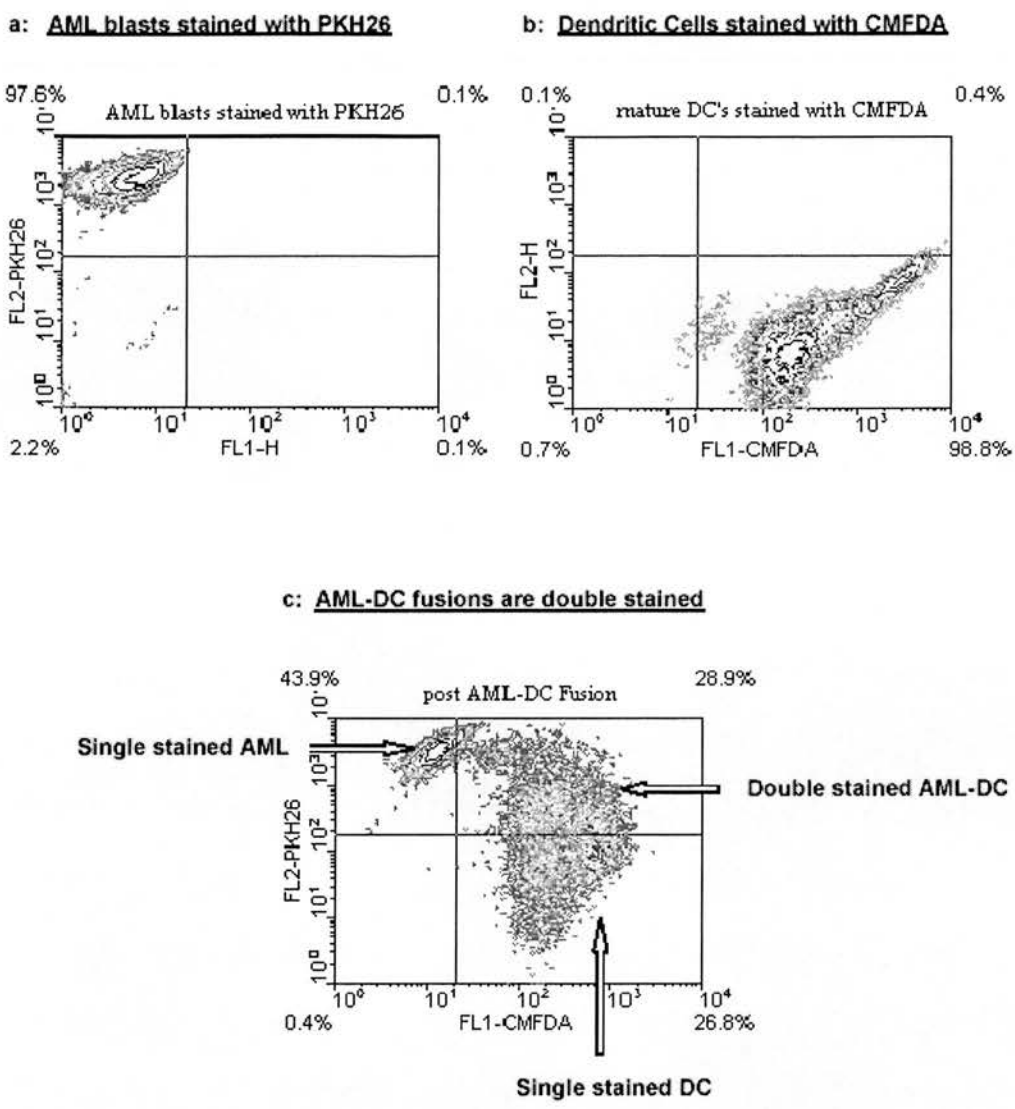


Fig. 4.1: Flow-cytometric analysis of fusion efficiency

Fluorescence microscopy provides confirmation of membrane derived from both fusion partners on the membrane stained heterokaryons generated. Motorised micro-focussing sections through fusion hybrids on cytospin preparations were visualised. Fig.4.2 shows sections through a fusion hybrid. As the common membrane of the Fusion hybrid comes into focus, red (AML) and green (mDC) stained membrane elements are seen.

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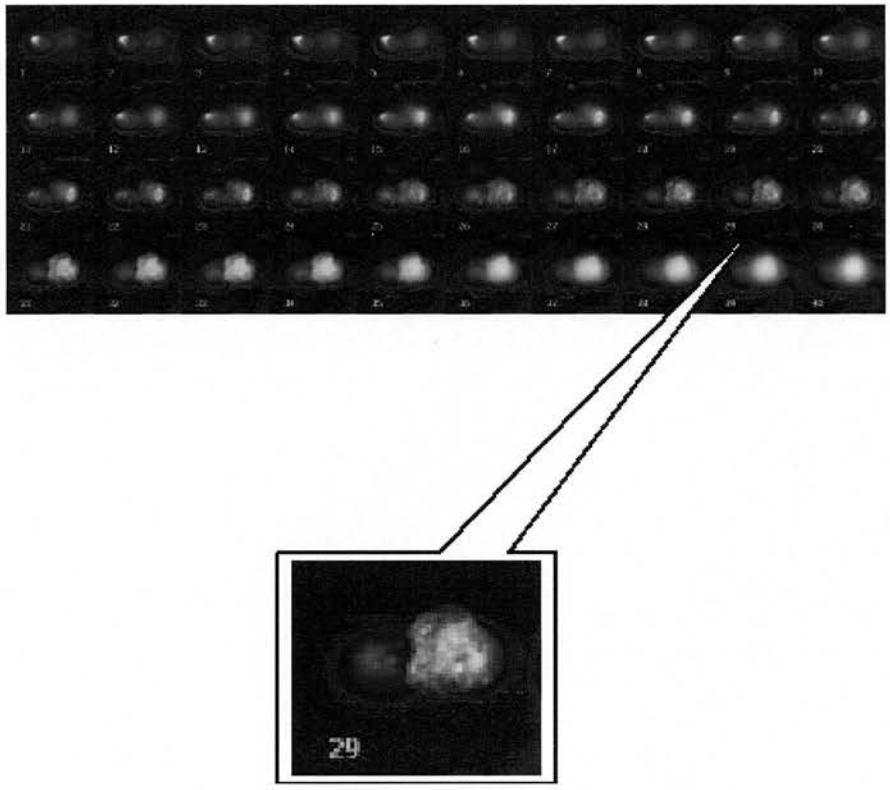

Fig.4.2: Fluorescent microscopic sections through an AML-DC fusion cell. A series of fluorescent microscopic images are cross-sectioning a membrane-stained AML-DC fusion cell. Motorised focussing through the heterokaryon reveals dual membrane staining from both fusion partners. The Dendritic Cell was stained with the green fluorescent membrane dye CMFDA. The AML blast was stained with the red membrane dye PKH26. Nuclei are stained with DAPI (blue).

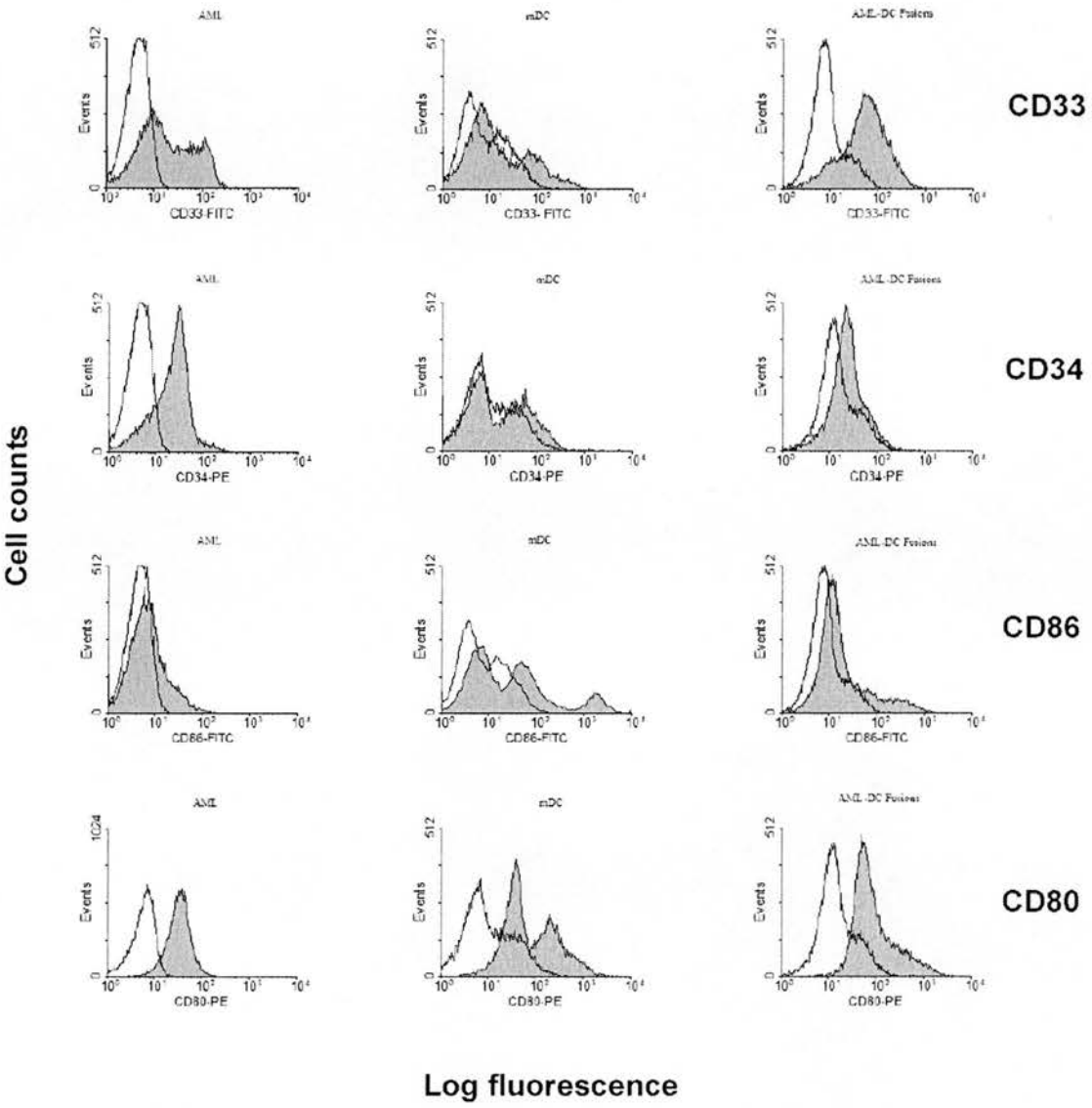

Fig.4.3: AML-DC fusion hybrids express a bi-phenotypic flow-cytometric profile.
AML blasts and DCs were stained before and after fusion using a panel of monoclonal antibodies against AML and DC associated antigens. The fusion hybrids express AML derived antigens (CD34) as well as DC associated antigens and co-stimulatory molecules (CD 86,CD 80). Results of a representative experiment are shown. Fusion hybrids are not gated.

Additionally, a bi-phenotypic flow-cytometric profile of fusion hybrids was demonstrated by analysis of the fusion partners before as well as fusion hybrids after fusion. Fig.4.3 shows representative results of one patient. Un-fused AML blasts and mDC are stained with mABs against the leukaemia associated antigens CD34 and CD33 and the DC associated antigens CD86 and CD80 prior to PEG fusion. Immediately after fusion the Hybrids express both AML and DC associated membrane antigens.

4.3 Fusion Hybrids induce AML-directed T-cell responses

^{51}Cr-release cytotoxicity assays were performed in two patients, where good ^{51}Cr uptake by autologous AML blast targets was shown (UPN1, UPN2).

In the remaining four patients (UPN 3-6) an interferon-γ ELISPOT assay was used to demonstrate T-cell responses against native, autologous AML blasts induced by priming co-culture with the various autologous stimulators (Table 2).

Table 4.2: Immuno-assays performed and results generated

Patient	Immuno-assay performed	Best Stimulator Construct	Results
UPN 1	51-Chromium release	AML-DC Fusion Hybrids	Fig.4.4
UPN 2	51-Chromium release	AML-DC Fusion Hybrids	Fig.4.4
UPN 3	Interferon-γ ELISPOT	AML-DC Fusion Hybrids	Fig.4.5a
UPN 4	Interferon-γ ELISPOT	AML pulsed mDC	Fig.4.6
UPN 5	Interferon-γ ELISPOT	AML pulsed mDC	Fig.4.6
UPN 6	Interferon-γ ELISPOT	No stimulators	Not shown

In three patients AML-mDC fusion hybrids proved the strongest inducers of anti-leukaemic T-cell responses, using ^{51}Cr-release cytotoxicity assays (UPN1, UPN2) and ELISPOT interferon-γ assay (UPN3), respectively. In UPN1 (Fig.4.4), PBMC primed with Fusion Hybrids, but not with either unfused AML blasts ($p<0.0001$) or mDC ($p<0.0001$), resulted in 47% target lysis at the Effector:Target ratio of 50:1 in a ^{51}Cr-release assay. Cytotoxicity induced by mDC pulsed with tumour-cell bodies was significantly lower at 24% at the same Effector: Target ratio ($p=0.0005$).

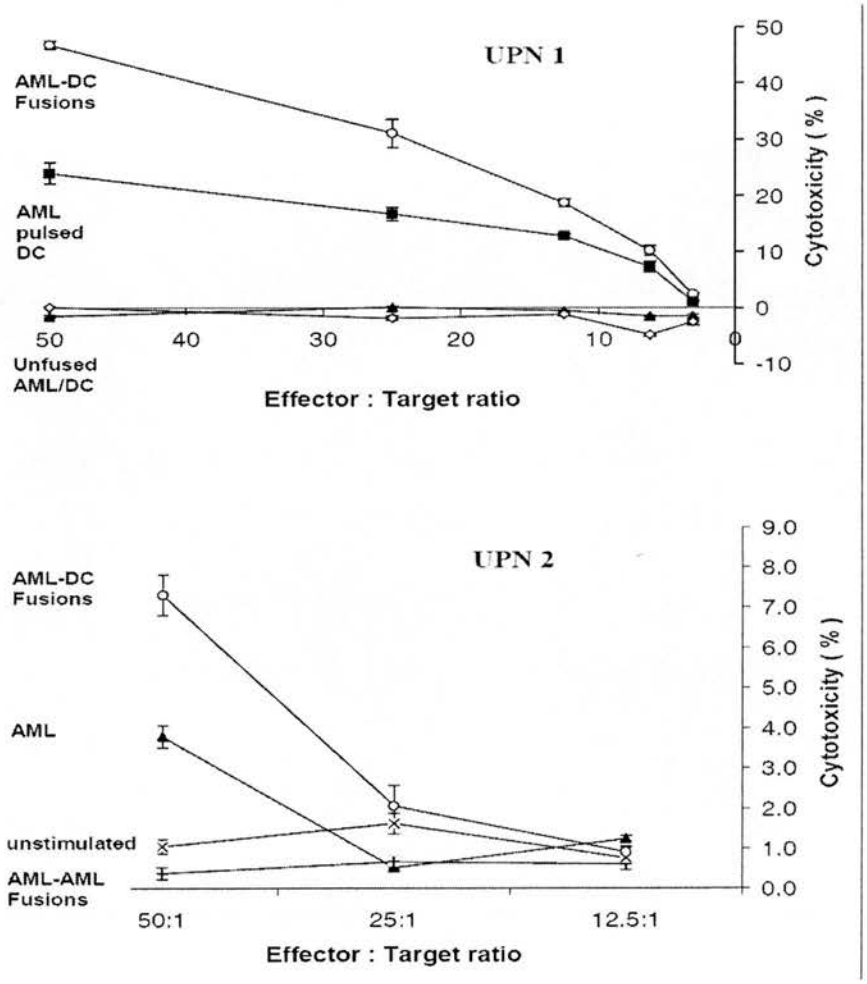

Fig.4.4: AML-mDC Fusion Hybrids generate anti-leukaemic cytotoxicity in co-culture with autologous remission PBMCs.
Cr^{51}-release assays of UPN 1 and UPN 2 are shown

In UPN2 Fusion Hybrids generated 7.3% AML directed cytotoxicity, compared to 3.8% induced by to un-fused AML (p =0.005), 1% cytotoxicity induced by unstimulated PBMC (p=0.001) and 0.4% by inappropriate AML-AML fusion hybrids (p=0.003) at the highest Effector/ Target ratio of 50:1 (Fig. 4.4).

In a third patient (UPN3) Interferon-γ responses to autologous AML blasts increased more than 3.5 fold in effectors primed by co-culture with Fusion Hybrids compared to those primed with AML blasts alone (p=0.0002), DLLC (p=0.0003) or un-primed effectors (p=0.0002) at the highest Effector:Stimulator ratio of 20:1 (Fig.4.5a).

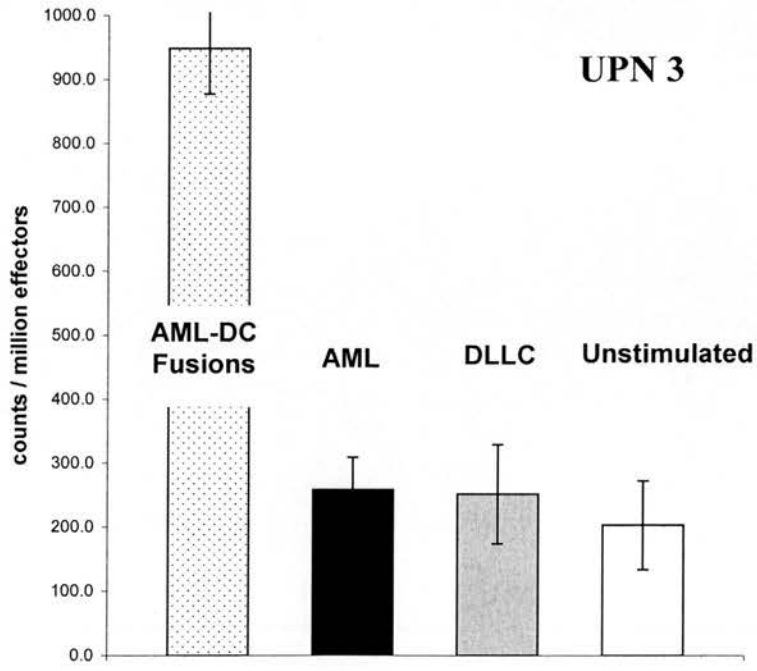

Fig.4.5a: Anti-leukaemic T-cell responses induced by AML-DC Fusion Hybrids (UPN3) exceed those of other autologous stimulators in an Interferon-γ ELISPOT assay.
Results of triplicate wells at the Effector /Stimulator ratio of 20:1 are shown. Co-culture with AML-DC fusions (⊡) induces a more than 3-fold increase in T-cell Intereron-γ release, compared to T-cells stimulated by AML (■), DLLC (▩) and unstimulated (□) T-cells.

Flow-cytometric characteristics of remission PBMC used in the ELISPOT Interferon- γ release assay for patient UPN3 are demonstrated in Fig.4.5b.

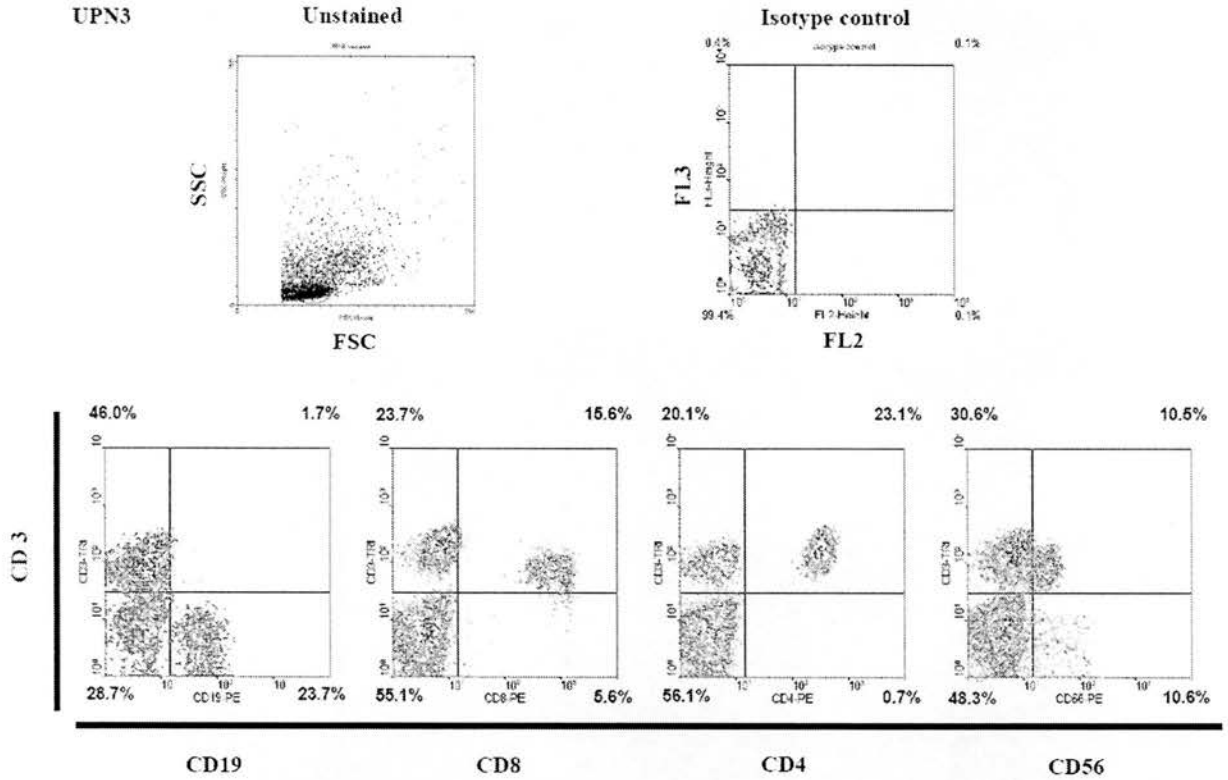

Fig.4.5b: Flow-cytometric characteristics of PBMC responders used in ELISPOT Interferon-γ release immuno-assay (UPN3). A lymphocyte gate is applied, cells are incubated with monoclonal antibodies against CD3 (Tricolor-conjugated) and additionally with either anti-CD19, CD8, CD4 or CD56 (PE-conjugated) to characterize the remission lymphocytes used.

4.4 mDC co-cultured with irradiated whole tumour cell bodies may provide an alternative cellular vaccine construct

In two patients (UPN4, UPN5) AML-pulsed mDC proved better stimulators of anti-leukaemic T-cell responses than AML-DC Fusion Hybrids in Interferon- γ ELISPOT assays (Fig.4.6). The stimulatory differences observed between these constructs were significant in both patients (p=0.003 in UPN4, p=0.009 in UPN5).

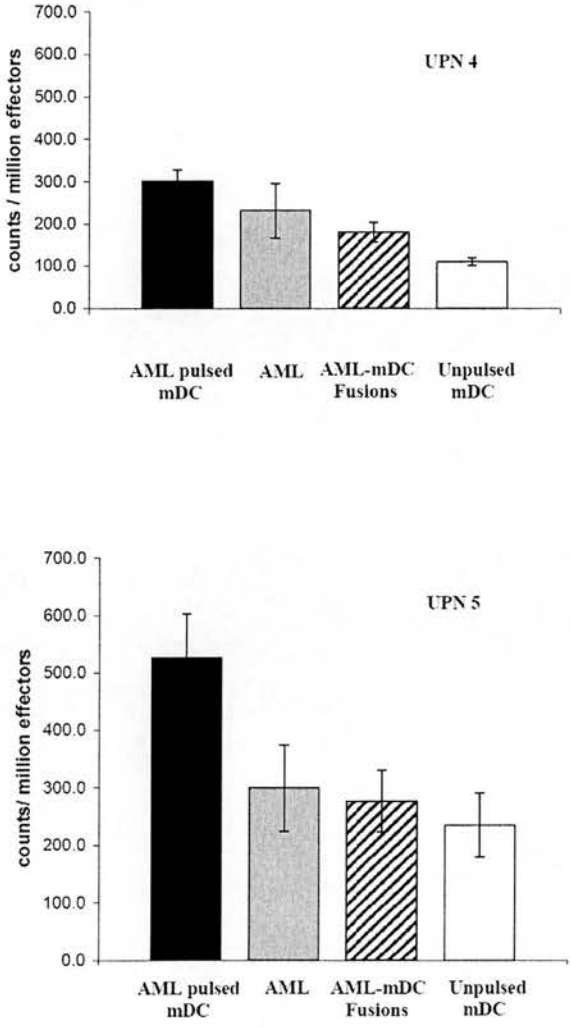

Fig.4.6: In two patients (UPN4, UPN5) AML pulsed mDC (■) prove better stimulators of anti-leukaemic T-cell responses than AML-DC fusion hybrids (▨), autologous AML blasts (▩) and unpulsed mDC (□). ELISPOT Interferon-γ release assay counts at the Effector:Target ratio of 20:1 are shown.

However, mDC pulsed with tumour cell bodies showed a significant advantage over immune responses induced by unmodified AML blasts only in UPN 5 ($p=0.02$), whereas the differences observed did not reach statistical significance in UPN 4 ($p=0.15$). In both patients Fusion Hybrids failed to induce T-cell responses exceeding those to unmodified AML blasts.

4.5 Suppression of lymphocyte proliferation by Fusion Hybrids is not specific to fusion partners

Assessing the immune-stimulatory properties of fusion hybrids it was observed that the proliferation of lymphocytes was suppressed by AML-DC fusion hybrids in [^{3}H]-thymidine uptake assays, where AML blasts allogeneic to the mDC and responder lymphocytes were used. A representative result of three experiments is shown in Fig.4.7a. This anti-proliferative property, however, was shown to be not specific to AML-DC fusion, but was also induced by allogeneic AML-AML as well as autologous mDC-mDC heterokaryons (Fig.4.7b,c). Polyethyleneglycol did not contribute to this effect (results not shown). Importantly, the induction of tumour specific T-cell cytotoxicity by AML-DC fusion hybrids was maintained regardless, further emphasizing their potency as cellular stimulators of anti-leukaemic responses.

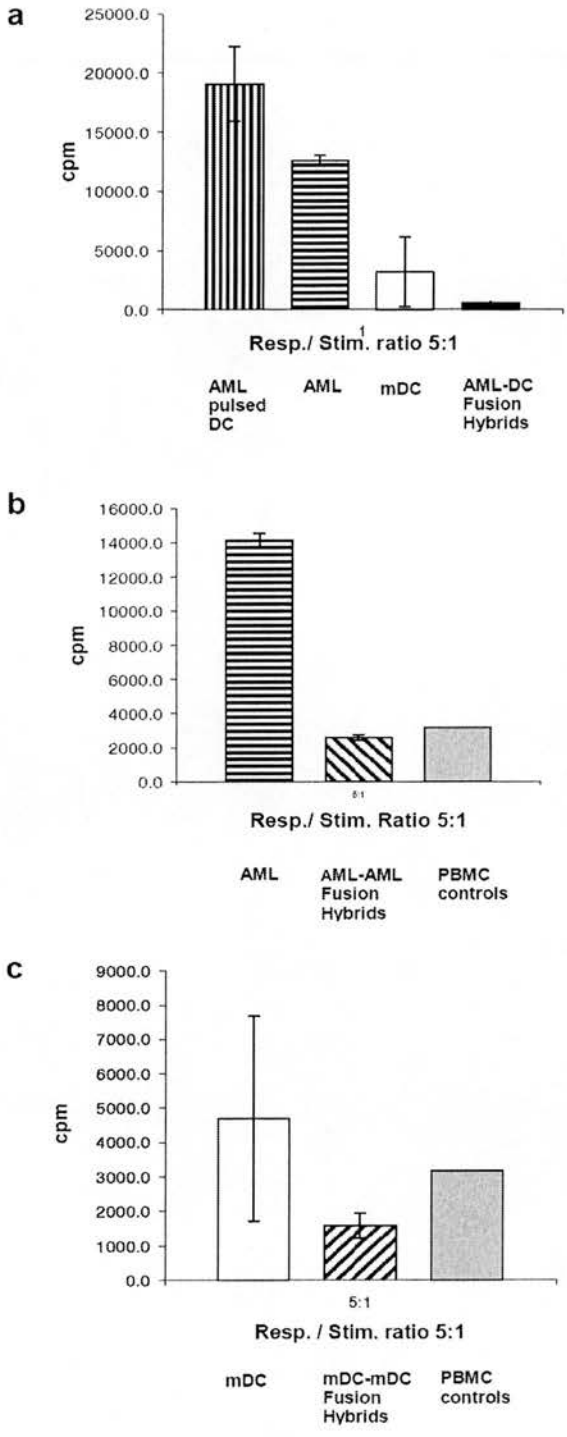

Fig.4.7: a) AML pulsed mDC (▥), but not AML-mDC Fusion Hybrids (■) stimulate proliferation of T-cells autologous to the mDC and allogeneic to the AML blasts. **b)** A similar antiproliferative effect is induced by allogeneic AML-AML fusion (▨) and **c)** autologous mDC-mDC hybrids (▨), suggesting a mechanism not specific to individual fusion partners. PBMC controls (□) are cultured without stimulators.

4.6 Discussion of laboratory studies

The heterogeneity of the Acute Myeloid Leukaemias poses a challenge for the generation of a dendritic cell based vaccine, as few leukaemia specific or tumour associated antigens can be expected to be expressed in all cases and remain stable over the course of the disease and chemotherapy treatment courses. Strategies focussing on the generation of CTL responses against single, specific tumour antigens are therefore fraught with the risk of possible tumour immune escape due to clonal evolution and tolerance to known and also unknown tumour antigens, resulting in relapse originating from minimal residual disease.

Whole tumour based vaccine strategies attempt to circumvent this problem by inducing polyvalent immune responses against a large number of antigens expressed by leukaemic blasts. DLLC derived from AML blasts by sequential cytokine incubation have been demonstrated to acquire co-stimulatory molecules that are mostly lacking in native AML blasts and are necessary to induce CTL responses. This approach to generation of a cellular vaccine is, however limited by the resistance to cytokine maturation in a significant number of cases (Roddie *et al*, 2002;Choudhury *et al*, 1997;Harrison *et al*, 2001).

In this pre-clinical study the approach of fusing whole tumour cells to autologous DC was investigated. Thus hybridomas were constructed which express both tumour derived antigens and DC-derived co-stimulatory molecules. It was shown that AML-mDC fusion hybrids and, to a lesser degree, mDC co-cultured with irradiated AML blasts are capable of inducing leukaemia directed *in vitro* cytotoxic responses in autologous remission PBMC. Both of these vaccine constructs can be readily

generated with little technical difficulty and are therefore attractive alternatives to DLLC.

Spisek *et al* have previously described the induction of leukaemia-specific response by cross-presentation of late-apoptotic leukaemic blasts by autologous dendritic cells of non-leukaemic origin (Spisek *et al*, 2002). Tumour-mDC fusion hybrids have been generated and successfully used as vaccines in the prevention and treatment in murine models of breast carcinoma (Gong *et al*, 1997;Gong *et al*, 1998;Gong *et al*, 2000;Chen *et al*, 2003) and induction of *in vitro* cytotoxicity has been demonstrated in B-CLL (Kokhaei *et al*, 2003) and Multiple Myeloma (Raje *et al*, 2004). Evidence for their effectiveness as vaccine constructs in AML, however, is limited. Galea-Lauri *et al* have previously reported one case of successful *in vitro* induction of CTL responses to AML blasts by autologous Fusion Hybrids and supported this finding by assessment of DC loading strategies with antigens derived from the U937 leukaemic cell line, including U937-DC Fusion Hybrids (Galea-Lauri *et al*, 2002).

In the pre-clinical assessment conducted for this thesis it was possible to elicit leukaemia directed T-cell responses in three out of six patients whose remission PBMC were co-cultured with autologous AML-mDC fusion hybrids. In two cases anti-leukaemic cytotoxicity generated by fusion hybrids was demonstrated by Cr^{51}-release assay (Fig4.4), in a third case T-cell responses to fusion hybrids exceeded those to other autologous stimulators in an Interferon-γ ELISPOT assay (Fig.4.5a).

In a further two out of three patients, where AML-mDC Fusion Hybrids proved ineffective in stimulating leukaemia directed cytotoxicity above the level induced by native AML blasts, however, mDC co-cultured with irradiated AML blasts did induce anti-leukaemic T-cell responses (Fig.4.6).

The determinants of successful CTL generation by either strategy require further investigation. MLLR experiments showed that PEG generated Fusion Hybrids exert anti-proliferative effects on co-cultured lymphocytes. This appeared to be independent of the fusion partners and was not induced by titrated addition of PEG to cultured PBMC alone. In spite of this anti-proliferative property, AML-mDC Fusion Hybrids remained capable of generating potent anti-leukaemic cytotoxic responses, further emphasizing the potential of these heterokaryons as cellular vaccines. Their efficacy might be further enhanced by exclusion of non-viable Hybrids by means of immuno-magnetic selection prior to co-culture, while positive selection of fused from un-fused cells based on their characteristic forward and side scatter profile (Galea-Lauri *et al*, 2002) could be achieved using a cell sorting facility. Purified hybrid cells from dendritic cell and tumour cell fusions have previously been shown to be more effective in inducing an immune response (IFN-γ) when compared to fusion mixture (Li *et al*, 2001). Furthermore residual un-hybridised AML blasts may anergise T-cells. All these parameters could affect the proliferation response when using DC Fusion Hybrids to stimulate autologous anti-leukaemic T-cells.

A recent study in a murine model confirmed that DCs fused or pulsed with AML cellular antigen provide comparable *in vivo* anti-tumour protective responses (Weigel *et al*, 2006) .

In conclusion this laboratory study provides pre-clinical data on developing a DC based vaccination strategy for Acute Myeloid Leukaemia. AML-mDC Fusion Hybrids can be generated with relative ease and hold promise as a novel potential cellular vaccine, providing an alternative to whole tumour cell-pulsed mDC or DLLC.

Chapter 5: Concluding Remarks

This study has investigated the feasibility and safety of generating a cellular vaccine for the treatment of AML derived from autologous myeloid blasts by means of *ex-vivo* cytokine-maturation to Dendritic-like Leukaemic Cells in a Phase I/II clinical trial. The study suggests a body of tests to monitor immune responses to the vaccination and to measure the impact on minimal residual disease. It explores the possible role of regulatory T-cells in the eventual dampening of anti-tumour cytotoxicity following DLLC vaccination.

Pre-clinical laboratory investigations aimed to develop the next generation of "whole cell" DC-based vaccine constructs:

- The use of the agents Bryostatin-1 and Calcium-Ionophore, in an attempt to overcome blast resistance to cytokine-mediated DLLC differentiation,
- Co-culture of monocyte-derived DC with autologous, irradiated blasts, and
- Generation of fusion hybrids from AML blasts and autologous monocyte-derived DC

In my opinion "whole cell" based DC vaccines, e.g. fusion hybrids, non-leukaemic DC pulsed with autologous tumour lysates or apoptotic tumour cell bodies provide the most promising therapeutic strategy in the immediate future. These constructs circumvent the obstacle of differentiation resistance encountered when using AML-DC and provide "polyvalent" immune stimulation, making tumour immune escape by antigen drift less likely. They do not require detailed analysis of the individual

leukaemic antigens presented and are not HLA restricted, thereby representing an easier, albeit individualised vaccine design.

The clinical study of DLLC vaccination confirmed feasibility and safety of these constructs, however, the approach is limited by the resistance to cytokine induced AML blast differentiation in the majority of patients. Previous investigators (Harrison *et al*, 2001) have attempted to individualise and optimise cytokine cocktails and sequencing of agents. Our study could not demonstrate that such modifications reliably convert differentiation resistant cases into permissive ones. Likewise, the use of Bryostatin-1 and Calcium Ionophore, with or without other cytokines, has not overcome differentiation resistance in our hands. Questions about the functional ability of AML blast derived DLLC remain. In some cases a not fully mature phenotype is generated, that might not induce clinically beneficial anti-tumour cytotoxicity. The heterogeneity of AML contributes to the complexity of the task, and the response to cytokine maturation remains essentially unpredictable in individual cases.

Immune monitoring of vaccine responses is essential in order to improve the next generation of AML vaccines. The combination of assessing minimal residual disease with quantitative PCR for WT1 or leukaemia-specific chromosomal translocations, sensitive and specific Interferon-γ and Granzyme-B immune assays and HLA-tetramer studies of tumour-specific CTL in the peripheral blood in patients with a suitable HLA type, proved fruitful in evaluating the vaccine impact in our clinical study, but has highlighted the short-lasting nature of immune responses seen in the vaccinated patients. The analysis of T_{reg}, gated on the basis of CD4 positivity and

bright expression of CD25, showed a dip in their frequency at the height of measurable anti-tumour responses in two patients (UPN18, UPN20) and a subsequent rise in T_{reg} numbers, as CTL responses weakened.

T_{reg} have been investigated in many recent studies because of their apparent immuno-modulatory role in infection, auto-immunity and inhibitory effect on anti-cancer immune responses (Casares *et al*, 2003a;Mesel-Lemoine *et al*, 2005;Morse *et al*, 2002;Wolf *et al*, 2003a). Conversely, evidence has emerged that effective anti-cancer immunity can be restored by suppression of T_{reg} numbers (Ghiringhelli *et al*, 2004). A better characterisation of T_{reg}, using the now available marker FOXP3 may help correlate the number of T_{reg} cells before, during and following DC vaccination with the variation of measurable anti-leukaemic cytotoxicity and MRD burden. This may provide insight into the role of these cells in dampening anti-leukaemic immune responses.

The challenge for future vaccine approaches will be to translate the anti-leukaemic immune responses observed into demonstrable clinical benefit. This may be achieved by optimizing dosing, timing and sequencing of vaccine administration as well as potentiating the vaccine through use of adjuvants and addressing the suppressive effect of T_{reg}. Any future vaccination strategy will have to address the possibility of various immunological tumour escape mechanisms and the problem of targeting dormant tumour cells (Saudemont & Quesnel, 2004). It remains uncertain, if the anti-leukaemic role of NK cells observed in allogeneic stem cell transplantation can be replicated in autologous vaccine constructs, but a recent study in a murine model suggests that NK cells activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis (Saudemont *et al*, 2005).

Approaches based on transducing an autologous cellular vaccine with genes encoding co-stimulatory molecules and IL-2 (Rousseau *et al*, 2006;Chan *et al*, 2005) also appear promising, provided safety concerns about using a virally transduced vaccine can be addressed. Looking further ahead, the emerging knowledge of important, immunogenic leukaemia associated antigens, in particular those that are integral to the leukaemogenic process, might well reveal a number of peptides loadable to a wide range of HLA types, making this vaccine approach more widely applicable. Ultimately, *in vivo* loading of leukaemic antigens and achieving activation of the patient's own DCs by targeting specific DC toll-like receptors (TLRs) would seem an ideal mode of vaccine delivery. New, emerging adjuvants might greatly enhance the immune stimulation achieved by *in vivo* DC targeting.

Regardless of the chosen vaccine design, standardization of DC generation, antigen loading and maturation as well as evaluation of the vaccination response will be essential to allow observations from different clinical studies to be compared. Only then might an effective cellular vaccine, delivering a sustained clinical benefit to patients with low tumour burden AML post chemotherapy, emerge from the plethora of current hypotheses.

References:

Altman,J.D., Moss,P.A., Goulder,P.J., Barouch,D.H., McHeyzer-Williams,M.G., Bell,J.I., McMichael,A.J., & Davis,M.M. (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science*, **274**, 94-96.

Ambe,K., Mori,M., & Enjoji,M. (1989) S-100 protein-positive dendritic cells in colorectal adenocarcinomas. Distribution and relation to the clinical prognosis. *Cancer*, **63**, 496-503.

Avigan,D., Vasir,B., Gong,J., Borges,V., Wu,Z., Uhl,L., Atkins,M., Mier,J., McDermott,D., Smith,T., Giallambardo,N., Stone,C., Schadt,K., Dolgoff,J., Tetreault,J.C., Villarroel,M., & Kufe,D. (2004) Fusion cell vaccination of patients with metastatic breast and renal cancer induces immunological and clinical responses. *Clin.Cancer Res.*, **10**, 4699-4708.

Banchereau,J., Palucka,A.K., Dhodapkar,M., Burkeholder,S., Taquet,N., Rolland,A., Taquet,S., Coquery,S., Wittkowski,K.M., Bhardwaj,N., Pineiro,L., Steinman,R., & Fay,J. (2001) Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res.*, **61**, 6451-6458.

Banchereau,J. & Steinman,R.M. (1998) Dendritic cells and the control of immunity. *Nature*, **392**, 245-252.

Bender,A., Sapp,M., Schuler,G., Steinman,R.M., & Bhardwaj,N. (1996) Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J.Immunol.Methods*, **196**, 121-135.

Beyer,M., Kochanek,M., Darabi,K., Popov,A., Jensen,M., Endl,E., Knolle,P.A., Thomas,R.K., Bergwelt-Baildon,M., Debey,S., Hallek,M., & Schultze,J.L. (2005) Reduced frequencies and suppressive function of CD4+CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood*, **106**, 2018-2025.

Boyer,M.W., Vallera,D.A., Taylor,P.A., Gray,G.S., Katsanis,E., Gorden,K., Orchard,P.J., & Blazar,B.R. (1997) The role of B7 costimulation by murine acute myeloid leukemia in the generation and function of a CD8+ T-cell line with potent in vivo graft-versus-leukemia properties. *Blood*, **89**, 3477-3485.

Brossart,P., Wirths,S., Stuhler,G., Reichardt,V.L., Kanz,L., & Brugger,W. (2000) Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. *Blood*, **96**, 3102-3108.

Brouwer,R.E., van der,H.M., Kluin-Nelemans,H.C., Zelderen-Bhola,S., Willemze,R., & Falkenburg,J.H. (2000) The generation of dendritic-like cells with increased allostimulatory function from acute myeloid leukemia cells of various FAB subclasses. *Hum.Immunol.*, **61**, 565-574.

Buggins,A.G., Lea,N., Gaken,J., Darling,D., Farzaneh,F., Mufti,G.J., & Hirst,W.J. (1999) Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood*, **94**, 3479-3490.

Buhmann,R., Nolte,A., Westhaus,D., Emmerich,B., & Hallek,M. (1999) CD40-activated B-cell chronic lymphocytic leukemia cells for tumor immunotherapy: stimulation of allogeneic versus autologous T cells generates different types of effector cells. *Blood*, **93**, 1992-2002.

Burnett,A.K., Goldstone,A.H., Stevens,R.M., Hann,I.M., Rees,J.K., Gray,R.G., & Wheatley,K. (1998) Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. UK Medical Research Council Adult and Children's Leukaemia Working Parties. *Lancet*, **351**, 700-708.

Cardoso,A.A., Seamon,M.J., Afonso,H.M., Ghia,P., Boussiotis,V.A., Freeman,G.J., Gribben,J.G., Sallan,S.E., & Nadler,L.M. (1997) Ex vivo generation of human anti-pre-B leukemia-specific autologous cytolytic T cells. *Blood*, **90**, 549-561.

Casares,N., Arribillaga,L., Sarobe,P., Dotor,J., Lopez-Diaz,d.C., Melero,I., Prieto,J., Borras-Cuesta,F., & Lasarte,J.J. (2003a) CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J.Immunol.*, **171**, 5931-5939.

Casares,N., Arribillaga,L., Sarobe,P., Dotor,J., Lopez-Diaz,d.C., Melero,I., Prieto,J., Borras-Cuesta,F., & Lasarte,J.J. (2003b) CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J.Immunol.*, **171**, 5931-5939.

Cella,M., Sallusto,F., & Lanzavecchia,A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr.Opin.Immunol.*, **9**, 10-16.

Chan,L., Hardwick,N., Darling,D., Galea-Lauri,J., Gaken,J., Devereux,S., Kemeny,M., Mufti,G., & Farzaneh,F. (2005) IL-2/B7.1 (CD80) fusagene transduction of AML blasts by a self-inactivating lentiviral vector stimulates T cell responses in vitro: a strategy to generate whole cell vaccines for AML. *Mol.Ther.*, **11**, 120-131.

Charbonnier,A., Gaugler,B., Sainty,D., Lafage-Pochitaloff,M., & Olive,D. (1999) Human acute myeloblastic leukemia cells differentiate in vitro into mature dendritic cells and induce the differentiation of cytotoxic T cells against autologous leukemias. *Eur.J.Immunol.*, **29**, 2567-2578.

Chaux,P., Favre,N., Martin,M., & Martin,F. (1997) Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. *Int.J.Cancer*, **72**, 619-624.

Chaux,P., Moutet,M., Faivre,J., Martin,F., & Martin,M. (1996) Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7-1 and B7-2 costimulatory molecules of the T-cell activation. *Lab Invest*, **74**, 975-983.

Chen,D., Xia,J., Tanaka,Y., Chen,H., Koido,S., Wernet,O., Mukherjee,P., Gendler,S.J., Kufe,D., & Gong,J. (2003) Immunotherapy of spontaneous mammary carcinoma with fusions of dendritic cells and mucin 1-positive carcinoma cells. *Immunology*, **109**, 300-307.

Choudhury,A., Gajewski,J.L., Liang,J.C., Popat,U., Claxton,D.F., Kliche,K.O., Andreeff,M., & Champlin,R.E. (1997) Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood*, **89**, 1133-1142.

Choudhury,B.A., Liang,J.C., Thomas,E.K., Flores-Romo,L., Xie,Q.S., Agusala,K., Sutaria,S., Sinha,I., Champlin,R.E., & Claxton,D.F. (1999) Dendritic cells derived in vitro from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. *Blood*, **93**, 780-786.

Cignetti,A., Bryant,E., Allione,B., Vitale,A., Foa,R., & Cheever,M.A. CD34(+) acute myeloid and lymphoid leukemic blasts can be induced to differentiate into dendritic cells.

Clark,R.E., Dodi,I.A., Hill,S.C., Lill,J.R., Aubert,G., Macintyre,A.R., Rojas,J., Bourdon,A., Bonner,P.L., Wang,L., Christmas,S.E., Travers,P.J., Creaser,C.S., Rees,R.C., & Madrigal,J.A. (2001) Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. *Blood*, **98**, 2887-2893.

Collins,R.H., Jr., Shpilberg,O., Drobyski,W.R., Porter,D.L., Giralt,S., Champlin,R., Goodman,S.A., Wolff,S.N., Hu,W., Verfaillie,C., List,A., Dalton,W., Ognoskie,N., Chetrit,A., Antin,J.H., & Nemunaitis,J. (1997) Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J.Clin.Oncol.*, **15**, 433-444.

de Bueger,M., Bakker,A., van Rood,J.J., Van der,W.F., & Goulmy,E. (1992) Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens. *J.Immunol.*, **149**, 1788-1794.

De Vries,I.J., Krooshoop,D.J., Scharenborg,N.M., Lesterhuis,W.J., Diepstra,J.H., Van Muijen,G.N., Strijk,S.P., Ruers,T.J., Boerman,O.C., Oyen,W.J., Adema,G.J., Punt,C.J., & Figdor,C.G. (2003) Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res.*, **63** , 12-17.

Dilloo,D., Bacon,K., Holden,W., Zhong,W., Burdach,S., Zlotnik,A., & Brenner,M. (1996) Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nat.Med.*, **2**, 1090-1095.

Eibl,B., Ebner,S., Duba,C., Bock,G., Romani,N., Erdel,M., Gachter,A., Niederwieser,D., & Schuler,G. (1997) Dendritic cells generated from blood precursors of chronic myelogenous leukemia patients carry the Philadelphia translocation and can induce a CML-specific primary cytotoxic T-cell response. *Genes Chromosomes.Cancer*, **20**, 215-223.

Elisseeva,O.A., Oka,Y., Tsuboi,A., Ogata,K., Wu,F., Kim,E.H., Soma,T., Tamaki,H., Kawakami,M., Oji,Y., Hosen,N., Kubota,T., Nakagawa,M., Yamagami,T., Hiraoka,A., Tsukaguchi,M., Udaka,K., Ogawa,H., Kishimoto,T., Nomura,T., & Sugiyama,H. (2002) Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies. *Blood*, **99**, 3272-3279.

Espinoza-Delgado,I., Bosco,M.C., Musso,T., Mood,K., Ruscetti,F.W., Longo,D.L., & Varesio,L. (1994) Inhibitory cytokine circuits involving transforming growth factor-beta, interferon-gamma, and interleukin-2 in human monocyte activation. *Blood*, **83**, 3332-3338.

Falkenburg,J.H., Smit,W.M., & Willemze,R. (1997) Cytotoxic T-lymphocyte (CTL) responses against acute or chronic myeloid leukemia. *Immunol.Rev.*, **157**, 223-230.

Farag,S.S., Fehniger,T.A., Ruggeri,L., Velardi,A., & Caligiuri,M.A. (2002) Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood*, **100**, 1935-1947.

Ferlazzo,G., Klein,J., Paliard,X., Wei,W.Z., & Galy,A. (2000) Dendritic cells generated from CD34+ progenitor cells with flt3 ligand, c-kit ligand, GM-CSF, IL-4, and TNF-alpha are functional antigen-presenting cells resembling mature monocyte-derived dendritic cells. *J.Immunother.*, **23**, 48-58.

Fox,S.B., Jones,M., Dunnill,M.S., Gatter,K.C., & Mason,D.Y. (1989) Langerhans cells in human lung tumours: an immunohistological study. *Histopathology*, **14**, 269-275.

Fujii,S., Fujimoto,K., Shimizu,K., Ezaki,T., Kawano,F., Takatsuki,K., Kawakita,M., & Matsuno,K. (1999) Presentation of tumor antigens by phagocytic dendritic cell clusters generated from human CD34+ hematopoietic progenitor cells: induction of autologous cytotoxic T lymphocytes against leukemic cells in acute myelogenous leukemia patients. *Cancer Res.*, **59**, 2150-2158.

Galea-Lauri,J., Darling,D., Mufti,G., Harrison,P., & Farzaneh,F. (2002) Eliciting cytotoxic T lymphocytes against acute myeloid leukemia-derived antigens: evaluation of dendritic cell-leukemia cell hybrids and other antigen-loading strategies for dendritic cell-based vaccination. *Cancer Immunol.Immunother.*, **51**, 299-310.

Gao,L., Bellantuono,I., Elsasser,A., Marley,S.B., Gordon,M.Y., Goldman,J.M., & Stauss,H.J. (2000) Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood*, **95**, 2198-2203.

Gastl,G.A., Abrams,J.S., Nanus,D.M., Oosterkamp,R., Silver,J., Liu,F., Chen,M., Albino,A.P., & Bander,N.H. (1993) Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. *Int.J.Cancer*, **55**, 96-101.

Ghiringhelli,F., Larmonier,N., Schmitt,E., Parcellier,A., Cathelin,D., Garrido,C., Chauffert,B., Solary,E., Bonnotte,B., & Martin,F. (2004) CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur.J.Immunol.*, **34**, 336-344.

Gong,J., Apostolopoulos,V., Chen,D., Chen,H., Koido,S., Gendler,S.J., McKenzie,I.F., & Kufe,D. (2000) Selection and characterization of MUC1-specific CD8+ T cells from MUC1 transgenic mice immunized with dendritic-carcinoma fusion cells. *Immunology*, **101**, 316-324.

Gong,J., Chen,D., Kashiwaba,M., & Kufe,D. (1997) Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat.Med.*, **3**, 558-561.

Gong,J., Chen,D., Kashiwaba,M., Li,Y., Chen,L., Takeuchi,H., Qu,H., Rowse,G.J., Gendler,S.J., & Kufe,D. (1998) Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proc.Natl.Acad.Sci.U.S.A*, **95**, 6279-6283.

Haining,W.N., Cardoso,A.A., Keczkemethy,H.L., Fleming,M., Neuberg,D., DeAngelo,D.J., Stone,R.M., Galinsky,I., Silverman,L.B., Sallan,S.E., Nadler,L.M., & Guinan,E.C. (2005) Failure to define window of time for autologous tumor vaccination in patients with newly diagnosed or relapsed acute lymphoblastic leukemia. *Exp.Hematol.*, **33**, 286-294.

Harrison,B.D., Adams,J.A., Briggs,M., Brereton,M.L., & Yin,J.A. (2001) Stimulation of autologous proliferative and cytotoxic T-cell responses by "leukemic dendritic cells" derived from blast cells in acute myeloid leukemia. *Blood*, **97**, 2764-2771.

Helg,C., Starobinski,M., Jeannet,M., & Chapuis,B. (1998) Donor lymphocyte infusion for the treatment of relapse after allogeneic hematopoietic stem cell transplantation. *Leuk.Lymphoma*, **29**, 301-313.

Hernando,J.J., Park,T.W., Kubler,K., Offergeld,R., Schlebusch,H., & Bauknecht,T. (2002) Vaccination with autologous tumour antigen-pulsed dendritic cells in advanced

gynaecological malignancies: clinical and immunological evaluation of a phase I trial. *Cancer Immunol.Immunother.*, **51**, 45-52.

Hirst,W.J., Buggins,A., Darling,D., Gaken,J., Farzaneh,F., & Mufti,G.J. (1997) Enhanced immune costimulatory activity of primary acute myeloid leukaemia blasts after retrovirus-mediated gene transfer of B7.1. *Gene Ther.*, **4**, 691-699.

Inoue,K., Ogawa,H., Sonoda,Y., Kimura,T., Sakabe,H., Oka,Y., Miyake,S., Tamaki,H., Oji,Y., Yamagami,T., Tatekawa,T., Soma,T., Kishimoto,T., & Sugiyama,H. (1997) Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. *Blood*, **89**, 1405-1412.

Kharfan-Dabaja,M., Ayala,E., Lindner,I., Cejas,P.J., Bahlis,N.J., Kolonias,D., Carlson,L.M., & Lee,K.P. (2005) Differentiation of acute and chronic myeloid leukemic blasts into the dendritic cell lineage: analysis of various differentiation-inducing signals. *Cancer Immunol.Immunother.*, **54**, 25-36.

Kohler,T., Plettig,R., Wetzstein,W., Schmitz,M., Ritter,M., Mohr,B., Schaekel,U., Ehninger,G., & Bornhauser,M. (2000) Cytokine-driven differentiation of blasts from patients with acute myelogenous and lymphoblastic leukemia into dendritic cells. *Stem Cells*, **18**, 139-147.

Kokhaei,P., Rezvany,M.R., Virving,L., Choudhury,A., Rabbani,H., Osterborg,A., & Mellstedt,H. (2003) Dendritic cells loaded with apoptotic tumour cells induce a stronger T-cell response than dendritic cell-tumour hybrids in B-CLL. *Leukemia*, **17**, 894-899.

Kolb,H.J., Schattenberg,A., Goldman,J.M., Hertenstein,B., Jacobsen,N., Arcese,W., Ljungman,P., Ferrant,A., Verdonck,L., Niederwieser,D., van Rhee,F., Mittermueller,J., de Witte,T., Holler,E., & Ansari,H. (1995) Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood*, **86**, 2041-2050.

Korngold,R., Leighton,C., & Manser,T. (1994) Graft-versus-myeloid leukemia responses following syngeneic and allogeneic bone marrow transplantation. *Transplantation*, **58**, 278-287.

Lee,J.J., Kook,H., Park,M.S., Nam,J.H., Choi,B.H., Song,W.H., Park,K.S., Lee,I.K., Chung,I.J., Hwang,T.J., & Kim,H.J. (2004) Immunotherapy using autologous monocyte-derived dendritic cells pulsed with leukemic cell lysates for acute myeloid leukemia relapse after autologous peripheral blood stem cell transplantation. *J.Clin.Apher.*, **19**, 66-70.

Li,J., Holmes,L.M., Franek,K.J., Burgin,K.E., Wagner,T.E., & Wei,Y. (2001) Purified hybrid cells from dendritic cell and tumor cell fusions are superior activators of antitumor immunity. *Cancer Immunol.Immunother.*, **50**, 456-462.

Luscher,U., Filgueira,L., Juretic,A., Zuber,M., Luscher,N.J., Heberer,M., & Spagnoli,G.C. (1994) The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes. *Int.J.Cancer*, **57**, 612-619.

Ma,L., Delforge,M., van,D., V, Verhoef,G., Emanuel,B., Boogaerts,M., Hagemeijer,A., & Vandenberghe,P. (2004) Circulating myeloid and lymphoid precursor dendritic cells are clonally involved in myelodysplastic syndromes. *Leukemia*, **18**, 1451-1456.

Mackinnon,S., Papadopoulos,E.B., Carabasi,M.H., Reich,L., Collins,N.H., Boulad,F., Castro-Malaspina,H., Childs,B.H., Gillio,A.P., Kernan,N.A., & . (1995) Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood*, **86**, 1261-1268.

Maeda,H. & Shiraishi,A. (1996) TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J.Immunol.*, **156**, 73-78.

Mailander,V., Scheibenbogen,C., Thiel,E., Letsch,A., Blau,I.W., & Keilholz,U. (2004) Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT1 peptide in the absence of hematological or renal toxicity. *Leukemia*, **18** , 165-166.

Marchand,M., van Baren,N., Weynants,P., Brichard,V., Dreno,B., Tessier,M.H., Rankin,E., Parmiani,G., Arienti,F., Humblet,Y., Bourlond,A., Vanwijck,R., Lienard,D., Beauduin,M., Dietrich,P.Y., Russo,V., Kerger,J., Masucci,G., Jager,E., De Greve,J., Atzpodien,J., Brasseur,F., Coulie,P.G., van der,B.P., & Boon,T. (1999) Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int.J.Cancer*, **80**, 219-230.

Marijt,W.A., Heemskerk,M.H., Kloosterboer,F.M., Goulmy,E., Kester,M.G., van der Hoorn,M.A., Luxemburg-Heys,S.A., Hoogeboom,M., Mutis,T., Drijfhout,J.W., van Rood,J.J., Willemze,R., & Falkenburg,J.H. (2003) Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc.Natl.Acad.Sci.U.S.A*, **100**, 2742-2747.

MartIn-Fontecha,A., Sebastiani,S., Hopken,U.E., Uguccioni,M., Lipp,M., Lanzavecchia,A., & Sallusto,F. (2003) Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J.Exp.Med.*, **198**, 615-621.

Matzinger,P. (1998) An innate sense of danger. *Semin.Immunol.*, **10**, 399-415.

Menssen,H.D., Renkl,H.J., Rodeck,U., Maurer,J., Notter,M., Schwartz,S., Reinhardt,R., & Thiel,E. (1995) Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. *Leukemia*, **9**, 1060-1067.

Mesel-Lemoine,M., Cherai,M., Le Gouvello,S., Guillot,M., Leclercq,V., Klatzmann,D., Thomas-Vaslin,V., & Lemoine,F.M. (2005) Initial depletion of regulatory T-cells: the missing solution to preserve the immune functions of T lymphocytes designed for cell-therapy. *Blood.*

Mintz,P.J., Kim,J., Do,K.A., Wang,X., Zinner,R.G., Cristofanilli,M., Arap,M.A., Hong,W.K., Troncoso,P., Logothetis,C.J., Pasqualini,R., & Arap,W. (2003) Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nat.Biotechnol.*, **21**, 57-63.

Mohty,M., Isnardon,D., Charbonnier,A., Lafage-Pochitaloff,M., Merlin,M., Sainty,D., Olive,D., & Gaugler,B. (2002) Generation of potent T(h)1 responses from patients with lymphoid malignancies after differentiation of B lymphocytes into dendritic-like cells. *Int.Immunol.*, **14**, 741-750.

Mohty,M., Jarrossay,D., Lafage-Pochitaloff,M., Zandotti,C., Briere,F., de Lamballeri,X.N., Isnardon,D., Sainty,D., Olive,D., & Gaugler,B. (2001) Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. *Blood*, **98**, 3750-3756.

Molldrem,J., Dermime,S., Parker,K., Jiang,Y.Z., Mavroudis,D., Hensel,N., Fukushima,P., & Barrett,A.J. (1996) Targeted T-cell therapy for human leukemia: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells. *Blood*, **88**, 2450-2457.

Molldrem,J.J., Lee,P.P., Kant,S., Wieder,E., Jiang,W., Lu,S., Wang,C., & Davis,M.M. (2003) Chronic myelogenous leukemia shapes host immunity by selective deletion of high-avidity leukemia-specific T cells. *J.Clin.Invest*, **111**, 639-647.

Molldrem,J.J., Lee,P.P., Wang,C., Felio,K., Kantarjian,H.M., Champlin,R.E., & Davis,M.M. (2000) Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat.Med.*, **6**, 1018-1023.

Morse,M.A., Clay,T.M., Mosca,P., & Lyerly,H.K. (2002) Immunoregulatory T cells in cancer immunotherapy. *Expert.Opin.Biol.Ther.*, **2**, 827-834.

Moser,M. & Murphy,K.M. (2000) Dendritic cell regulation of TH1-TH2 development. *Nat.Immunol.*, **1**, 199-205.

Muller,C.I., Trepel,M., Kunzmann,R., Lais,A., Engelhardt,R., & Lubbert,M. (2004) Hematologic and molecular spontaneous remission following sepsis in acute monoblastic leukemia with translocation (9;11): a case report and review of the literature. *Eur.J.Haematol.*, **73**, 62-66.

Murphy,G., Tjoa,B., Ragde,H., Kenny,G., & Boynton,A. (1996) Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate*, **29**, 371-380.

Mutis,T., Gillespie,G., Schrama,E., Falkenburg,J.H., Moss,P., & Goulmy,E. (1999a) Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat.Med.*, **5**, 839-842.

Mutis,T., Schrama,E., Melief,C.J., & Goulmy,E. (1998) CD80-Transfected acute myeloid leukemia cells induce primary allogeneic T-cell responses directed at patient specific minor histocompatibility antigens and leukemia-associated antigens. *Blood*, **92**, 1677-1684.

Mutis,T., Verdijk,R., Schrama,E., Esendam,B., Brand,A., & Goulmy,E. (1999b) Feasibility of immunotherapy of relapsed leukemia with ex vivo-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood*, **93**, 2336-2341.

Nagy,N. & Vanky,F. (1998) Transforming growth factor beta, (TGFbeta) secreted by immunogenic ex vivo human carcinoma cells, counteracts the activation and inhibits the function of autologous cytotoxic lymphocytes. Pretreatment with interferon gamma and tumor necrosis factor alpha reduces the production of active TGFbeta. *Cancer Immunol.Immunother.*, **45**, 306-312.

Nakase,K., Kita,K., Otsuji,A., Anazawa,H., Hoshino,K., Sekine,T., Shirakawa,S., Tanaka,I., Nasu,K., Tsutani,H., & . (1992) Diagnostic and clinical importance of interleukin-2 receptor alpha chain expression on non-T-cell acute leukaemia cells. *Br.J.Haematol.*, **80**, 317-326.

Nakazaki,Y., Tani,K., Lin,Z.T., Sumimoto,H., Hibino,H., Tanabe,T., Wu,M.S., Izawa,K., Hase,H., Takahashi,S., Tojo,A., Azuma,M., Hamada,H., Mori,S., & Asano,S. (1998) Vaccine effect of granulocyte-macrophage colony-stimulating factor or CD80 gene-transduced murine hematopoietic tumor cells and their cooperative enhancement of antitumor immunity. *Gene Ther.*, **5**, 1355-1362.

Napolitani,G., Rinaldi,A., Bertoni,F., Sallusto,F., & Lanzavecchia,A. (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol.*, **6**, 769-776.

Nestle,F.O., Alijagic,S., Gilliet,M., Sun,Y., Grabbe,S., Dummer,R., Burg,G., & Schadendorf,D. (1998) Vaccination of melanoma patients with pep. *Nat.Med.*, **4**, 328-332.

Nestle,F.O., Burg,G., Fah,J., Wrone-Smith,T., & Nickoloff,B.J. (1997) Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am.J.Pathol.*, **150**, 641-651.

Ogawa,H., Tamaki,H., Ikegame,K., Soma,T., Kawakami,M., Tsuboi,A., Kim,E.H., Hosen,N., Murakami,M., Fujioka,T., Masuda,T., Taniguchi,Y., Nishida,S., Oji,Y., Oka,Y., & Sugiyama,H. (2003) The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. *Blood*, **101**, 1698-1704.

Ohminami,H., Yasukawa,M., & Fujita,S. (2000) HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. *Blood*, **95**, 286-293.

Ohminami,H., Yasukawa,M., Kaneko,S., Yakushijin,Y., Abe,Y., Kasahara,Y., Ishida,Y., & Fujita,S. (1999) Fas-independent and nonapoptotic cytotoxicity mediated by a human CD4(+) T-cell clone directed against an acute myelogenous leukemia-associated DEK-CAN fusion peptide. *Blood*, **93**, 925-935.

Oka,Y., Tsuboi,A., Taguchi,T., Osaki,T., Kyo,T., Nakajima,H., Elisseeva,O.A., Oji,Y., Kawakami,M., Ikegame,K., Hosen,N., Yoshihara,S., Wu,F., Fujiki,F., Murakami,M., Masuda,T., Nishida,S., Shirakata,T., Nakatsuka,S., Sasaki,A., Udaka,K., Dohy,H., Aozasa,K., Noguchi,S., Kawase,I., & Sugiyama,H. (2004) Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc.Natl.Acad.Sci.U.S.A*, **101**, 13885-13890.

Osman,Y., Takahashi,M., Zheng,Z., Toba,K., Liu,A., Furukawa,T., Narita,M., Aizawa,Y., Koike,T., & Shibata,A. (1999) Dendritic cells stimulate the expansion of PML-RAR alpha specific cytotoxic T-lymphocytes: its applicability for antileukemia immunotherapy. *J.Exp.Clin.Cancer Res.*, **18**, 485-492.

Panoskaltsis,N. (2005) Dendritic cells in MDS and AML--cause, effect or solution to the immune pathogenesis of disease? *Leukemia*, **19**, 354-357.

Panoskaltsis,N., Belanger,T.J., Liesveld,J.L., & Abboud,C.N. (2002) Optimal cytokine stimulation for the enhanced generation of leukemic dendritic cells in short-term culture. *Leuk.Res.*, **26**, 191-201.

Portielje,J.E., Kruit,W.H., Eerenberg,A.J., Schuler,M., Sparreboom,A., Lamers,C.H., Gratama,J.W., Stoter,G., Huber,C., & Hack,C.E. (2005) Subcutaneous injection of interleukin 12 induces systemic inflammatory responses in humans: implications for the use of IL-12 as vaccine adjuvant. *Cancer Immunol.Immunother.*, **54**, 37-43.

Qazilbash,M.H., Wieder,E., Rios,R., Lu,S., Kant,S., Giralt,S., Estey,E.H., Thall,P., de Lima,M., Couriel,D., Champlin,R.E., Komanduri,K., & Molldrem,J.J. (2004) Vaccination with the PR1 Leukemia-Associated Antigen Can Induce Complete Remission in Patients with Myeloid Leukemia. *ASH Annual Meeting Abstracts*, **104**, 259.

Qu,C., Edwards,E.W., Tacke,F., Angeli,V., Llodra,J., Sanchez-Schmitz,G., Garin,A., Haque,N.S., Peters,W., van Rooijen,N., Sanchez-Torres,C., Bromberg,J., Charo,I.F., Jung,S., Lira,S.A., & Randolph,G.J. (2004) Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J.Exp.Med.*, **200**, 1231-1241.

Raje,N., Hideshima,T., Davies,F.E., Chauhan,D., Treon,S.P., Young,G., Tai,Y.T., Avigan,D., Gong,J., Schlossman,R.L., Richardson,P., Kufe,D.W., & Anderson,K.C. (2004) Tumour cell/dendritic cell fusions as a vaccination strategy for multiple myeloma. *Br.J.Haematol.*, **125**, 343-352.

Ratzinger,G., Stoitzner,P., Ebner,S., Lutz,M.B., Layton,G.T., Rainer,C., Senior,R.M., Shipley,J.M., Fritsch,P., Schuler,G., & Romani,N. (2002) Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J.Immunol.*, **168**, 4361-4371.

Ravindranath,Y., Yeager,A.M., Chang,M.N., Steuber,C.P., Krischer,J., Graham-Pole,J., Carroll,A., Inoue,S., Camitta,B., & Weinstein,H.J. (1996) Autologous bone marrow transplantation versus intensive consolidation chemotherapy for acute myeloid leukemia in childhood. Pediatric Oncology Group. *N.Engl.J.Med*, **334**, 1428-1434.

Robinson,S.P., English,N., Jaju,R., Kearney,L., Knight,S.C., & Reid,C.D. (1998) The in-vitro generation of dendritic cells from blast cells in acute leukaemia. *Br.J.Haematol.*, **103**, 763-771.

Roddie,P.H., Horton,Y., & Turner,M.L. (2002) Primary acute myeloid leukaemia blasts resistant to cytokine-induced differentiation to dendritic-like leukaemia cells can be forced to differentiate by the addition of bryostatin-1. *Leukemia*, **16**, 84-93.

Rousseau,R.F., Biagi,E., Dutour,A., Yvon,E.S., Brown,M.P., Lin,T., Mei,Z., Grilley,B., Popek,E., Heslop,H.E., Gee,A.P., Krance,R.A., Popat,U., Carrum,G., Margolin,J.F., & Brenner,M.K. (2006) Immunotherapy of high-risk acute leukemia with a recipient (autologous) vaccine expressing transgenic human CD40L and IL-2 after chemotherapy and allogeneic stem cell transplantation. *Blood*, **107**, 1332-1341.

Ruggeri,L., Capanni,M., Casucci,M., Volpi,I., Tosti,A., Perruccio,K., Urbani,E., Negrin,R.S., Martelli,M.F., & Velardi,A. (1999) Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood*, **94**, 333-339.

Ruggeri,L., Capanni,M., Urbani,E., Perruccio,K., Shlomchik,W.D., Tosti,A., Posati,S., Rogaia,D., Frassoni,F., Aversa,F., Martelli,M.F., & Velardi,A. (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*, **295**, 2097-2100.

Sallusto,F. & Lanzavecchia,A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J.Exp.Med.*, **179**, 1109-1118.

Saudemont,A., Buffenoir,G., Denys,A., Desreumaux,P., Jouy,N., Hetuin,D., Bauters,F., Fenaux,P., & Quesnel,B. (2002) Gene transfer of CD154 and IL12 cDNA induces an anti-leukemic immunity in a murine model of acute leukemia. *Leukemia*, **16**, 1637-1644.

Saudemont,A., Jouy,N., Hetuin,D., & Quesnel,B. (2005) NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells. *Blood* , **105**, 2428-2435.

Saudemont,A. & Quesnel,B. (2004) In a model of tumor dormancy, long-term persistent leukemic cells have increased B7-H1 and B7.1 expression and resist CTL-mediated lysis. *Blood*, **104**, 2124-2133.

Scheibenbogen,C., Letsch,A., Thiel,E., Schmittel,A., Mailaender,V., Baerwolf,S., Nagorsen,D., & Keilholz,U. (2002) CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. *Blood*, **100**, 2132-2137.

Schroder,S., Schwarz,W., Rehpenning,W., Loning,T., & Bocker,W. (1988) Dendritic/Langerhans cells and prognosis in patients with papillary thyroid carcinomas. Immunocytochemical study of 106 thyroid neoplasms correlated to follow-up data. *Am.J.Clin.Pathol.*, **89**, 295-300.

Schui,D.K., Singh,L., Schneider,B., Knau,A., Hoelzer,D., & Weidmann,E. (2002) Inhibiting effects on the induction of cytotoxic T lymphocytes by dendritic cells pulsed with lysates from acute myeloid leukemia blasts. *Leuk.Res.*, **26**, 383-389.

Sousa,C., Sher,A., & Kaye,P. (1999) The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Curr.Opin.Immunol.*, **11**, 392-399.

Spisek,R., Chevallier,P., Morineau,N., Milpied,N., Avet-Loiseau,H., Harousseau,J.L., Meflah,K., & Gregoire,M. (2002) Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. *Cancer Res.*, **62**, 2861-2868.

Staveley-O'Carroll,K., Sotomayor,E., Montgomery,J., Borrello,I., Hwang,L., Fein,S., Pardoll,D., & Levitsky,H. (1998) Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc.Natl.Acad.Sci.U.S.A*, **95**, 1178-1183.

Steinman,R.M. (1991) The dendritic cell system and its role in immunogenicity. *Annu.Rev.Immunol.*, **9**, 271-296.

Stripecke,R., Cardoso,A.A., Pepper,K.A., Skelton,D.C., Yu,X.J., Mascarenhas,L., Weinberg,K.I., Nadler,L.M., & Kohn,D.B. (2000) Lentiviral vectors for efficient delivery of CD80 and granulocyte-macrop. *Blood*, **96**, 1317-1326.

Takasugi,M., Ramseyer,A., & Takasugi,J. (1977) Decline of natural nonselective cell-mediated cytotoxicity in patients with tumor progression. *Cancer Res.*, **37**, 413-418.

Tanaka,M., Suda,T., Haze,K., Nakamura,N., Sato,K., Kimura,F., Motoyoshi,K., Mizuki,M., Tagawa,S., Ohga,S., Hatake,K., Drummond,A.H., & Nagata,S. (1996) Fas ligand in human serum. *Nat.Med.*, **2**, 317-322.

Therasse,P., Arbuck,S.G., Eisenhauer,E.A., Wanders,J., Kaplan,R.S., Rubinstein,L., Verweij,J., Van Glabbeke,M., van Oosterom,A.T., Christian,M.C., & Gwyther,S.G. (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J.Natl.Cancer Inst.*, **92**, 205-216.

Thurner,B., Roder,C., Dieckmann,D., Heuer,M., Kruse,M., Glaser,A., Keikavoussi,P., Kampgen,E., Bender,A., & Schuler,G. (1999) Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J.Immunol.Methods*, **223**, 1-15.

Thurnher,M., Radmayr,C., Ramoner,R., Ebner,S., Bock,G., Klocker,H., Romani,N., & Bartsch,G. (1996) Human renal-cell carcinoma tissue contains dendritic cells. *Int.J.Cancer*, **68**, 1-7.

Valmori,D., Dutoit,V., Lienard,D., Lejeune,F., Speiser,D., Rimoldi,D., Cerundolo,V., Dietrich,P.Y., Cerottini,J.C., & Romero,P. (2000) Tetramer-guided analysis of TCR beta-chain usage reveals a large repertoire of melan-A-specific CD8+ T cells in melanoma patients. *J.Immunol.*, **165**, 533-538.

Vasir,B., Borges,V., Wu,Z., Grosman,D., Rosenblatt,J., Irie,M., Anderson,K., Kufe,D., & Avigan,D. (2005) Fusion of dendritic cells with multiple myeloma cells results in maturation and enhanced antigen presentation. *Br.J.Haematol.*, **129**, 687-700.

Vinante,F., Rigo,A., Tecchio,C., Morosato,L., Nadali,G., Ricetti,M.M., Krampera,M., Zanolin,E., Locatelli,F., Gallati,H., Chilosi,M., & Pizzolo,G. (1998) Serum levels of p55 and p75 soluble TNF receptors in adult acute leukaemia at diagnosis: correlation with clinical and biological features and outcome. *Br.J.Haematol.*, **102**, 1025-1034.

Waclavicek,M., Berer,A., Oehler,L., Stockl,J., Schloegl,E., Majdic,O., & Knapp,W. (2001) Calcium ionophore: a single reagent for the differentiation of primary human acute myelogenous leukaemia cells towards dendritic cells. *Br.J.Haematol.*, **114**, 466-473.

Weigel,B.J., Panoskaltsis-Mortari,A., Diers,M., Garcia,M., Lees,C., Krieg,A.M., Chen,W., & Blazar,B.R. (2006) Dendritic cells pulsed or fused with AML cellular antigen provide comparable in vivo antitumor protective responses. *Exp.Hematol.*, **34**, 1403-1412.

Wolf,A.M., Wolf,D., Steurer,M., Gastl,G., Gunsilius,E., & Grubeck-Loebenstein,B. (2003a) Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin.Cancer Res.*, **9**, 606-612.

Wolf,A.M., Wolf,D., Steurer,M., Gastl,G., Gunsilius,E., & Grubeck-Loebenstein,B. (2003b) Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin.Cancer Res.*, **9**, 606-612.

Ye,Z., Gee,A.P., Bowers,W.E., Lamb,L.S., Turner,M.W., & Henslee-Downey,P.J. (1996) In vitro expansion and characterization of dendritic cells derived from human bone marrow CD34+ cells. *Bone Marrow Transplant.*, **18**, 997-1008.

Yin,J.A. & Tobal,K. (1999) Detection of minimal residual disease in acute myeloid leukaemia: methodologies, clinical and biological significance. *Br.J.Haematol.*, **106**, 578-590.

Zeis,M., Siegel,S., Wagner,A., Schmitz,M., Marget,M., Kuhl-Burmeister,R., Adamzik,I., Kabelitz,D., Dreger,P., Schmitz,N., & Heiser,A. (2003) Generation of cytotoxic responses in mice and human individuals against hematological malignancies using survivin-RNA-transfected dendritic cells. *J.Immunol.*, **170**, 5391-5397.

APPENDIX I: INFORMATION FOR PATIENTS - Version Two 16/7/01

Hospital Headed Paper

A STUDY OF USING CHEMOTHERAPY AND A VACCINE DEVELOPED FROM PATIENTS' OWN LEUKAEMIA CELLS TO TREAT ACUTE myeloid leukaemia

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you would like to take part.

What is the purpose of the study?
The study is part of a research project involving 50 patients aimed at the development of a new type of treatment for Acute Myeloid Leukaemia. Treatment of leukaemia with special drugs (chemotherapy) is often successful in making patients better for a period of time, this is called remission. However, small numbers of leukaemia cells are able to survive and these may cause the leukaemia to come back. We are trying to develop an alternative form of treatment which aims to use your body's own defences to destroy these residual leukaemia cells. The new treatment is therefore like a vaccine against leukaemia and will be used in combination with standard drug treatments.

Why am I being asked to take part in this study?
You are being asked to participate in this study as you have acute myeloid leukaemia that may be difficult to cure by chemotherapy alone.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be give this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will it involve?
You will have some of your leukaemia cells removed either by taking a blood sample or, if this is not possible because of low numbers of leukaemia cells in your blood, by taking a sample of your bone marrow or by going onto a cell separator machine. If

you need to go on the cell separator machine your doctor will give you a separate leaflet explaining what this involves.

A portion of the leukaemia cells that we collect will be tested to see if they are suitable to be used as a vaccine for you. The remaining leukaemia cells will be stored down for later use. Your blood will also be tested for HIV and the viruses that cause hepatitis as this is routinely required for this type of treatment.

You will then receive drugs aimed at achieving remission of the leukaemia. The consultant in charge of your care will make the decision as to which treatment you receive. If this treatment is successful we will take some of the leukaemia cells that were stored at the start of the study and grow them under special conditions in the laboratory to make them able to help your body recognise any remaining leukaemia cells in order to destroy them. These cells are then specially treated to prevent them growing again once they are given back. The cells will be given to you by a series of four injections at weekly intervals under the skin. Following each injection you will need to stay in the ward for four hours for routine monitoring.
Before you receive the first injection you will be given the opportunity to consider whether you wish to continue in the study and receive the injections. If you do wish to receive the injections you will be asked to sign a form consenting to this procedure.
A blood sample (15mL/3 teaspoons) will be taken before each of these injections and at 1 week, 3 weeks and 9 weeks after the last injection to count your blood cells and check that your liver and kidneys are working properly.
In addition, to check how the treatment is working, you will have the following samples taken:-

- A blood sample (50mL/10 teaspoons) before the first injection of the cells and at 2 weeks, 4 weeks, 6 weeks and then every 12 weeks after the first injection.
- A sample of bone marrow (5-10mL/1-2 teaspoons) before the first injection and at 2 weeks, 4 weeks and 12 weeks after the first injection.

You will also be given a simple patient diary, asking questions about discomfort at the injection site, which you will be asked to fill in daily for seven days after each injection.

After the treatment you will have blood samples (10mL/2 teaspoons) taken at regular intervals and bone marrow samples every three months to check that the leukaemia has not come back.

If all your cells are not used for your treatment, with your permission they will be destroyed.

You will be given a card saying that you are taking part in a research project and giving details of the study. You will be asked to carry this card with you at all times.

What treatment will I receive if I do not take part in the study?
You will receive drugs aimed at achieving remission of the leukaemia. The consultant in charge of your care will make the decision as to which treatment you

receive. You will not have some of your leukaemia cells stored at the start of the study for growing in the laboratory to try and make a vaccine, so you will not receive the four injections at weekly intervals. You will have blood samples (10mL/2 teaspoons) taken at regular intervals and bone marrow samples every three months to check that the leukaemia has not come back.

What risks are involved in taking part in the study?
Your own doctor will not admit you to the study unless he/she thinks you are fit enough to have the cell harvest procedure performed and undergo treatment.

There is a small risk of allergic reactions, dizziness or tingling sensations associated with having the cell harvest procedure but a doctor will assess you beforehand to ensure that you are fit enough to undergo it. In common with all people who give blood donations you will be tested for viruses such as HIV and those causing hepatitis. In the event that you test positive for any of the viruses you will be informed about the results and their potential significance.

We cannot guarantee that you will be able to receive your cells back. Your leukaemia cells may not be capable of undergoing the change needed to help your body recognise any remaining leukaemia cells. Also, there is a risk that your cells could become infected while being grown in the laboratory, in which case it would be dangerous for you to receive them back. However, every effort is made to prevent such infections occurring.

You may experience some temporary discomfort and redness at the injection site.

What are the possible benefits of taking part in the study?
We hope the treatment will help you. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients with leukaemia better.

Will my taking part in this study be kept confidential?
All information obtained from this study which can be identified with you will remain confidential. Although you will be named in internal study documentation, your name will not be disclosed in any external reports or publications.
Your medical notes may be examined by responsible staff from the hospital, study team or from regulatory authorities where it is relevant to the research but all information in your records will be treated in strict confidence.
Your GP will be informed about your taking part in this study with your permission.

What if something goes wrong?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached

or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

What will happen if new information becomes available during the study?

Sometimes during the course of a study new information becomes available. Your doctor will talk to you about this and discuss with you whether you want to continue in the study. If you decide to withdraw, your doctor will make arrangements for your care to continue. If you decide to stay in the study you may be asked to sign an updated consent form.

Will taking part in this study affect my insurance?

If you have private medical insurance you should let the insurers know you intend to take part in a research project. They will be able to tell you if this will affect your medical insurance.

Who is organising the study?

The study is being organised by the Department of Oncology, University of Edinburgh, the Scottish National Blood Transfusion Service (SNBTS) Cell Therapeutics Group and the Department of Clinical and Laboratory Haematology Oncology, Western General Hospital, Edinburgh in collaboration with your local hospital.

Who has reviewed the study?

The study protocol has been reviewed by the Leukaemia Research Fund Clinical Trials Committee, by the Medicines Control Agency and by the Scottish Multicentre Research Ethics Committee and Local Research Ethics Committee.

Can I discuss the study with a Doctor who is not involved with running the study?

Dr *(name of local contact)* is a Doctor working within the Department of Haematology who is not directly involved in the running of the study but who is available to answer questions about the study if required.

How can I find out the results of the study?

Once the study is complete the results will be published in a medical journal. Your doctor will be able to tell you whether the new treatment works better than drugs alone and will let you have a copy of the published results if you wish to see them.

Contact for further information

(Insert name of local investigator)
(Insert contact details of local investigator)

You can contact your local clinical investigator or a member of his/her team at any time, the staff will be able to help you.

Thank you for taking the time and trouble to read this information sheet and consider taking part in the study, your co-operation is appreciated.

If you agree to take part, you should sign the standard consent form that is presented with this document. You will be given a copy of this information sheet and the signed consent form to keep.

TO BE KEPT BY THE PATIENT

APPENDIX II - PATIENT CONSENT FORM

Hospital Headed Paper

A STUDY OF USING CHEMOTHERAPY AND A VACCINE DEVELOPED FROM PATIENTS' OWN LEUKAEMIA CELLS TO TREAT ACUTE myeloid leukaemia

Please initial box

1. I confirm that I have read and understood the information sheet dated 16/7/01 (version Two) on the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and without my medical care or legal rights being affected. ☐
3. I understand that sections of my medical notes may be looked at by responsible staff hospital, study team or from regulatory authorities where it is relevant to my taking part in research.
   I give my permission for these individuals to have access to my records, and understand that any
   information in my records will be treated in strict confidence ☐
4. I agree to my GP being notified of my participation in this study ☐
5. I agree to take part in the above study ☐

_____ _____ _____
Name of Patient (Block Capitals) Signature Date

_____ _____ _____
Name of Doctor Signature
Date

Telephone number(s) where patient can be contacted:

Home: (_____) _____ Work: (_____)
STD Code STD Code

Mobile: _____

Copies to:

- patient
- investigator
- hospital notes

APPENDIX III - PATIENT CONSENT FORM – REINJECTION OF LEUKAEMIA CELLS

Hospital Headed Paper

A STUDY OF USING CHEMOTHERAPY AND A VACCINE DEVELOPED FROM PATIENTS' OWN LEUKAEMIA CELLS TO TREAT ACUTE myeloid leukaemia

Please initial box

1. I confirm that I am still content to participate in the above study and have had the opportunity to ask further questions. ☐
2. I consent to re-injection of my cells ☐

Name of Patient (Block Capitals)	Signature	Date

Name of Doctor	Signature	Date

Copies to:

- patient
- investigator
- hospital notes

APPENDIX IV - GP LETTER

Hospital Headed Paper

Dear Dr _____

RE: A PHASE I/II Study of vaccination with dendritic-like leukaemia cells generated from primary acute myeloid leukaemia blasts as immunotherapy for acute myeloid leukaemia

I am writing to inform you that the person named below, who is registered with you, has agreed to take part in this research project as a patient, and has consented to my contacting you.

Please keep this as a permanent record of their involvement. I will also inform you later if there are any abnormal findings or possible adverse events noted in the subject.

Name of subject _____

Date of birth _____

Date of consent _____

The study aims to examine the utility of autologous cultured dendritic-like leukaemia cells as adoptive immunotherapy in patients over the age of 60 with newly presenting, or over 50 with relapsed, acute myeloid leukaemia.

The patient's own leukaemia cells are cultured ex vivo in order to induce their differentiation into dendritic-like cells, which can then be used as autologous vaccines. Clinical studies using dendritic cells to treat a variety of malignant disease have not shown any serious adverse events.

Your patient will be asked to donate either a 100mL sample of blood or 5mL of bone marrow aspirate or have a leucapheresis procedure performed, depending on peripheral blood blast cell count. Leukaemia cells derived from this sample will be cultured in the laboratory to see if they undergo dendritic cell differentiation. If they do, the patient will be eligible for the vaccination part of the study. Patients will be treated with chemotherapy to induce remission using treatment regimens decided by the physician in overall charge of the patient's care. The patient will then be vaccinated with autologous dendritic-like leukaemia cells that have been prepared in a tissue culture environment dedicated to the use of human cells, using standard aseptic techniques. Infection of the product during the culture period is a potential risk, and sterility and close monitoring will be employed.

This is a phase I/II study designed to assess feasibility and tolerability of this treatment approach. If you would like to discuss the study with one of the principal investigators or the local investigator please contact them as detailed below:

Yours sincerely,

(Insert local investigator details)

APPENDIX V: Induction of dendritic cell differentiation in primary AML blasts derived from patients with Acute Myeloid leukaemia

Peripheral blood or bone marrow samples were obtained from 42 patients presenting with newly diagnosed or relapsed Acute Myeloid Leukaemia.
Leukaemia cells were cultured with GM-CSF (50ng/mL) and IL-4 (15ng/Ll) for 4 days followed by a further 3-7 days culture with GM-CSF (50ng/mL) and TNFα (15ng/mL) or CD40 ligand (1μg/mL). Evidence for dendritic cell differentiation was based on changes in morphology and flow cytometric analysis of costimulatory molecule and dendritic cell associated marker expression.
Of 42 patients in 4 cases poor viability over the culture period prevented further evaluation. 22 cases showed evidence for dendritic cell like differentiation based on their acquisition of characteristic dendritic cell morphology and expression of costimulatory molecules CD40, CD80, CD86 and the dendritic cell associated marker CD83. (Fig 1+2). In 19 patients the leukaemia cells appeared to be resistant to dendritic cell differentiation.

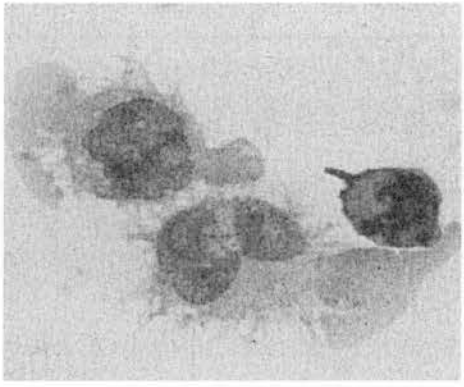

Figure 1.

Characteristic dendritic cell morphology following cytokine-induced differentiation in primary leukaemia cells

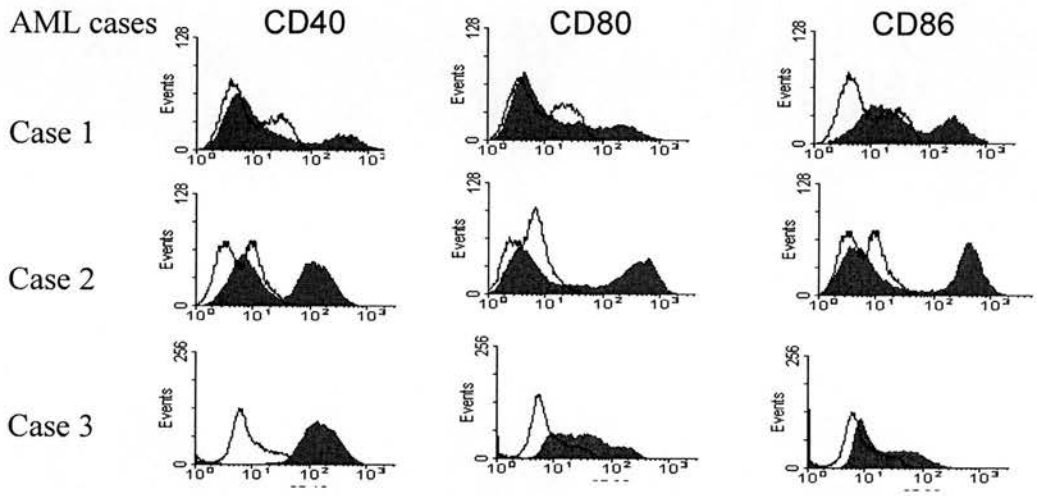

Figure 2. Expression of costimulatory molecules following dendritic cell differentiation on leukaemia cells derived from three individual AML cases

APPENDIX VI: Functional analysis of dendritic-like leukaemia cells

Dendritic-like leukaemia cells derived from primary AML blasts by cytokine induced differentiation were tested for their ability to stimulate allogeneic T lymphocytes in mixed leukaemia lymphocyte reactions.

Mitomycin-C treated dendritic-like leukaemia cells were cultured in wells of a 96 well plate at various responder:stimulator ratios with lymphocytes derived from allogeneic normal volunteer donors. As controls, freshly thawed leukaemia cells, derived from the same patient as the dendritic cell like leukaemia cells, were used as stimulators in the same way. After 4 days culture 1μCi of thymidine was added to each well and the cells were cultured for a further 18 hours. Activity per well as counts per minute was measured on a β scintillation counter.

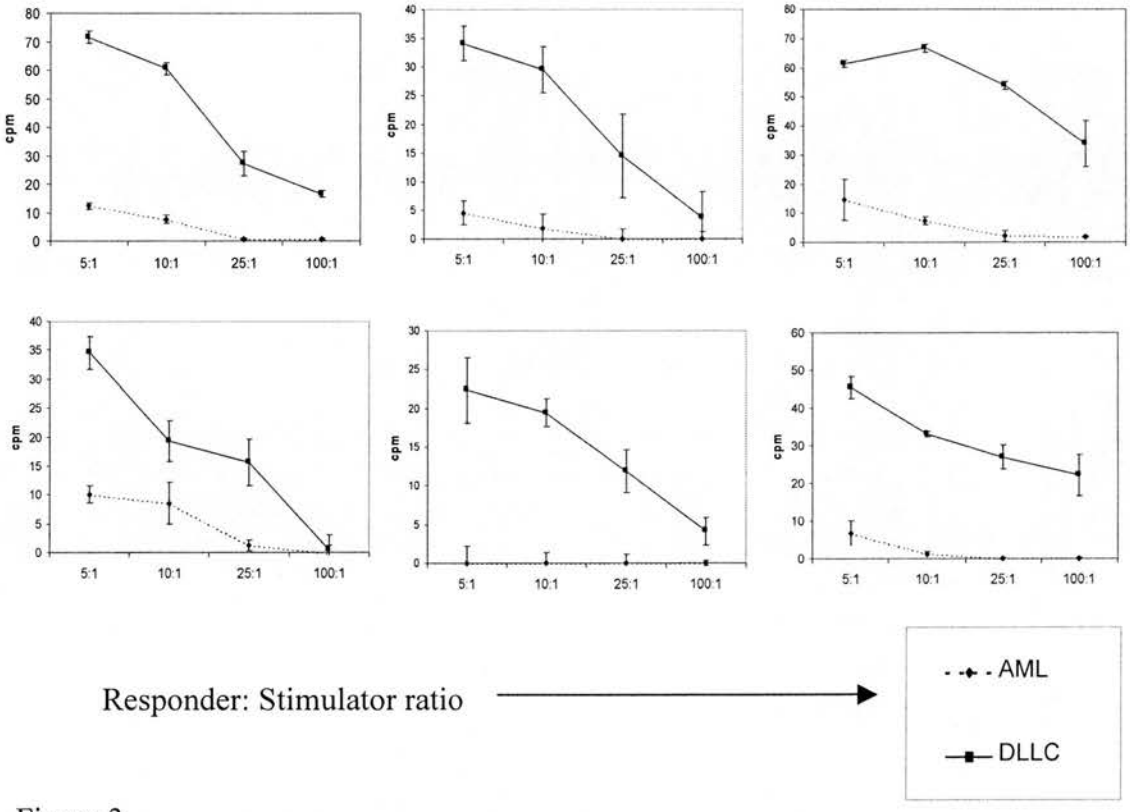

Figure 3.
Allogeneic mixed leukaemia lymphocyte reactions for 6 AML cases in which the leukaemia cells underwent dendritic cell differentiation. Responders: allogeneic peripheral blood lymphocytes. Stimulators: leukemic cells either unmodified (AML) or following DLLC differentiation (DLLC) derived from the same patient.

Conclusions

The data from the allogeneic mixed leukaemia lymphocyte reactions demonstrates that, in contrast to unmodified leukaemia cells, dendritic-like leukaemia cells are potent stimulators of allogeneic lymphocytes.

Dendritic-like leukaemia cells can induce the development of autologous cytotoxic T lymphocytes that can recognise and destroy unmodified leukaemia cell targets

Co-culture of mitomycin-C treated dendritic-like leukaemia cells with autologous T lymphocytes could induce the development of cytotoxic T lymphocytes that were capable of destroying freshly thawed leukaemia cells in a LDH release cytotoxicity assay.

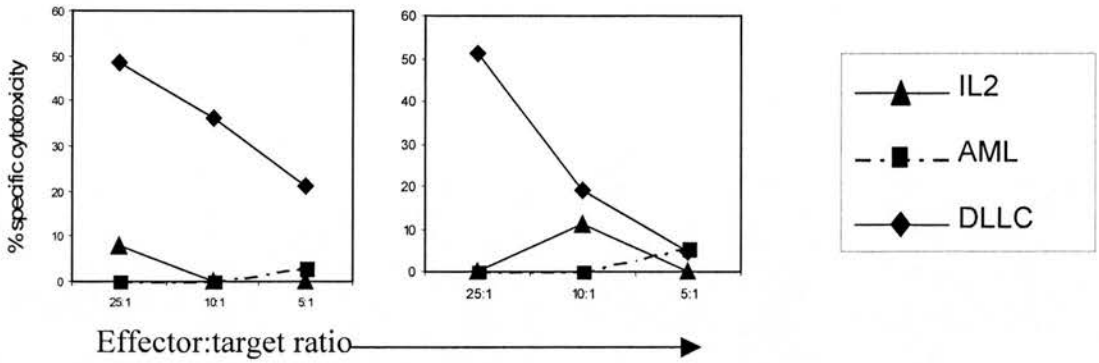

Figure 4.
LDH release cytotoxicity assay for 2 AML cases. Autologous T lymphocytes co-cultured for 3 days with dendritic-like leukaemia cells are capable of destroying unmodified leukaemia cells targets. Controls are T cells that have been cultured either with IL2 alone or with unmodified autologous leukaemia cells.

Conclusions

Dendritic-like leukaemia cells have the ability to prime autologous T lymphocytes such that they are able to specifically recognise and destroy unmodified leukaemia cell targets.

APPENDIX VII: Tissue Bank Standard Operating Procedures

SOP Document 1
SCOTTISH NATIONAL BLOOD TRANSFUSION SERVICE
SOUTH EAST REGIONAL CENTRE & DEPARTMENT OF TRANSFUSION MEDICINE

SOP NoTSD 256 01

Tissue Service
Master Document
Preparation of Autologous Dendritic-like Leukaemia Cells for Infusion into Acute Myeloid Leukaemia Patients

1. OBJECTIVE

To generate dendritic-like leukaemia cells from acute myeloid leukaemia blasts.

2. EQUIPMENT

Microbiological Safety Cabinet (MSC)
CO_2 incubator (LEEC) at 37°C
Inverted microscope (Leitz)
Benchtop centrifuge, Model BR401 (Denley)
Neubauer haemocytometer and coverslip
Automatic pipetter
Mr Frosty cell freezer (Nalgene)
A range of Gilson pipettes
A range of pipettes
A shaking table (Denley)
Gammacell 3000 Elan Irradiator with double caesium –137 source

3. MATERIALS

RPMI 1640 without phenol red (Sigma and PFC)
L-glutamine, 200mM , (Sigma)
Sterile sachets containing Tryptic Soy Broth (TSB) and Thioglycollate (THIO) universals (Cherwell)
Nigrosin (5% w/v) (NRU)
Sterile AB serum (SEBTS)
15% (w/v) di-potassium EDTA (BDH)
IL-4, 5υg vial, (R&D Systems), catalogue no 204-IL/CF
Saline for injection (Baxter)
GM-CSF, 400υg vial, (Sandoz)
Lymphoprep (Nycomed), 300ml vial
TNF-α (R&D systems)
IFN-γ 0.5 ml vial (Boerhinger Ingelheim)

Poly:IC (Sigma)
DMSO, sterile filtered and tissue culture tested (Sigma)
Settle Plates (Cherwell)

4. DISPOSABLES

Accuspin tubes, c/n A2055, (Sigma)
Selection of syringes and needles
50ml sterile universals
15ml sterile centrifuge tubes
sleeve of 25cm^2 flasks with filter cap (Costar)
sleeve of 25cm^2 flasks, non TC treated (Iwaki)
sleeve of 75cm^2 flasks with filter cap (Costar)
selection of single wrap pipettes and pastettes
2ml sterile Cryovials

white and yellow Gilson tips (autoclaved in-house)
Sterets
Sterile drapes
Sterile syringe caps (Avon)
selection of Sterile surgeons' gloves
Sterile disposable scalpel blades

5. PROCEDURE

5.1 ***PREPARATION OF REAGENTS***

5.1.1. **RPMI-1640 + 5% AB serum**

5.1.1.1 This medium bought from Sigma (500ml bottle) is used as diluent for the stock cytokine solutions and for the growth medium base.

5.1.1.2 Before use, 25ml of AB serum and 5ml of glutamine are added to each bottle in the MSC using 5 ml disposable pipettes.

5.1.1.3 This medium once made up is used in the following steps and referred to as complete RPMI.

5.1.1.4. *Where **RPMI** is referred to, then PFC RPMI 1640 without phenol red or ANY OTHER ADDITIONS is to be used.*

GM-CSF is prepared as stock and kept frozen (-70°C) until required for use. IL-4 is reconstituted when required

5.1.2. **IL-4**

5.1.2.1 IL4, supplied by R&D Systems, is presented as sterile 5υg vial.

5.1.2.2 A stock solution must be made at 500ng/ml.

5.1.2.3 The solution is prepared within the MSC in the General Laboratory.

5.1.2.4 1ml of sterile complete RPMI is added using Gilson and sterile tip to the vial to dissolve the powder.

5.1.2.5 This solution is removed and transferred to a universal container. Steps 5.1.2.4 and .5 are then repeated until the IL4 vial has been rinsed out with a total of 5ml complete RPMI.

5.1.2.6 A further 5ml complete RPMI is added to the universal container, and the contents mixed by gentle swirling.

5.1.2.7 2 x 250μl aliquots are removed into TSB and Thioglycollate for sterility testing.

5.1.2.8 The remainder is used immediately and any small residue discarded for R&D use (dispensed as 1ml aliquots into 2ml sterile cryovials)

5.1.2.9 The vial must be labelled with the following:

- name of solution
- concentration
- date prepared
- date of expiry (one year from date of formulation)
- batch number

The stock solutions must be stored at -70°C prior to use.

5.1.2.10 Batch formulation record must be completed.

5.1.3 **GM-CSF**

5.1.3.1 GM-CSF (Leucomax), supplied by Sandoz, is presented as a vial containing 400μg powder.

5.1.3.2 A stock solution must be made at 10,000ng/ml.

5.1.3.3 The solution is prepared within the MSC in the General Laboratory.

5.1.3.4 1ml of sterile complete RPMI is added to the vial to dissolve the powder.

5.1.3.5 The solution is removed and a further 39ml of complete RPMI is added to a universal container.

5.1.3.6 2 x 1ml aliquots are removed into TSB and THIO for sterility testing

5.1.3.7 1ml aliquots are then dispensed into 2ml sterile cryovials.

5.1.3.8 The vial must be labelled with the following:

- name of solution
- concentration
- date prepared

date of expiry (one year from date of formulation)
batch number

5.1.3.9 The tubes are stored at -70°C until required.

5.1.3.10 Batch formulation record must be completed.

5.1.4 **TNF-α**

5.1.4.1 Add the appropriate volume of RPMI to the vial to give a working solution of 15μg/ml

5.1.4.2 Process as in 5.1.2.7 – 5.1.2.10.

5.1.5 **IFN-γ**

5.1.5.1 Add the appropriate volume of RPMI to the vial to give a working solution of 200μg/ml

5.1.5.2 Process as in 5.1.2.7 – 5.1.2.10.

5.1.6 **Poly I:C**

5.1.6.1 Add the appropriate volume of RPMI to give a working concentration of 1mg.ml

5.1.6.2 Process as in 5.1.2.7 – 5.1.2.10.

5.1.7 ***Growth Medium***

5.1.7.1 Calculate the volume required.

5.1.7.2 The basal medium used is complete RPMI prepared as in 5.1.1.

For each 10ml required take:

300μl IL-4 stock solution to give 15ng/ml final concentration
50μl GM-CSF stock solution to give 50ng/ml final concentration
10μl TNFα stock solution to give 15ng/ml final concentration

5.1.7.3. The vials of cytokines should be thawed immediately prior to use, and any unused reagents discarded, not refrozen.

5.2 ***REFERRALS AND VIROLOGY TESTING***

5.2.1 Blood samples will be withdrawn from the patient no more than seven days prior to the planned date of harvest.
Referring Consultant must complete a Donor Referral Form (see Appendix 2). which must accompany the blood samples to the relevant centre for the testing of mandatory virology markers.
Testing for mandatory markers will be repeated when the donation for dendritic cell preparation is withdrawn.

5.2.2 The blood will be processed as per SOP No TSD 22401 and a unique tissue number will be attached to the referral form.

5.3 ***Preparation of the Leukaemia cell harvest***

5.3.1 Leukaemia cell harvest will take the form of either an apheresis pack, or 100ml of peripheral blood or 10ml of bone marrow and clearly labelled with the patients name, donation number, date of birth, date and total volume withdrawn.

5.3.2 The donation will be collected by a member of staff and taken to Tissue Services.

5.3.3 On arrival the TS must verify the identity of the donor and enter the time of arrival in the Tissue Processing Log (see Appendix 3).

5.3.4 The donation must be sprayed with 70% (v/v) alcohol before placing it in the transfer hatch to the general laboratory.

5.3.5 Either the virology testing laboratory, or components production area should be telephoned to obtain the virology screening results on the patient's donation, and the results entered in the patient's records (Appendix 3)

5.4 ***MANIPULATION IN THE GENERAL LABORATORY***

5.4.1 Using a sterile medium sized drape, prepare a sterile field within the MSC.

5.4.2 Using a syringe and needle add 15ml of Lymphoprep to the Accuspin tubes. (the number required depends on the sample taken) .Ensure that the Lymphoprep is equilibrated to ambient temperature. When using an apheresis pack use a sterile plunger to depress the sinter in the Accuspin tube to the tube shoulder before adding lymphoprep.

5.4.3 Spin at 450 x g for 30 seconds to position the Lymphoprep at the base of the centrifuge tube.

5.4.4 Swab one access port of the donation several times using fresh sterets.

5.4.5 If the donation is in a blood pack use a sterile scalpel blade and with very great care, cut the tail and dispense the donation equally

between the four tubes. Make up each tube up to 50ml with RPMI if required (*which has not had serum or glutamine added).*

5.4.6 Retain 2 x 1ml aliquots for sterility testing and place in TSB and Thio respectively.

5.4.7 Centrifuge the Accuspin for 30 minutes at 450 x g with thermostat set to control the temperature at 18°C.

5.4.8 Remove 2 x 1ml aliquots of supernatant for sterility testing and place in TSB and Thio respectively.

5.4.9 Using the automatic pipettor remove supernatant to within 0.5cm of the leukocyte layer and discard the supernatant.

5.4.10 Using a plastic pastette loosen any cells adherent to the walls of the tubes.

5.4.11 Pour the leukocyte suspension from the Accuspin tubes into 2 x 50ml centrifuge tubes.

5.4.12 Make up to 50ml per tube with RPMI.

5.4.13 Wash by centrifugation for 5 minutes at 160 x g and remove supernatant to waste.

5.4.14 Retain 2 x 1ml aliquots for sterility testing and place in TSB and Thio respectively. If there appears to be a white platelet layer on the pellet, resuspend in RPMI and centrifuge as before.

5.4.15 Re-suspend in 10ml volume complete RPMI per tube.

5.4.16 Disaggregate the pellet by pipetting up and down as 5.4.14 and transfer the contents to one tube.

5.4.17 Perform a viable cell count:

Make a 1 in 20 dilution of the cell suspension by removing 50μl and adding it to 950μl RPMI in a small tube. Add one volume (eg.100μl) of this to the same volume of Nigrosin stain in a fresh tube (this need not be done under sterile conditions). Mix and count this suspension on a Neubauer Haemocytometer.
Count the number of lymphocytes in the 25 small squares in the centre of the counting chamber. The cell count is calculated as follows:

Cells per ml = Number of cells in 25 squares x 10^4 x dilution factor 20 x 2 (addition of nigrosin and record in the patient notes –appendix 3)

The mononuclear cells will then be cryopreserved (see section 5.6)

When the dendritic-like leukaemia cells are required the cryopreserved cells will be thawed and a viable cell count performed

5.4.18 From the cell count the number of flasks required must be calculated. A total of 180 x 10^6 cells are required to seed a 75cm^2 flask, 360 x 10^6 cells for a 175cm^2 flask and 60 x 10^6 cells for a 25cm^2 flask. 1 x 25cm^2 is required for FACS analysis.

5.4.19 Dispense the appropriate volume of cells into the flasks and label the flasks with the unique number, date and patient's name.
The leukaemia cells will be cultured with 1000ng/ml GM-CSF and 20ng/ml IL-4. After 4 days the TNFα will be added at 25ng/ml. The cells will then be cultured for a further 3 days. IFN-γ (30ng/ml) and polyI;C (12.5μg/ml) will be added 24 hours prior to the end of culture. At the end of the culture period the cells will be cryopreserved.

Refer to SOP NO TSD 236 01

5.4.20 Observe the cells on a daily basis and note their integrity. Also, note any signs of contamination. Note any observations in appendix 4.

5.4.21 Record batch numbers of all reagent used on appendix 3.

5.5 ***HARVESTING AND PREPARING THE CELLS FOR INJECTION AND CRYOPRESERVATION***

Each patient will received four escalating dose injections at weekly intervals. The doses will be 0.125x10^6/kg, 0.25x10^6/kg, 0.5x10^6/kg and 1x10^6/kg.

5.5.1 Check that the individual flasks show no signs of overt infection. The 25cm^2 flask is transported to John Hughes Bennett.Laboratory, Western General Hospital. This flask will be evaluated using the FACS machine to establish the percentage dual positive CD40/CD83 cells present in the population. The WGH staff will phone the Tissue Bank with the results which must be entered in the Tissue Processing Log (appendix 5).

5.5.2 Using a sterile pipette, sample and count a 50μl volume of cell suspension from each 75cm^2 flask. Calculate the number of viable cells per ml of culture as in 5.4.18. (record on appendix 4).

5.5.3 The cell number for vaccination is to be based on total nos. of cells NOT on CD40/83 percentages.

5.5.4 Remove from one flask the volume containing the required number of cells and transfer to one or more sterile centrfuge tubes as appropriate.

5.5.5 Centrifuge as 5.4.13. Sample 2x 1ml of the supernatant for bacteriology testing. Remove and keep aside at least 5ml of the supernatant. Decant the rest of the supernatant to waste.

5.5.6 Place the tube in the clean room hatch.

5.5.7 Take samples of 2nd wash supernatant for bacteriology and 1ml for IL-4 determination and remove the remainder of the supernatant.

5.5.8 Disaggregate the cell pellet by gently flicking the base of the universal.

5.5.9 Re-suspend the cells in 0.5ml saline, draw the cell suspension into a 2ml syringe securing the port with a syringe cap. Record the batch number of saline on appendix 5.

5.5.10 Place in the syringe transport container.

5.5.11 Stick a pre-prepared label onto the syringe container and arrange delivery to the clinic.

5.5.12 The label must contain the following information:
Patient name and date of birth.
Unique Tissue Number bar-code
Total number of cells for injection.
Date of Preparation and the dosing regimen.

Prior to vaccination cells need to be irradiated to minimum 25Gy using a local MCA accredited irradiation source. Refer to SOP No 99 327 013.

5.6 ***CRYOPRESERVING THE CELLS***

5.6.1 Calculate and then dispense the appropriate volumes of cells to give the cell numbers required into universals and label with appropriate cell number. Pre-chill the required volume of cryoprotectant medium.

5.6.2 Centrifuge the cells as 5.4.13.

5.6.3 Decant the supernatant to waste.

5.6.4 Disaggregate the cell pellet by gently flicking the base of the universal.

5.6.5 Add 0.5ml AB serum. Leave the resuspended cells on ice for 10-15 minutes.

5.6.6 Add 0.5ml cryoprotectant medium (90% AB serum, 10% DMSO), dropwise with gentle mixing.

5.6.7 Place in a cryovial previously labelled with
Patient name and date of birth.

Unique Tissue Number bar-code
Total number of cells for injection.
Date of Preparation and the dosing regimen.

5.6.8 Place the ampoules in the Mr Frosty cell freezer and store the -80°C freezer for at least 4 hours before moving the vials to the liquid nitrogen vapour phase of the 'Penguin' tank for long term storage.

5.6.9 Record the location in the inventory log and in processing record(appendix 5).

5.7 ***PREPARING THE CRYOPRESERVED CELLS FOR INJECTION***

5.7.1 Prepare 10ml complete RPMI, adding sufficient AB serum to make the concentration of serum to 10%.

5.7.2 Remove the appropriate ampoules from the nitrogen tank.

5.7.3 Allow the vial(s) of frozen cells to thaw by placing it/them in a tray of warm sterile water

5.7.4 Immediately after the vial(s) have thawed, transfer the contents to a 15ml centrifuge tube using a sterile pastette. Rinse out the vial(s) with 1.5ml of 10%AB serum in RPMI, so that the entire contents of the vial(s) have been transferred to the centrifuge tube. Take 100μl sample for sterility testing

5.7.5 Add the remainder of the RPMI with 10% AB serum to the tube, very gradually at first, using a pastette, and gently shaking the tube during addition of medium.

5.7.6 Centrifuge the tube as in 5.4.13. Sample 2 x 1ml of the supernatant for bacteriology testing and remove the remaining supernatant to waste.

5.7.7 Process as in 5.5.6 to 5.5.12 omitting 5.5.7.

5.7.8 Record batch numbers of reagents, cell numbers etc on appendix 6

SOP Document 2

TISSUE SERVICE
21 Ellen's Glen Road, Edinburgh EH17 7QT
0131 536 5751/6

Leukaemia cell harvest
Referral Form

Patient Details

Tissue No	Blood No

Surname: _____ Forename: _____

DOB: _____

Address: _____ GP: _____

_____ _____

Diagnosis: _____

Body weight: _____ kg

Referring Consultant: _____ Hospital:

_____

Comments: _____

_____

_____

SOP Document 3

Appendix 2

TISSUE SERVICE
SOUTH EAST SCOTLAND
Telephone No 0131-536 5751 Fax No: 0131-536-

Dendritic Cell Donor
Referral Form

Patient Details

Tissue No	Blood No

Surname_____ Forname_____

Address_____

_____

Diagnosis_____

Body weight: _____ kg

Referring Consultant_____ Hospital:_____

Comments_____

_____

Tissue Details

Volume of Apheresis/Whole Blood/Bone Marrow withdrawn: _____ ml

Date and Time of Retrieval: _____

Packaged :_____ Designation:_____

_____

Tissue Despatch

Transport by_____ Time Dispatched:_____

Received at Tissue Services by:_____ Time:_____

SOP Document 4

TISSUE SERVICE

21 Ellen's Glen Road, Edinburgh EH17 7QT
0131 536 5751/6

Dendritic Cell Culture History

Donor Unique Number:

Donor Name:

Tissue Details

Volume of Leukaemia cell harvest Withdrawn:_____ml
Source of leukaemia cells: Peripheral blood/apheresis/bone marrow
Date and Time of Retrieval:

Tissue Despatch

Transport by:_____ Time Dispatched:_____

Received at Tissue Services by:_____Time:_____

Confirm Donor's Identity: Sign _____ Date: _____

Enter Buffy Coat unit no here:

Phone Virology to confirm negative results on buffy coat/apheresis unit virology

Confirmed verbally: Y/N by _____ TS staff Sign here: _____

Time and Date Processed:_____

Volume of Buffy Coat or Apheresis Pack Received: ___ ml Cabinet Safe/Unsafe: Sign: ___

Batch Record of Reagents & Equipment Used:-

Reagent	*Batch No*	*Expiry Date*
RPMI 1640 (PFC)		
L-glutamine		
RPMI 1640 (sigma)		
IL-4		
GM-CSF		
TNF-α		
PolyI;C		
IFN-γ		
Lymphoprep		

EDTA 15%		
Accuspin tubes		
AB serum		
Theatre gown		
Laboratory gown		

Operator	
Assistant	

Cell Count

Perform Calculations here:-
Vol
Dilution:

Total Cell Count
$25cm^2$ flasks n=
$75cm^2$ flasks n= ____

Growth medium added: check here ☐ _____ Sign

Growth Phase

Observation Day 2 Date: _____ Initials: _____

Observation Day 3 Date: _____ Initials: _____

Observation Day 4 Date: _____ Initials: _____

Observation Day 5 Date: _____ Initials: _____

Observation Day 6 Date: _____ Initials: _____

Observation Day 7 Date: _____ Initials: _____

Cell Harvest and Cryopreservation

SOP Document 5

Cabinet Safe/Unsafe: Sign: _____

Batch Record of Reagents & Equipment Used:-

Reagent	*Batch No*	*Expiry Date*
RPMI 1640 (PFC)		
L-glutamine		
RPMI 1640 (sigma)		
IL-4		
GM-CSF		
TNF-α		
PolyI;C		
IFN-γ		
Saline		
DMSO		
AB serum		
Theatre gown		
Laboratory gown		

Operator	
Assistant	

Date: _____
Check the flasks to ensure there are no signs of overt infection:
Comments:

_____

_____

Send 25cm^2 flask to John Hughes Bennett Laboratories, WGH. Time and Sign:

Results:

% CD40/83+ cells	
FACS Analysis Operator	

Cell Count on Pooled Cells
Volume= Counting dilution =

Cell Count = /ml culture

CDla Count = %

Number Cells Required		Volume of culture to be removed	Number of cells
Dose 1	0.125x10^6/kg		
Dose 2	0.25 x 10^6/kg		
Dose 3	0.5 x 10^6/kg		
Dose 4	1 x 10^6/kg		

Sign _____ Date _____

Location of Cells

	Tank	Location	Sign	Removal	Date/Time
Week 2					
Week 3					
Week 4					

SOP Document 6

Recovery of Cells for Giveback

Dose No:-

Patient Name:-

Tissue No : Date :

Batch Record of Reagents & Equipment Used:-

Reagent	*Batch No*	*Expiry Date*
RPMI 1640 (PFC)		
L-glutamine		
RPMI 1640 (sigma)		
IL-4		
GM-CSF		
TNF-α		
PolyI;C		
IFN-γ		
Saline		
AB serum		
Theatre gown		
Laboratory gown		

Operator	
Assistant	

Count on Recovered Cells

Counting dilution:

% Recovery/Viability:

Actual dose given:

Sign _____ Date _____

APPENDIX VIII Validation of irradiation as a means of removing the engraftment potential of primary AML blasts

Principle

To establish that γ irradiation of primary leukaemia cells will remove any potential for leukaemia re-engraftment following vaccination of patients with autologous dendritic-like leukaemia cells

1.1. Method

Cryopreserved leukaemia cells derived from 10 patients with AML were thawed rapidly. Following washing with complete medium a viable cell count was performed.
For each patient $5x10^7$-10^8 leukaemia cells were resuspended in 1 mL phosphate buffered saline (PBS) and then added to 2mL sterile syringes. The remaining cells were resuspended in PBS and incubated at 37°C.
The 10 syringes were then irradiated to 25Gy using a Caesium 137 source.
A viable cell count was then performed for both the irradiated and non-irradiated leukaemia cells. The cell density was adjusted to $2x10^6$/mL in PBS
250μL of the cell suspension was added to 2.5mL of Methocult GF H4535 (Stemcell Technologies) in 15mL conical tubes and mixed by vortexing. The cell suspension was allowed to stand for 10 minutes and then 1mL was added in duplicate for each patient, both irradiated and non-irradiated leukaemia cells, to the wells of a 4-well plate.
The plates were then incubated for 14 days at 37°C, 5%CO_2 in a humidified atmosphere.
The number of colonies per well was counted and the mean value for the two wells recorded

1.2. Results

The mean colony number for the 10 individual samples of non-irradiated leukaemia cells was 46 (range 3-114). Irradiation of leukaemia cells completely abolished their clonogenic potential with no colonies seen in any wells that had been seeded with leukaemia cells that had received 25Gy irradiation.

APPENDIX IX: Data on adverse events from the Phase I clinical trial of MUC-1 pulsed dendritic cells as immunotherapy for patients with metastatic carcinoma

Patient no.	CTC grade 1	Duration	CTC grade 2	Duration
1	Fatigue Runny nose	3 days 1 day		
2				
3	Fever Pain at injection site Chills Fatigue	Recorded once 4 days 1 day 28 days	Hb 98 Myalgia Fatigue	3 months 2 days 2 days
4	Weight loss Hb 105	 14 days		
5	Fatigue	7 days		
6	Fatigue	14 days		
7	Fatigue	3 months	Fatigue	14 days
8				
9	? fever	1 night		
10	Dry throat	24 hours		
11				
12				

N.B. There were no grade 3 or 4 toxicities noted

APPENDIX X: Monitoring of immune function in patients with AML

Patients fulfilling the entry criteria for the study will be pre-screened to ensure that it is possible to generate cytokine differentiated DLLC from their peripheral blood.

The phenotype of undifferentiated AML cells and cytokine differentiated AML cells will be assessed by flow cytometry using combinations of fluorochrome conjugated antibodies to CD1a, CD14, CD80, CD86, CD83, CD40, CD33, CD54 and CD95. IL-12 (p70) production by DLLC in response to various maturational agents will be measured by ELISA.

Patients will donate 50mL peripheral blood at a number of time points through out the study: at the time of the leukaemia cell harvest for the pre-screen, following completion of chemotherapy (i.e. shortly before vaccination with autologous DLLC) and at 2, 4, 6 and 12 weeks following vaccination, according to the Trial Schedule (Appendix XII). Patients remaining in remission at 12 weeks post vaccination will be monitored at 12 weekly intervals thereafter until relapse. This will allow for investigation of patients' immune responses in general and specifically to AML cells before and after receiving immunotherapy.

Blood samples will be diluted 1:1 with RPMI culture medium before separation of PBMC on Ficoll/Hypaque. Following centrifugation plasma will be collected and stored for analysis for immunosuppressive factors. Recovered PBMC will be washed twice, where necessary separated from AML cells and counted before use. The phenotype of isolated AML cells will be assessed by flow cytometry.

The phenotype and activation state of peripheral blood mononuclear cells (PBMC), freshly isolated and following 7 days culture in the presence of culture medium (CM, control), ConA and PHA (mitogen), autologous AML cells, autologous cytokine differentiated AML cells and allogeneic cytokine differentiated AML cells will be measured by flow cytometric analysis (FACS).

Cells will be stained with combinations of antibodies to CD19 (B lymphocytes), CD3 (total T lymphocytes), CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes) and the activation markers CD25 (IL-2 receptor), CD45R0 (activation memory marker), CD95L, HLA Class I and HLA-DR (MHC class II). Samples will be analysed using a Becton Dickinson FACSCaliber.

The proliferative responses of patients' T cells to mitogens, autologous undifferentiated leukaemic cells and autologous DLLC will be assessed by performing standard tritiated thymidine uptake assays.

To assess the generation of cytotoxic T lymphocytes PBMC will be cultured for 5 days with culture medium alone, or medium supplemented with cytokines IL-2 and IL-7, autologous DLLC plus IL-2 and IL-7 or allogeneic cytokine differentiated AML cells plus IL-2 and IL-7. Cultured cells (effector cells) will be harvested and titrated at various effector cell to target cell (AML cells) ratios, treated to remove dead cells prior to use. Their ability to kill autologous AML cells will be measured after 3 hours incubation using the LDH assay. In addition an assay of T cell interferon gamma production will be used.

SUMMARY OF IMMUNE FUNCTION MONITORING

Immunophenotypic analysis of AML and DLLC

AML	Cell surface molecules	CD1a, CD14, CD33, CD40, CD54, CD80, CD83, CD86, CD95, HLA Class I and II
	Cytokine secretion	IL-12 (p70), IL-10, VEGF, TGFβ
DLLC	Cell surface molecules	CD1a, CD14, CD33, CD40, CD54, CD80, CD83, CD86, CD95, HLA Class I and II
	Cytokine secretion	IL-12 (p70), IL-10, VEGF, TGFβ

Immunophenotypic analysis of lymphocyte numbers and activation status of T cells

T cells	Numbers and proportions	CD3 positive, CD4:CD8 ratio
	Activation status	CD25, CD45RO
	Response to mitogens	CD25, CD45RO
	Others	CD95, HLA Class I and II
B cells	Numbers	CD19 positive

Proliferation assays

PBMNCs used as a source of autologous T cells

Mitogens	ConA, PHA	Proliferation measured by 3HTdR uptake
MLLR	autologous AML	Proliferation measured by 3HTdR uptake
	autologous DLLC	

Cytotoxicity assays

PBMNCs used as a source of autologous T cells

Co-culture	autologous AML	CTL generation measured by Cr 51-release or
	autologous DLLC	ELISPOT assay

Abbreviations: AML=freshly thawed unmodified leukaemia cell
DLLC=leukaemia cells following cytokine induced differentiation
PBMNCs=peripheral blood mononuclear cells

APPENDIX XI: Monitoring of Minimal Residual Disease (MRD)

Disappearance or reduction in the level of MRD in AML patients undergoing vaccination with dendritic cells may provide evidence of an anti-leukaemic activity. There is increasing evidence that during clinical remission, high or increasing levels of MRD, as measured by quantitation of specific fusion transcripts, are predictive of impending relapse. Conversely absent or very low levels of MRD correlate with durable remission. Monitoring of MRD will be carried out using real-time quantitative RT-PCR. This sensitive technique will allow the accurate quantitation of low levels of MRD in over 80% of AML patients. The bone marrow karyotypes of patients in the pilot study will be known at presentation. Patients with t(8;21) and inv 16 will be monitored by quantitation of AML 1-MTG8 and CBFB-MYH 11 transcripts respectively. However the majority of AML patients will not have specific chromosomal translocations and therefore the WT1 gene, which is over expressed in 80% of AML patients, will be used as a marker of MRD. The WT1 gene is being increasingly found to be a useful predictor of clinical relapse and survival.

In this study all patients (*de novo* or at relapse) will have their DNA stored at the time of screening for their suitability to enter the study. Those who become eligible for the vaccination programme will have the WT1 gene expression assessed and/or checked for the presence of specific fusion transcripts e.g. AML 1-MTG8 and CBFB/MYH 11. Levels of the appropriate transcripts in both peripheral blood and bone marrow will be quantitated using the Taqman machine (real-time RT-PCR) at specific time points (pre-vaccination, day +14, day +28, day +84), as shown on the trial schedule and subsequently at 3 monthly intervals during the first year and at 4 monthly intervals during the second year of remission. The MRD work will be carried out in the Department of Haematology at the Manchester Royal Infirmary.

Appendix XII: Trial Schedule

Time in days in relation to first DLLC vaccination										
	At study entry	Pre Vacc	D+0 **DLLC injection #1**	D+7 **DLLC injection #2**	D+14 **DLLC injection #3**	D+21 **DLLC injection #4**	D+ 28	D+ 42	D+ 84	Notes
History	X									
Examination	X	X	X	X	X	X	X	X	X	
Observation			X	X	X	X				
Weight	X	X								
Height	X									
Karnofsky score	X	X	X	X	X	X	X	X	X	
Adverse events			X	X	X	X	X	X	X	C.T.C grading
HLA type	X									
Virology/VDRL	X									HIV/HBV/HCV/VDRL
ECG	X	X								
Urinalysis	X	X								
Drugs in the last 7 days	X	X	X	X	X	X	X	X	X	Patient diary
BM aspirate	X	X			X		X		X	Then at 3 monthly intervals
FBC	X	X	X	X	X	X	X	X	X	
Flow cytometry	X									T&B lymphocytes, CD4:CD8
U&Es/LFTs	X	X	X	X	X	X	X	X	X	
Immunoglobulins	X	X	X				X		X	
Complement	X	X	X				X		X	
CRP	X	X	X				X	X	X	
ANF	X	X					X		X	
Skin testing		X					X			10^6 γ-irradiated autologous leukaemia cells
Immune function monitoring		X			X		X	X	X	50mL peripheral blood
MRD monitoring		X			X		X		X	3 monthly after D+84
Injection site			X	X	X	X	X			

APPENDIX XIII: DECLARATION OF HELSINKI

Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly Helsinki, Finland, June 1964 and amended by the 29th WMA General Assembly, Tokyo, Japan, October 1975 35th WMA General Assembly, Venice, Italy, October 1983 41st WMA General Assembly, Hong Kong, September 1989 48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996 and the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000

A. INTRODUCTION

1. The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. Medical research involving human subjects includes research on identifiable human material or identifiable data.

2. It is the duty of the physician to promote and safeguard the health of the people. The physician's knowledge and conscience are dedicated to the fulfillment of this duty.

3. The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."

4. Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects.

5. In medical research on human subjects, considerations related to the well-being of the human subject should take precedence over the interests of science and society.

6. The primary purpose of medical research involving human subjects is to improve prophylactic, diagnostic and therapeutic procedures and the understanding of the aetiology and pathogenesis of disease. Even the best proven prophylactic, diagnostic, and therapeutic methods must continuously be challenged through research for their effectiveness, efficiency, accessibility and quality.

7. In current medical practice and in medical research, most prophylactic, diagnostic and therapeutic procedures involve risks and burdens.

8. Medical research is subject to ethical standards that promote respect for all human beings and protect their health and rights. Some research populations are vulnerable and need special protection. The particular needs of the economically and medically disadvantaged must be recognized. Special attention is also required for those who cannot give or refuse consent for themselves, for those who may be subject to giving consent under duress, for those who will not benefit personally from the research and for those for whom the research is combined with care.

9. Research Investigators should be aware of the ethical, legal and regulatory requirements for research on human subjects in their own countries as well as applicable international requirements. No national ethical, legal or regulatory requirement should be allowed to reduce or eliminate any of the protections for human subjects set forth in this Declaration.

B. BASIC PRINCIPLES FOR ALL MEDICAL RESEARCH

10. It is the duty of the physician in medical research to protect the life, health, privacy, and dignity of the human subject.

11. Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and on adequate laboratory and, where appropriate, animal experimentation.

12. Appropriate caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.

13. The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol. This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be in conformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.

14. The research protocol should always contain a statement of the ethical considerations involved and should indicate that there is compliance with the principles enunciated in this Declaration.

15. Medical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given consent.

16. Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others. This does not preclude the participation of healthy volunteers in medical research. The design of all studies should be publicly available.

17. Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits or if there is conclusive proof of positive and beneficial results.

18. Medical research involving human subjects should only be conducted if the importance of the objective outweighs the inherent risks and burdens to the subject. This is especially important when the human subjects are healthy volunteers.

19. Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.

20. The subjects must be volunteers and informed participants in the research project.

21. The right of research subjects to safeguard their integrity must always be respected. Every precaution should be taken to respect the privacy of the subject, the confidentiality of the patient's information and to minimize the impact of the study on the subject's physical and mental integrity and on the personality of the subject.

22. In any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely-given informed consent, preferably in writing. If the consent cannot be obtained in writing, the non-written consent must be formally documented and witnessed.

23. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship with the physician or may consent under duress. In that case the informed consent should be obtained by a well-informed physician who is not engaged in the investigation and who is completely independent of this relationship.

24. For a research subject who is legally incompetent, physically or mentally incapable of giving consent or is a legally incompetent minor, the investigator must obtain informed consent from the legally authorized representative in accordance with applicable law. These groups should not be included in research unless the research is necessary to promote the health of the population represented and this research cannot instead be performed on legally competent persons.

25. When a subject deemed legally incompetent, such as a minor child, is able to give assent to decisions about participation in research, the investigator must obtain that assent in addition to the consent of the legally authorized representative.

26. Research on individuals from whom it is not possible to obtain consent, including proxy or advance consent, should be done only if the physical/mental condition that prevents obtaining informed consent is a necessary characteristic of the research population. The specific reasons for involving research subjects with a condition that renders them unable to give informed consent should be stated in the experimental protocol for consideration and approval of the review committee. The protocol should state that consent to remain in the research should be obtained as soon as possible from the individual or a legally authorized surrogate.

27. Both authors and publishers have ethical obligations. In publication of the results of research, the investigators are obliged to preserve the accuracy of the results. Negative as well as positive results should be published or otherwise publicly available. Sources of funding, institutional affiliations and any possible conflicts of interest should be declared in the publication. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

C. ADDITIONAL PRINCIPLES FOR MEDICAL RESEARCH COMBINED WITH MEDICAL CARE

28. The physician may combine medical research with medical care, only to the extent that the research is justified by its potential prophylactic, diagnostic or therapeutic value. When medical research is combined with medical care, additional standards apply to protect the patients who are research subjects.

29. The benefits, risks, burdens and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic

methods. This does not exclude the use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic or therapeutic method exists.

30. At the conclusion of the study, every patient entered into the study should be assured of access to the best proven prophylactic, diagnostic and therapeutic methods identified by the study.

31. The physician should fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study must never interfere with the patient-physician relationship.

32. In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgement it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published. The other relevant guidelines of this Declaration should be followed.

bjh research paper

Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia

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Summary

Twenty-two patients with acute myeloid leukaemia were recruited into a phase I/II clinical trial investigating the vaccination of patients in complete remission (CR) with autologous dendritic-like leukaemia cells (DLLC). At trial entry, leukaemia cells were harvested and tested for their ability to undergo cytokine-induced dendritic cell differentiation. Patients were then treated with intensive chemotherapy. Five patients achieved both CR and had leukaemia cells that successfully underwent differentiation and therefore proceeded to vaccination. Four escalating doses of DLLC were administered weekly by subcutaneous injection. Vaccination was generally well tolerated although one patient developed extensive eczema and an increased antinuclear factor titre possibly indicating induction of autoimmunity. Development of anti-leukaemic T-cell responses was assessed by enzyme-linked immunospot analysis of gamma-interferon secreting T lymphocytes and by human leucocyte antigen tetramer analysis for WT1-specific T cells. Increases in anti-leukaemic T-cell responses were demonstrated in four patients, but only two of the five remained in remission more than 12 months postvaccination. The study has demonstrated that generation of DLLC is feasible in only a subgroup of patients and is currently neither broadly applicable or clinically effective.

Keywords: acute myeloid leukaemia, immunotherapy, dendritic cells, minimal residual disease.

Despite recent advances in the treatment of acute myeloid leukaemia (AML), cure is only possible in the minority of patients treated with intensive chemotherapy. This is despite the fact that clinical complete remission (CR) rates are high, amounting to around 80% in patients under 60 years (Hann *et al*, 1997). The reason for this discrepancy is that relapse is common, which is a consequence of the failure of chemotherapy to eradicate minimal residual disease (MRD). Therefore there is a need to develop new approaches to tackling MRD that circumvent leukaemia cell resistance to cytotoxic agents. One potential strategy is to stimulate, through vaccination, the patient's own immune system to recognise leukaemic antigens leading to subsequent killing of the leukaemia cells. Previous studies have shown that it is possible to differentiate leukaemia cells into dendritic-like cells *in vitro* and that these dendritic-like leukaemia cells (DLLC) have enhanced immunostimulatory capacity and therefore might be suitable to act as cellular vaccines. DLLC are capable of stimulating the *in vitro* development of cytotoxic T lymphocytes (CTL) that have the ability to destroy unmodified autologous leukaemia cells (Menssen *et al*, 1995; Charbonnier *et al*, 1999; Choudhury *et al*, 1999). One advantage of using DLLC, rather than targeting immunity to a specific leukaemia peptide, is that there is potential to develop immunity to multiple leukaemia antigens. This avoids one method of immune escape whereby the leukaemia cells down-regulation expression of a specific leukaemia antigen. However, one obstacle to the use of DLLC as a broadly applicable leukaemia vaccine is that the leukaemia cells are able to undergo DLLC differentiation in only a subgroup of patients. Previous studies have shown that only 25–40% of AML cases have leukaemic blasts that successfully undergo DLLC differentiation (Robinson *et al*, 1998; Cignetti *et al*, 1999; Brouwer *et al*, 2000; Roddie *et al*, 2002). In order to establish whether DLLC vaccination was feasible and safe we undertook a phase I/II clinical trial of vaccination with autologous DLLC in patients with AML who had achieved prior CR following intensive combination chemotherapy.

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Patients and methods

Patients

The trial protocol received approval from the local institutional review board and the UK Medicines and Healthcare products Regulatory Authority (MHRA). Patients were recruited from five UK centres. Patients enrolled into the study had leukaemia cells harvested prior to the start of induction chemotherapy. The method of harvest was dependent upon the peripheral blood (PB) blast cell count at presentation. If the PB blast count was $>10 \times 10^9$/l, isovolaemic venesection of 100 ml PB was performed, >1 to $<10 \times 10^9$/l, leucaphaeresis on a COBE spectra aphaeresis machine (Gambro BCT, Lakewood, CO, USA) was performed, and for those with PB blast cell count was $<1 \times 10^9$/l, 20 ml of bone marrow (BM) was aspirated. Patients were then treated with intensive combination chemotherapy administered at the referring hospital. Only patients who achieved CR (as defined by a blast cell count of <5% in a normocellular BM) were considered as potentially eligible to proceed to vaccination (Fig 1).

Vaccine production

Following collection the leukaemia cell harvest was transferred immediately to a Good Manufacturing Practice accredited cell manipulation facility where a mononuclear cell fraction (MC) was prepared by density centrifugation over Ficoll (Histopaque 1077; Sigma Diagnostics, St Louis, MO, USA). An aliquot of the harvest was tested in a prescreen to establish whether the leukaemia cells would undergo differentiation to DLLC. The remainder of the cell harvest was then cryopreserved for later vaccine generation (Fig 1). The process used in the prescreen and for generating the final cell vaccine product followed the same standard operating procedure. In summary, leukaemia cells were cultured in flasks in complete Roswell Park Memorial Institute (RPMI) medium (CM) with 5% human AB serum, 1% Glutamine (Gibco, Paisley, UK) and cytokines granulocyte–macrophage colony stimulating factor (GM-CSF) at 100 ng/ml and interleukin (IL)-4 (both R&D systems, Abingdon, UK) at 20 ng/ml for 4 d. Medium was then supplemented with a further 100 ng/ml of GM-CSF and tumour necrosis factor-alpha (Peprotech, Rocky Hill, NJ, USA) at 25 ng/ml and cells were cultured for further 3 d. In the final 24 h of culture interferon-gamma (IFN-γ; Boehringer, Ingelheim, Germany) at 30 ng/ml and polyIC (Sigma-Aldrich, Gillingham, UK) 12·5 μg/ml were added.

Confirmation of successful DLLC differentiation was determined by flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) following staining of the cells with the following murine monoclonal antibodies (mab): CD40, CD80, CD86 and CD83 (all Caltag, Burlingame, CA, USA). DLLC differentiation was deemed to have occurred if >20% of cells expressed two or more of the above markers. This phenotype was based on previous studies that demonstrated that this correlated with acquisition of functional dendritic cell characteristics, such as increased allostimulatory capacity and production of IL-12 (Roddie *et al*,

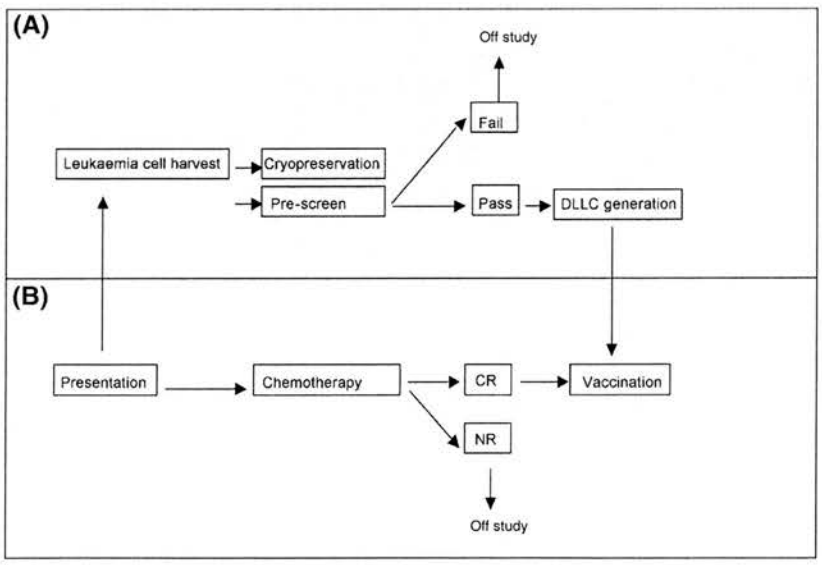

Fig 1. Clinical trial outline. (A) Laboratory protocol: at trial entry an aliquot of the leukaemia cell harvest was tested for their ability to undergo successful dendritic-like leukaemia cells (DLLC) differentiation with the remainder being cryopreserved. In those patients eligible for vaccination sufficient DLLC were generated to complete the vaccination programme. (B) Clinical protocol: following the leukaemia cell harvest patients were treated with intensive chemotherapy aimed at achieving complete remission (CR). If remission was achieved, and their leukaemia cells were successful at undergoing DLLC differentiation, vaccination was performed following haematopoietic recovery after the final cycle of chemotherapy. NR, no response.

2002). DLLC were generated just prior to the planned vaccination programme. Confirmation of DLLC phenotype was confirmed prior to vaccination. The first dose was provided fresh with subsequent doses being cryopreserved and then thawed prior to injection. DLLC were irradiated with 25–50 Gy from an MHRA accredited Caesium 137 source prior to administration.

DLLC vaccination schedule

Vaccination was performed between 2 and 8 weeks following haematopoietic recovery after the final cycle of chemotherapy. Patients received four escalating doses of DLLC of $0{\cdot}125 \times 10^6$/kg, $0{\cdot}25 \times 10^6$/kg, $0{\cdot}5 \times 10^6$/kg and 1×10^6/kg by subcutaneous injection at weekly intervals.

Immunological evaluation

Autoimmunity. This was monitored by clinical evaluation and by serial measurement of antinuclear factor (ANF).

Delayed-type hypersensitivity reaction. Intradermal injection of 1×10^6 autologous irradiated leukaemia cells was performed pre- and at day 28 postvaccination.

Measurement of IFN-γ secreting CTL by enzyme-linked immunospot assay. Peripheral blood mononuclear cells (PBMC) were used as a source of T cells for the purposes of the assay; 10^7 cells were co-cultured with 10^6 irradiated autologous leukaemia cells in CM supplemented with IL-2 5 ng/ml and IL-7 5 ng/ml (both Peprotech). Cells were harvested on day 7 for subsequent enzyme-linked immunospot (ELISPOT) assay. Sterile Multiscreen-IP plates (Millipore, Watford, UK) were coated overnight at 4°C with 50 μl/well anti-human IFN-antibody (Bender MedSystems, Vienna, Austria) diluted to 10 μg/ml in carbonate buffer. Coated plates were washed four times with phosphate-buffered saline/0·05% Tween 20 and blocked with CM for 2 h at 37°C/5% CO_2. Effector cells (100 μl) were added to triplicate wells at graded concentrations, followed by 1×10^4 target cells/well (100 μl) to E:T ratios of 20:1, 10:1, 5:1 and 2·5:1. Target cells were either unmodified autologous leukaemia cells or remission PBMC. After 36 h cells were removed and biotinylated anti-IFN avidin-conjugated alkaline phosphatase and 5-bromo-4-chloro-3-indolyl-phosphate/nitro-Blue Tetrazolium (BCIP/NBT) substrate (Sigma-Aldrich) were used for colorimetric reactions. Spots were quantified using an automated ELISPOT reader (AID Systems, Strassberg, Germany).

WT1/HLA-A2 tetramer positive CTL analysis. Numbers of WT1-specific CTL were measured in the PB of unique patient number (UPN)18, who was HLA-A*0201 positive. WT1-HLA-A2 tetramer (Beckman Coulter, Fullerton, CA, USA) was custom-made for the WT126 peptide (sequence RMFPNAPYL) (Gao *et al*, 2000). Phycoerythrin (PE)-conjugated WT1/HLA-A2 tetramer was incubated with 1×10^6 PBMC at final concentration of 50 μg/ml. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated CD8 mab (Caltag) and subsequently analysed by flow cytometry.

Measurement of regulatory T-cell numbers. Peripheral blood mononuclear cells were stained with the following combinations of mab; CD3-FITC alone, CD4-FITC + CD25-PE and CD8-FITC + CD25-PE (all Caltag) and cells were analysed by flow cytometry. Regulatory T cells were gated on the basis of CD4 positivity and bright expression of CD25.

Minimal residual disease

Monitoring for MRD was performed by real-time quantitative polymerase chain reaction (RT-PCR) for WT1 gene expression prior to, and then at 14, 28 and 84 d following vaccination. In four patients WT1 expression was measured in BM, with the remaining patient having PB WT1 expression measured, due to practical considerations. Total RNA was extracted from MCs using TRI-Reagent (Sigma-Aldrich). Reverse transcription was carried out using random hexamers (GE Healthcare, Amersham, UK) and Superscript II (Invitrogen, Paisley, UK). Relative quantitation of WT1 expression was performed by RT-PCR using the ΔΔ-Ct method on an ABI 7900 HT thermal cycler (Applied Biosystems, Foster City, CA, USA). cABL was used as the endogenous control. The WT1 primers and Taqman probe were as previously described (Ogawa *et al*, 2003). The c*ABL* primers and probe were ABL-146F; GATACGAAGG-GAGGGTGTACCA, ABL-240R; CTCGGCCAGGGTGTTGAA and ABL-183T; 5′-FAM-TGCTTCTGATGGCAAGCTCTA-CGTCTCCT-TAMRA-3′. Fifty cycles of PCR were performed. Absolute quantitation of WT1 transcript was performed using the Profile*Quant* (Ipsogen, Marseille, France).

Results

Feasibility and safety

The clinical details of the 22 patients recruited into the study are summarised in Table I; five patients were eligible to enter the vaccination program. The reason for patients being unable or unsuitable to proceed to DLLC vaccination was as a consequence of one or more of the following: induction death or failure to achieve CR (27%), insufficient cell numbers in the initial leukaemia harvest (22%) (five of six marrow harvests), and failure of leukaemia cells to undergo DLLC differentiation (68%).

Vaccination was generally well tolerated. One patient, UPN 20, developed side effects likely to be attributable to the vaccination. At day 14 postvaccination she had an extensive recurrence of eczema. In addition, ANF that was positive at a titre of 1/40 prevaccination, increased to a titre of 1/160 (nucleolar pattern) at day 84 and has remained at this level. Two of the patients, UPN12 and UPN14, had clinical relapse of

Table I. Clinical details of the patients recruited into the study.

Patient identifier	Age (years)	Sex	Presentation status	WHO type	Cytogenetics	PB blast cell count ($\times 10^9$/l)	Type of harvest	DLLC differentiation	Treatment outcome	Eligible for vaccination
UPN1	67	M	*De novo*	M4	46, XY	5·7	PB	N	CR	N
UPN2	70	M	Relapse	M1	Complex	14·2	PB	N	ID	N
UPN3	64	F	*De novo*	M1	46, XX	<1	BM	N	CR	N
UPN4	65	M	tMDS	M2	Complex	17	PB	Y	RD	N
UPN5	62	M	*De novo*	M5	46, XY	md	PB	N	RD	N
UPN6	61	M	tMDS	M4	47, XY, +8	26·8	PB	Y	RD	N
UPN7	67	F	Relapse	M1	46, XY	3·98	BM	N	CR	N
UPN8	74	F	*De novo*	M1	46, XX	2·9	BM	N	CR	N
UPN9	52	F	Relapse	M2	46, XX	<1	BM	N	CR	N
UPN10	62	M	*De novo*	M0	46, XY	<1	BM	N	CR	N
UPN11	62	M	Relapse	AML with mld	46, XY	3·6	PB	N	ID	N
UPN12	60	M	Relapse	M0	46, XY	3·1	PB	Y	CR	Y
UPN13	63	M	*De novo*	AML with mld	46, XY	<1	BM	N	CR	N
UPN14	61	F	*De novo*	M5	46, XX	50	PB	Y	CR	Y
UPN15	69	M	*De novo*	M5	del 9	<1	BM	N	RD	N
UPN16	40	M	*De novo*	M5	46, XY	26	PB	Y	CR	Y
UPN17	30	F	*De novo*	M4	46, X	13·8	PB	N	CR	N
UPN18	67	F	*De novo*	M1	46, XX	36·8	PB	Y	CR	Y
UPN19	65	M	*De novo*	AML with mld	47, XY, +8	1·5	LP	N	CR	N
UPN20	43	F	*De novo*	M5	46, XY	37	PB	Y	CR	Y
UPN21	70	M	Relapse	M1	46, XY, add(8)	5·8	LP	N	CR	N
UPN22	42	M	*De novo*	AML with mld	46, XX	7	PB	N	CR	N

Patient and leukaemia characteristics; blast count in peripheral blood prior to the leukaemia cell harvest and method used for harvesting; success of dendritic-like leukaemia cells (DLLC) differentiation in pre-screen; outcome of intensive therapy; eligibility for DLLC vaccination.
WHO, World Health Organization; tMDS, transformed myelodysplasia; mld, multilineage dysplasia; md, missing data; PB, peripheral blood; BM, bone marrow; LP, leucaphaeresis product; CR, complete remission; ID, induction death; RD, primary refractory disease.

leukaemia shortly after the start of vaccination. The clinical details of the five vaccinated patients are summarised in Table II.

Immunological responses

Delayed-type hypersensitivity reaction. Delayed-type hypersensitivity responses were minimal or absent in all patients.

Analysis of IFN-γ secreting CTLs by ELISPOT. In the first patient to be vaccinated, UPN14, anti-leukaemic T-cell responses were assessed by a cytotoxicity assay based on chromium[51] release. This assay was deemed to be too insensitive and therefore replaced by the ELISPOT assay for IFN-γ-secreting CTL in all subsequent patients tested. All four patients had measurable levels of anti-leukaemic CTL at some point within the monitoring period (Fig 2).

Table II. Clinical details on the five patients who received dendritic-like leukaemia cells vaccination.

Patient identifier	Age (years)	Sex	WHO type	Cytogenetics	Induction/consolidation therapy	Disease status at vaccination	Current status	LFS postvaccination (months)	OS postvaccination (months)
UPN12	60	M	M0	46, XY	FLAGx1	CR3	†	0	28
UPN14	62	F	M5	46, XX	FLAGx2/idACx2	CR1	†	2	3
UPN16	40	M	M5	46, XY	DAx2/hdACx2	CR1	CR1	16*	16*
UPN18	67	F	M1	46, XX	DAx2/MidAC + ICE	CR1	†	12	13
UPN20	43	F	M5	46, XY	DAx2/MACE + MidAC	CR1	CR1	13*	13*

Patients and leukaemia characteristics; chemotherapy regime; disease status at start of vaccination; current status; duration of leukaemia-free and overall survival from first vaccination.
WHO, World Health Organization; FLAG, fludarabine, cytarabine and granulocyte colony-stimulating factor; idAC, intermediate dose cytarabine; DA, daunorubicin and cytarabine; hdAC, high-dose cytarabine; MdAC, mitoxantrone and cytarabine; ICE, idarubicin, cytarabine and etoposide; MACE, amsacrine, cytarabine and etoposide; LFS, leukaemia-free survival from start of vaccination; OS, overall survival from start of vaccination.
*Alive in CR1; †patient died.

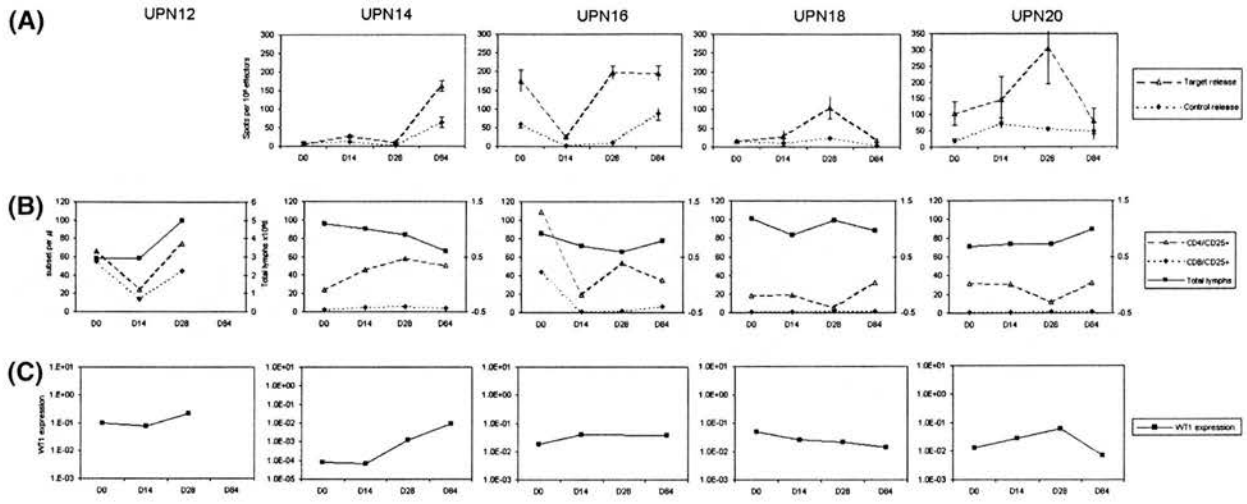

Fig 2. Results of monitoring for immunological responses and minimal residual disease in the five patients who underwent dendritic-like leukaemia cells vaccination. (A) Frequencies of interferon-gamma-secreting cytotoxic T lymphocytes measured by enzyme-linked immunospot analysis [unique patient number (UPN)12 did not have this analysis performed]. (B) Frequencies of regulatory T cells. Measurement of regulatory T cells was performed by flow cytometric analysis gating on the CD4/CD25 dual-positive population. Total lymphocyte and CD8/CD25 dual-positive population cell numbers are provided for comparison. (C) Levels of minimal residual disease as measured by gene expression of WT1 (reported as expression relative to K562 cell line in logarithmic scale).

Analysis of WT1-specific CTL. One of the five vaccination patients was HLA-A*0201 positive and therefore suitable for study. In this patient (UPN18) at the start of vaccination there was a very low frequency of WT1-specific CTL. Following vaccination there was a progressive rise in WT1-specific CTL PB with a maximum value of 0·35% of total T-cell numbers on day 84.

Regulatory T-cell numbers. There was no consistent pattern either in the regulatory T-cell frequency pre-vaccination or in the change in this frequency following vaccination in the five patients (Fig 2). In particular, initial regulatory T cells frequencies did not appear to be appreciably different between the two patients who had received fludarabine-based induction regimes (UPN12 and UPN14) compared with those that had received an anthracycline-based regime (UPN16, UPN18 and UPN20).

Monitoring of MRD

All patients were in morphological remission at the time of vaccination. However, two patients UPN12 and UPN 14 had morphological relapse of their leukaemia shortly after the start of the vaccination programme. In both patients this was mirrored by rises in the level of WT1 expression but particularly so in the case of UPN14 (Fig 2). In the remaining three patients WT1 expression remained relatively constant following vaccination.

Discussion

This study has demonstrated the feasibility of vaccinating patients with AML in CR with autologous DLLC. Vaccination was generally safe, with only one of five patients developing side effects that could be potentially attributable to DLLC vaccination. In this patient eczema and an increased ANF titre might indicate the induction of autoimmunity through presentation of self-antigens by the DLLC. Whilst DLLC vaccination was feasible it is not an approach that could be broadly applicable. Of the patients recruited into the study only 23% were eligible to proceed to vaccination. This was primarily as a consequence of a failure of leukaemia cells to differentiate into DLLC. Unless methods for improving the proportion of AML cases that are able to undergo DLLC differentiation can be found, different approaches to vaccination will need to be tried. In our group we are currently evaluating methods to load monocyte-derived dendritic cells with autologous leukaemia antigens as a means of developing a potentially more broadly applicable cell-based vaccine.

Four patients developed increased numbers of leukaemia-specific IFN-γ-secreting CTL following vaccination. In the one HLA-A*0201-positive patient this was also accompanied by an increase in WT1-specific CTL numbers. However, despite the development of these anti-leukaemic immune responses, evidence of clinical benefit has not been established. Indeed in one patient leukaemic relapse developed despite an increase in anti-leukaemic CTL activity. There could be a number of reasons why this increase in anti-leukaemic activity failed to prevent relapse in this patient. It is known that regulatory T cells inhibit anti-cancer immune responses (Morse *et al*, 2002; Casares *et al*, 2003; Wolf *et al*, 2003; Mesel-Lemoine *et al*, 2006). The rise in regulatory T-cell numbers seen in this patient may have abrogated the anti-leukaemic response to a degree that relapse could take place. Although regulatory T cells may have a role in suppression of anti-leukaemic

immunity a more likely explanation for the lack of clinical benefit is that DLLC vaccination, as performed in this study, failed to generate a clinically relevant level of anti-leukaemic immune response. If this is the case then it is necessary to develop ways to augment the vaccination method in order to generate clinically relevant immunity. Possible means to achieve this would be by improving the potency of the dendritic-cell-based vaccine through use of agents that promote dendritic cell maturation and/or polarisation or by supporting any anti-leukaemic T-cell responses by systemic administration of cytokines, such as IL-12 (Napolitani *et al*, 2005; Portielje *et al*, 2005). It might then be possible to convert subclinical immune responses into ones that are clinically effective, thus offering the promise of therapeutically beneficial vaccines.

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References

Brouwer, R.E., van der Hoorn, M., Kluin-Nelemans, H.C., Zelderen-Bhola, S., Willemze, R. & Falkenburg, J.H. (2000) The generation of dendritic-like cells with increased allostimulatory function from acute myeloid leukemia cells of various FAB subclasses. *Human Immunology*, **61,** 565–574.

Casares, N., Arribillaga, L., Sarobe, P., Dotor, J., Lopez-Diaz, D.C., Melero, I., Prieto, J., Borras-Cuesta, F. & Lasarte, J.J. (2003) CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *Journal of Immunology*, **171,** 5931–5939.

Charbonnier, A., Gaugler, B., Sainty, D., Lafage-Pochitaloff, M. & Olive, D. (1999) Human acute myeloblastic leukemia cells differentiate in vitro into mature dendritic cells and induce the differentiation of cytotoxic T cells against autologous leukemias. *European Journal of Immunology*, **29,** 2567–2578.

Choudhury, B.A., Liang, J.C., Thomas, E.K., Flores-Romo, L., Xie, Q.S., Agusala, K., Sutaria, S., Sinha, I., Champlin, R.E. & Claxton, D.F. (1999) Dendritic cells derived in vitro from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. *Blood*, **93,** 780–786.

Cignetti, A., Bryant, E., Allione, B., Vitale, A., Foa, R. & Cheever, M.A. (1999) CD34(+) acute myeloid and lymphoid leukemic blasts can be induced to differentiate into dendritic cells. *Blood*, **94,** 2048–2055.

Gao, L., Bellantuono, I., Elsasser, A., Marley, S.B., Gordon, M.Y., Goldman, J.M. & Stauss, H.J. (2000) Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood*, **95,** 2198–2203.

Hann, I.M., Stevens, R.F., Goldstone, A.H., Rees, J.K., Wheatley, K., Gray, R.G. & Burnett, A.K. (1997) Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML10). Adult and Childhood Leukaemia Working Parties of the Medical Research Council. *Blood*, **89,** 2311–2318.

Menssen, H.D., Renkl, H.J., Rodeck, U., Maurer, J., Notter, M., Schwartz, S., Reinhardt, R. & Thiel, E. (1995) Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. *Leukemia*, **9,** 1060–1067.

Mesel-Lemoine, M., Cherai, M., Le Gouvello, S., Guillot, M., Leclercq, V., Klatzmann, D., Thomas-Vaslin, V. & Lemoine, F.M. (2006) Initial depletion of regulatory T-cells: the missing solution to preserve the immune functions of T lymphocytes designed for cell-therapy. *Blood*, **107,** 381–388

Morse, M.A., Clay, T.M., Mosca, P. & Lyerly, H.K. (2002) Immunoregulatory T cells in cancer immunotherapy. *Expert Opinion on Biological Therapy*, **2,** 827–834.

Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. & Lanzavecchia, A. (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nature Immunology*, **6,** 769–776.

Ogawa, H., Tamaki, H., Ikegame, K., Soma, T., Kawakami, M., Tsuboi, A., Kim, E.H., Hosen, N., Murakami, M., Fujioka, T., Masuda, T., Taniguchi, Y., Nishida, S., Oji, Y., Oka, Y. & Sugiyama, H. (2003) The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. *Blood*, **101,** 1698–1704.

Portielje, J.E., Kruit, W.H., Eerenberg, A.J., Schuler, M., Sparreboom, A., Lamers, C.H., Gratama, J.W., Stoter, G., Huber, C. & Hack, C.E. (2005) Subcutaneous injection of interleukin 12 induces systemic inflammatory responses in humans: implications for the use of IL-12 as vaccine adjuvant. *Cancer Immunology Immunotherapy*, **54,** 37–43.

Robinson, S.P., English, N., Jaju, R., Kearney, L., Knight, S.C. & Reid, C.D. (1998) The in-vitro generation of dendritic cells from blast cells in acute leukaemia. *British Journal of Haematology*, **103,** 763–771.

Roddie, P.H., Horton, Y. & Turner, M.L. (2002) Primary acute myeloid leukaemia blasts resistant to cytokine-induced differentiation to dendritic-like leukaemia cells can be forced to differentiate by the addition of bryostatin-1. *Leukemia*, **16,** 84–93.

Wolf, A.M., Wolf, D., Steurer, M., Gastl, G., Gunsilius, E. & Grubeck-Loebenstein, B. (2003) Increase of regulatory T cells in the peripheral blood of cancer patients. *Clinical Cancer Research*, **9,** 606–612.

bjh research paper

Fusion hybrids of dendritic cells and autologous myeloid blasts as a potential cellular vaccine for acute myeloid leukaemia

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Summary

We assessed the potential of tumour cell/dendritic cell fusion hybrids to generate *in vitro* anti-leukaemic T-cell responses following co-culture with autologous remission lymphocytes in six patients with acute myeloid leukaemia (AML). Comparison was made to anti-leukaemic responses induced by mature dendritic cells (mDC) co-cultured with autologous, irradiated myeloid blasts. Fusion hybrids induced anti-leukaemic T-cell immune responses in three of six patients. Tumour-pulsed mDC induced T-cellular responses in two other patients. Only one of six patients remission lymphocytes failed to develop leukaemia-directed immune responses following stimulation with either construct. Anti-proliferative properties of fusion hybrids against allogeneic lymphocytes were observed in mixed lymphocyte-leukaemia reactions and were found not to be specific to the cell fusion partners and did not prevent the ability of AML–mDC heterokaryons to induce autologous anti-leukaemic cytotoxicity. We conclude that tumour cell/dendritic cell fusion hybrids hold promise as a cellular vaccine for AML.

Keywords: cellular therapies, dendritic cells, vaccines, acute myeloid leukaemia, cytotoxicity.

There is a need for novel treatment strategies for the acute myeloid leukaemias (AML), particularly in the older patient, where current approaches aimed at eradicating minimal residual disease (MRD), such as stem cell transplantation, are not feasible because of unacceptably high levels of treatment-related morbidity and mortality. Cellular therapy strategies aimed at eradicating MRD will have to overcome the immune escape mechanisms of leukaemic blasts, such as the low expression of co-stimulatory molecules (Hirano *et al*, 1996), Fas ligand expression associated with activated T-lymphocyte apoptosis (Buzyn *et al*, 1999) and secretion of cytokines inhibiting the development of efficient immune responses (Buggins *et al*, 1999).

Dendritic cell (DC)-based therapies hold promise for cancer immunotherapy, as DCs are the most efficient antigen-presenting cells and induce both primary and secondary immune responses (Banchereau & Steinman, 1998). After antigen uptake they migrate into secondary lymphoid organs, mature and activate both helper and cytotoxic T cells (Banchereau *et al*, 2000).

Dendritic-like leukaemic cells (DLLC) generated from primary AML blasts by cytokine-induced differentiation offer potential as autologous cellular vaccines, as they acquire co-stimulatory and adhesion molecules and thereby stimulate cytotoxic T-lymphocyte (CTL) responses to leukaemic tumour antigens (Choudhury *et al*, 1997; Charbonnier *et al*, 1999; Choudhury *et al*, 1999). In a significant number of cases, however, AML blasts prove resistant to cytokine-driven differentiation into DLLC (Robinson *et al*, 1998; Choudhury *et al*, 1999; Brouwer *et al*, 2000; Harrison *et al*, 2001), therefore limiting this approach, in our experience, to about 60% of cases (Roddie *et al*, 2002).

To overcome these limitations we investigated an alternative approach to generate potent anti-leukaemic cytotoxic responses by fusing autologous AML blasts to mature dendritic cells (mDC) derived from peripheral blood monocytes of six AML patients in complete morphological remission (CR), thus generating heterokaryons that express leukaemic antigens derived from the blast fusion partner as well as co-stimulatory and adhesion molecules of mDC. We demonstrate the potential of these AML–DC fusion hybrids to induce *in vitro* T-cellular responses against the patient's unmodified leukaemic cells. Where possible, the potency of these constructs was compared to mDC co-cultured with irradiated autologous leukaemic blasts,

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previously shown to generate leukaemia-specific T-cellular cytotoxicity (Galea-Lauri *et al*, 2002; Spisek *et al*, 2002).

Materials and Methods

Patient samples

Samples from six AML patients (median age: 54 years, range: 24–70 years) were used in this study. Table I shows relevant clinical and laboratory characteristics of these patients, in whom DLLC maturation was either not achievable ($n = 5$), or DLLC proved ineffective in stimulating anti-leukaemic CTL [unique patient number (UPN) 3].

Leukaemic blasts

Leukaemic blasts were taken with informed consent at the time of diagnosis. A highly purified population of leukaemic blasts (>95%) was generated through Ficoll (Histopaque 1077; Sigma Diagnostics, St Louis, MO, USA) density-gradient centrifugation and cells were immediately cryopreserved in 90% fetal calf serum (FCS; Perbio, Cheshire, UK) +10% dimethyl sulphoxide (DMSO; Sigma-Aldrich, Irvine, UK).

Remission samples

Remission samples were obtained after CR was confirmed (defined as <5% of blasts in the bone marrow). Ficoll density-gradient centrifugation was used to separate monocytes for the generation of mature mDC and isolate responder T cells for subsequent incubation with various autologous stimulators.

Generation of mDC from peripheral blood monocytes of patients in complete morphological remission

Mature DC were generated from the adherent fraction of peripheral blood mononuclear cells (PBMC) collected after the resolution of cytopenias following combination intravenous chemotherapy. Briefly, PBMC were suspended at 10^7/ml in complete medium (CM), RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin (Sigma-Aldrich) and 2 mmol/l L-glutamine (Sigma-Aldrich), and incubated for 2 h at 37°C/5% CO_2in 75 cm^2 culture flasks (Corning, Acton, MA, USA). Non-adherent lymphocytes were subsequently removed and cryopreserved. Adherent monocytes were resuspended with CM in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF; Sandoz, Surrey, UK) at 100 ng/ml and interleukin-4 (IL-4; R & D Systems, Abingdon, UK) at 15 ng/ml for 5 d. The DC were then matured for a further 2 days by the addition of tumour necrosis factor-alpha (TNF-α; Peprotech, Rocky Hill, NJ, USA) at 25 ng/ml, Poly I:C (Sigma-Aldrich) at 12·5 μg/l and interferon-gamma (IFN-γ; Boehringer, Ingelheim, Germany) at 30 ng/ml. Mature DCs were immunophenotyped by flow cytometry on day 7 and used fresh for the generation of fusion hybrids with thawed autologous AML blasts and co-incubation with irradiated AML blasts.

Table I. Patient characteristics.

Patient	Sex/age (years)	Sample origin	WHO/FAB classification	Karyotype	DLLC maturation
UPN1	M/66	PB	M4	46,XY	No
UPN2	M/48	PB	AML with trilineage dysplasia/M5a	46,XY	No
UPN3	F/61	PB	M5a	46,XX	Yes
UPN4	F/24	PB	M5a	46,XX	No
UPN5	M/39	PB	M4	46,XX	No
UPN6	F/70	PB	M5	46,XY	No

WHO, World Health Organization; FAB, French–American–British; PB, peripheral blood.

Generation of DLLC from AML blasts

Complete medium as described above was used. Cell density was adjusted to 1×10^6/ml and GM-CSF 100 ng/ml and IL-4 20 ng/ml were added. After 3 d of culture at 37°C/5% CO_2, fresh GM-CSF (100 ng/ml) and TNF-α 25 ng/ml was added. At day 6 of culture, further maturational agents Poly I: C at 12·5 μg/l and IFN-γ at 30 ng/ml were added and cells were harvested at day 7 of culture for immunophenotypic characterization by flow cytometry and microscopic analysis of cytospin preparations.

Immunophenotypic analysis of mDC, AML blasts, fusion hybrids and DLLC

A 2×10^5 cells were used for phenotypic analyses using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) against CD40, CD86, major histocompatibility complex (MHC) class I, CD33 and CD 14, phycoerythrin (PE)-conjugated mAbs against CD80, CD83, CD11c and CD34, and tricolour-conjugated mAbs against MHC class II (Caltag, Burlingame, CA, USA). Cells were incubated with antibodies for 20 min at 4°C, washed in phosphate-buffered saline (PBS) and resuspended in 300 μl PBS/sodium azide for analysis using a FACSCalibur flow-cytometer system (Becton Dickinson, San Jose, CA, USA) and WIN MDI analysis software (The Scripps Research Institute, La Jolla, CA, USA). Cell debris was excluded by establishing appropriate forward and side scatter gates and apoptotic cells were excluded on the basis of propidium iodide uptake before the analysis for expression of each phenotypic marker. Appropriate isotype-matched control mAbs were always used.

Generation of AML–mDC fusion hybrids

Thawed AML blasts and fresh mDC were mixed at a ratio of 1 : 1, not exceeding 5×10^6 each in CM at 37°C and centrifuged for 5 min at 400 ***g*** to pellet. After removal of

all supernatant, cells were fused by drop-wise addition of 500 μl of 50% Polyethylene Glycol (PEG)/10% DMSO (Sigma) over a period of 1 min in a 37°C waterbath. The reaction was stopped after a further minute by adding 1 ml of CM and further dilution to 15 ml with CM heated to 37°C prior to use as stimulators in autologous CTL assays and allogeneic mixed lymphocyte leukaemia reactions (MLLR).

Assessment of AML–mDC fusion efficiency

A series of experiments was conducted to establish the efficiency of PEG-induced hybrid formation. Briefly, prior to fusion the AML blasts were labelled with the red fluorescent linker dye PKH 26 (Sigma) according to the manufacturers instructions, whereas DC were labelled using the green Cell Tracker 5-chloromethylfluorescein diacetate (CMFDA). Unfused and fused cells were analysed by flow cytometry. Cells presenting both membrane dyes after fusion, i.e. fluorescing in both the FL1 and FL2 channels, were scored as fusion hybrids. In addition, fluorescent microscopy using motorized micro-focussing through the generated heterokaryons was undertaken to visualize the double-fluorescent membrane of AML–DC fusion hybrids. Separate images of cytospin preparations were created using FITC, Texas Red and 4,6-diamidino 2-phenly-indole (DAPI) excitation filters in a Zeiss Axioplan fluorescence microscope (Zeiss, Jena, Germany) with motorised focus and images were subsequently combined. Chroma filter sets, Vector shield mounting medium and a Princeton Instruments Micro-max digital camera were used.

Stimulation of leukaemia-specific lymphocytes by co-culture with autologous AML–mDC fusion hybrids, mDc cocultured with irradiated AML and DLLC

The non-adherent fraction of fresh or thawed remission blood samples provided the source of autologous responder cells. Unmodified AML blasts, AML–mDC fusion hybrids and mDC mixed 1 : 1 with irradiated (26 Gy) AML blasts were used as autologous stimulators. Dendritic-like differentiation was achieved from the leukaemic blasts of one of six patients. DLLC were generated as stimulators to autologous responder T cells in this case. PBMC cultured in the absence of stimulators or primed by unpulsed mDCs and AML–AML fusions served as negative controls, as these were not expected to stimulate leukaemia-directed cytotoxicity.

All stimulators received 26 Gy of irradiation from a ^{137}Cs source. Briefly, co-culture of 10^7 responder cells with 10^6 irradiated stimulators was prepared in 12-well plates at a cell density of 10^6/ml effectors. CM was supplemented with IL-2 5 ng/ml and IL-7 5 ng/ml (Peprotech, Rocky Hill, NJ, USA). Responder cells were re-stimulated at day 7 and day 14 using the same stimulators that had been added at the beginning of co-culture. CM and cytokines were half exchanged weekly. Effectors were harvested on day 21 for subsequent cytotoxicity assays against native, autologous AML blasts.

Allogeneic mixed leukaemia–lymphocyte reactions

Responder cells for allogeneic MLLR were obtained from the non-adherent lymphocytes of healthy, unrelated donors, whose PBMC had been separated by gradient density centrifugation followed by 2 h plastic adherence. Adherent donor monocytes were matured in a 7-day culture into mDC as described above and subsequently fused to patient AML blasts to generate AML–DC fusion hybrid stimulators. Unfused AML blasts and mDC as well as AML co-cultured with mDCs served as stimulator controls. AML–AML or mDC–mDC fusions and titrating concentrations of PEG were also assessed regarding their ability to stimulate the proliferation of lymphocytes allogeneic to the AML blasts used. Responder cells were plated at 1×10^5/well in CM in U-bottomed 96-well plates. Stimulators were added at graded responder/stimulator ratios of 5 : 1, 10 : 1, 25 : 1 and 100 : 1. The proliferation of lymphocytes was determined by the uptake of [^{3}H]-thymidine (ICN Biochemicals, Basingstoke, UK), added for the final 18 h of a 5-day co-culture at 37°C/5% CO_2. Results were expressed as mean counts per minute (cpm) ± standard deviation (SD) in triplicates.

^{51}Cr-release cytotoxicity assay

Following re-stimulation co-culture with a variety of autologous stimulators the resulting leukaemia directed *in vitro* cytotoxicity was measured in a 4-h ^{51}Cr-release cytotoxicity assay in two patients, where uptake of the isotope by their native AML blasts was demonstrable. Briefly, 1×10^6 AML blasts were incubated for 1 h at 37°C/5% CO_2 with 3·7 MBq ^{51}Cr (ICN Biochemicals, Basingstoke, UK), washed three times with CM and 1×10^4 labelled targets were subsequently co-cultured with autologously activated lymphocytes in 200 μl CM in 96-well U-bottomed plates at effector : target (E : T) ratios of 50 : 1, 25 : 1, 12·5 : 1 and 6·25 : 1. After 4 h, 25 μl of cell free supernatent were collected and mixed with 150 μl of scintillant (Ecolite+; ICN Biochemicals, Basingstoke, UK). Scintillation was analysed using a liquid scintillation and luminescence counter (1450 microbeta trilux; Wallac, Turku, Finland). Specific lysis was calculated using the following formula: (counts per minute) [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) ×100]. Spontaneous ^{51}Cr release was determined from wells containing target cells and medium only. Maximum ^{51}Cr release was gained by lysing target cells with 100 μl of 2% Triton × 100/sample well. Spontaneous release was <15% of maximum release. All assays were performed in triplicates and expressed as mean cpm ± SD.

IFN-γ enzyme-linked immunospot (ELISPOT) assay. IFN-ELISPOT assays were used to assess *in vitro* T-cell responses to autologous AML blasts in four patients (UPN3–UPN6). Sterile Multiscreen-IP plates [polyvinylidenedifluoride (PVDF) membranes; Millipore, Watford, UK] were coated overnight at 4°C with 50 μl/well anti-human IFN-antibody (Bender MedSystems, Vienna, Austria) diluted to 10 μg/ml in carbonate buffer.

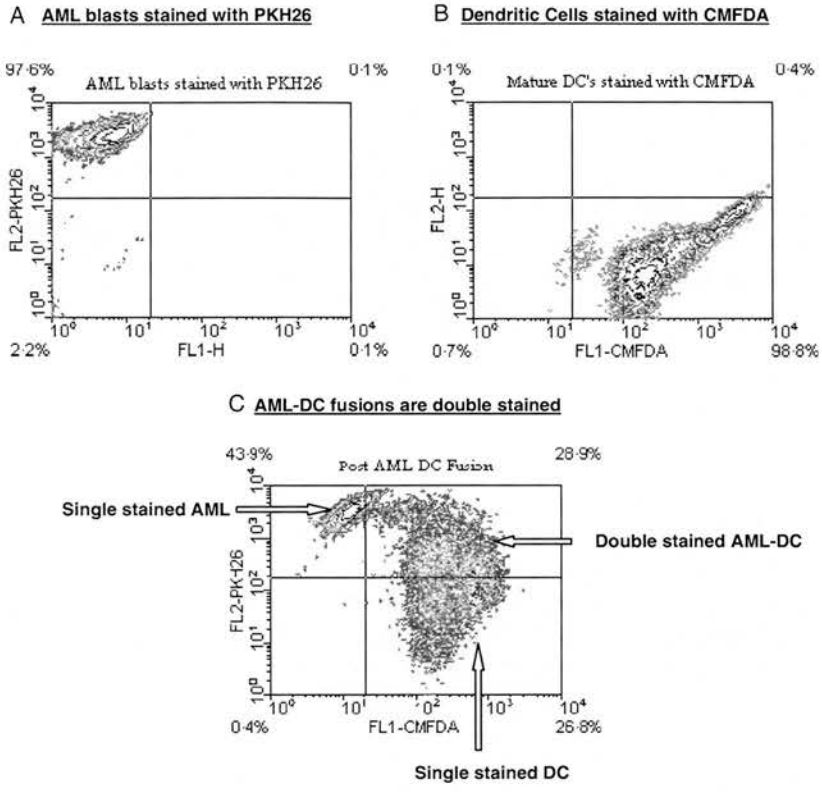


Fig 1. Flow-cytometric assessment of AML–DC fusion efficiency.

Coated plates were washed four times with PBS/0·05% Tween 20 and blocked with CM for 2 h at 37°C/5% CO_2. Effector cells (100 μl) were added to triplicate wells at graded concentrations, followed by 1×10^4target cells/well (100 μl) to E : T ratios of 20 : 1, 10 : 1, 5 : 1 and 2·5 : 1. After co-culture for 36 h at 37°C/5% CO_2 cells were removed from the assay and biotinylated anti-IFN–avidin conjugated alkaline phosphatase and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium) substrate (Sigma Aldrich) were used for the colorimetric reaction. Spots were quantified using an automated ELISPOT reader (AID Systems, Strassberg, Germany).

Statistical analysis

Statistical analysis of ^{51}Cr cytotoxicity and ELISPOT assay results was performed using Student's *t*-test. $P < 0{\cdot}05$ were considered significant.

Results

Flow cytometry and fluorescence microscopy confirm AML–mDC fusion

Before testing the immune-stimulatory function of AML–mDC fusion hybrids, a series of experiments was conducted to assess the efficiency of fusion using PEG/DMSO.

Flow-cytometric analysis following membrane staining of the fusion partners with different fluorescent dyes (CMFDA for DC and PKH 26 for AML blasts respectively) enabled the identification of fusion hybrids as double-stained cells. Fusion

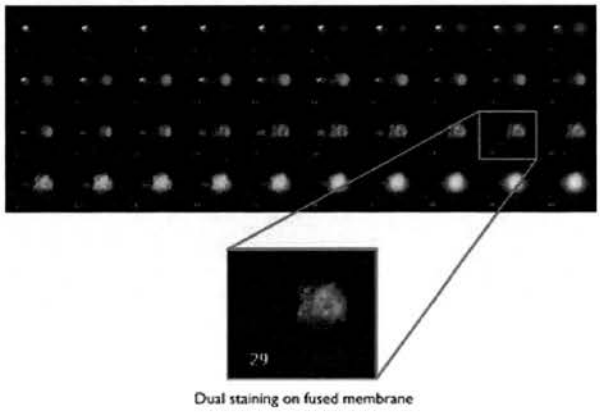


Fig 2. Fluorescent microscopic sections through an AML–DC fusion cell. A series of fluorescent microscopic images cross-section a membrane-stained AML–DC fusion cell. Motorized-focussing through the heterokaryon reveals dual membrane staining from both fusion partners. The dendritic cell was stained with the green fluorescent membrane dye CMFDA. The AML blast was stained with the red membrane dye PKH26. Additional DAPI staining was applied to outline both nuclei (blue).

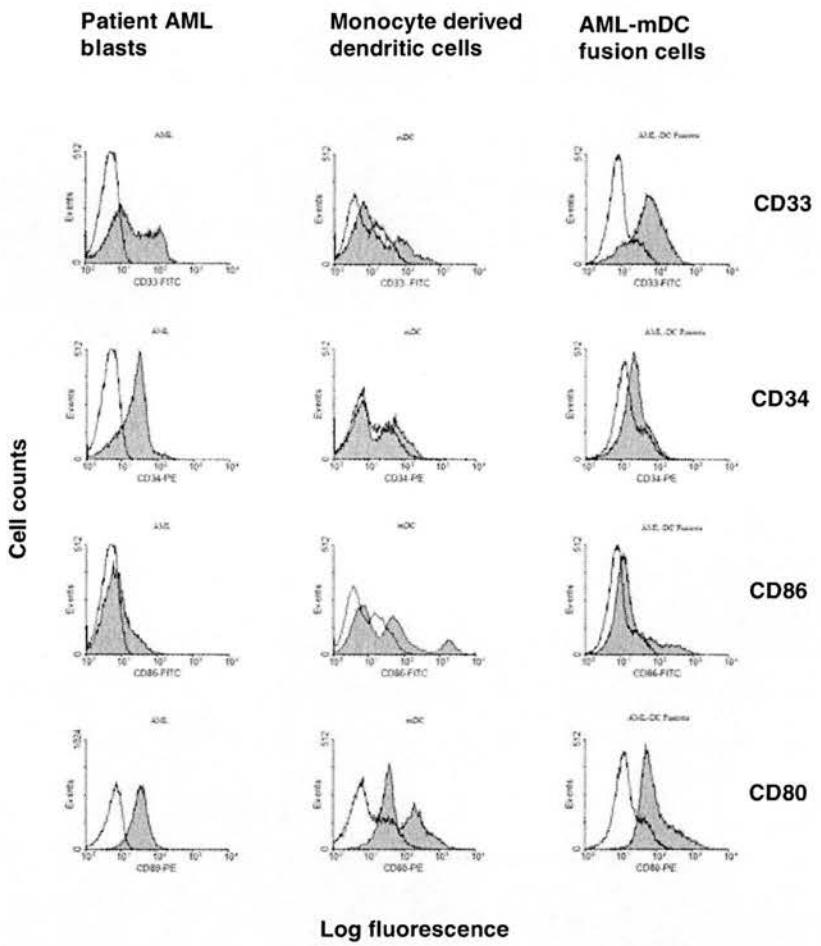


Fig 3. AML–DC fusion hybrids express a biphenotypic flow-cytometric profile. AML blasts and DCs were stained before and after fusion using a panel of monoclonal antibodies against AML and DC associated antigens. The fusion hybrids express AML restricted antigens (CD34) as well as DC associated antigens and co-stimulatory molecules (CD86,CD80). Results of a representative experiment are shown. Fusion hybrids were not gated.

efficiency was between 20% and 30% (mean 26%). Figure 1 shows a representative result of one experiment.

Fluorescence microscopy confirmed that the membrane was derived from both fusion partners on the membrane-stained heterokaryons that were generated. Motorized micro-focussing sections through fusion hybrids on cytospin preparations were visualized. Figure 2 shows sections through a fusion hybrid. As the common membrane of the fusion hybrid came into focus, red (AML) and green (mDC) stained membrane elements were seen.

Additionally, a bi-phenotypic flow-cytometric profile of the fusion hybrids was demonstrated by analysis of the fusion partners before and fusion hybrids after fusion. Figure 3 shows representative results of one patient. Unfused AML blasts and mDC were stained with mAbs against the leukaemia-associated antigens CD34 and CD33 and the DC-associated antigens CD86 and CD80 prior to PEG fusion. Immediately after fusion the hybrids expressed both AML- and DC-associated membrane antigens.

Fusion hybrids induce AML-directed T-cell responses

^{51}Cr-release cytotoxicity assays were performed in two patients, where good ^{51}Cr uptake by autologous AML blast targets was shown (UPN1, UPN2).

In the remaining four patients (UPN2–4) an IFN-γ ELISPOT assay was used to demonstrate T-cell responses against native, autologous AML blasts induced by priming co-culture with the various autologous stimulators (Table II). The ELISPOT assay has been shown to reliably detect the number of antigen-specific T cells in experiments in which known quantities of antigen-specific T cells were added to bulk PBMC preparations (Schmittel *et al*, 1997).

In three patients, AML–mDC fusion hybrids proved the strongest inducers of anti-leukaemic T-cell responses, using ^{51}Cr-release cytotoxicity assays (UPN1, UPN2) and ELISPOT IFN-γ assay (UPN3) respectively.

In UPN1 (Fig 4) PBMC primed with fusion hybrids, but not with either unfused AML blasts ($P < 0{\cdot}0001$) or mDC

Table II. Immunoassays performed and results generated.

Patient	Immunoassay performed	Best stimulator construct	Results shown
UPN1	^{51}Chromium release	AML–DC fusion hybrids	Fig 4
UPN2	^{51}Chromium release	AML–DC fusion hybrids	Fig 4
UPN3	IFN-γ ELISPOT assay	AML–DC fusion hybrids	Fig 5
UPN4	IFN-γ ELISPOT assay	AML-pulsed mDC	Fig 6
UPN5	IFN-γ ELISPOT assay	AML-pulsed mDC	Fig 6
UPN6	IFN-γ ELISPOT assay	No stimulators	Not shown

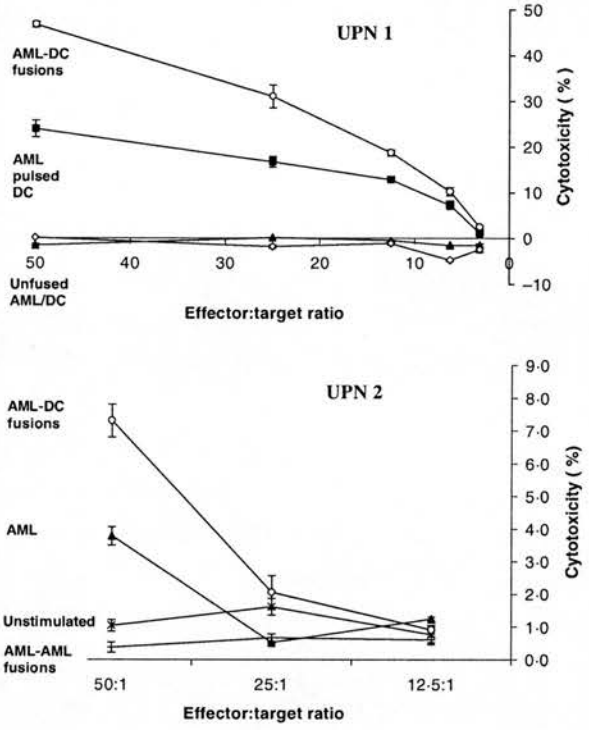


Fig 4. AML–mDC fusion hybrids generate anti-leukaemic cytotoxicity in co-culture with autologous remission PBMCs. ^{51}Cr-release assays of UPN1 and UPN2 are shown. Remission PBMCs (1×10^7) were stimulated weekly ×3, using 1×10^6 irradiated autologous AML–mDC fusion hybrids (○) and with either DC mixed with irradiated AML (■), unfused DC (◇) and AML (▲), or inappropriate AML–AML fusion hybrids (+) as negative controls.

($P < 0{\cdot}0001$), resulted in 47% target lysis at the E : T ratio of 50 : 1 in a ^{51}Cr-release assay. The cytotoxicity induced by mDC pulsed with tumour cell bodies was significantly lower at 24% at the same E : T ratio ($P = 0{\cdot}0005$).

In UPN2, fusion hybrids generated 7·3% AML-directed cytotoxicity, compared with 3·8% induced by unfused AML ($P = 0{\cdot}005$), 1% cytotoxicity induced by unstimulated PBMC ($P = 0{\cdot}001$) and 0·4% by inappropriate AML–AML fusion hybrids ($P = 0{\cdot}003$) at the highest E : T ratio of 50 : 1 (Fig 4).

In a third patient (UPN3), IFN-γ responses to autologous AML blasts increased more than 3·5-fold in effectors primed by co-culture with fusion hybrids compared with those primed with AML blasts alone ($P = 0{\cdot}0002$), DLLC ($P = 0{\cdot}0003$) or unprimed effectors ($P = 0{\cdot}0002$) at the highest effector : stimulator ratio of 20 : 1 (Fig 5A).

Flow-cytometric characteristics of remission PBMC used in this ELISPOT IFN-γ release assay are demonstrated in Fig 5B.

mDC co-cultured with irradiated whole tumour cell bodies may provide an alternative cellular vaccine construct

In two patients (UPN4, UPN5) AML-pulsed mDC proved better stimulators of anti-leukaemic T-cell responses than AML–DC fusion hybrids in IFN-γ ELISPOT assays (Fig 6). The stimulatory differences observed between these constructs were significant in both patients ($P = 0{\cdot}003$ in UPN4, $P = 0{\cdot}009$ in UPN5).

However, mDC pulsed with tumour cell bodies showed a significant advantage over immune responses induced by unmodified AML blasts only in UPN5 ($P = 0{\cdot}02$), whereas the differences observed did not reach statistical significance in UPN4 ($P = 0{\cdot}15$). In both patients, fusion hybrids failed to induce T-cell responses exceeding those to unmodified AML blasts.

Suppression of lymphocyte proliferation by fusion hybrids is not specific to fusion partners

Assessing the immune-stimulatory properties of fusion hybrids we observed that the proliferation of lymphocytes was suppressed by AML–DC fusion hybrids in [^{3}H]-thymidine uptake assays, where AML blasts allogeneic to the mDC and responder lymphocytes were used. A representative result of three experiments is shown in Fig 7A. This anti-proliferative property was, however, not specific to AML–DC fusion, but was also induced by allogeneic AML–AML as well as autologous mDC–mDC heterokaryons (Fig 7B,C). Importantly, the induction of tumour-specific T-cell cytotoxicity by AML–DC fusion hybrids was maintained regardless, further emphasizing their potency as cellular stimulators of anti-leukaemic responses.

Discussion

The heterogeneity of the AMLs poses a challenge for the generation of a DC-based vaccine, as few leukaemia-specific or tumour-associated antigens can be expected to be expressed in

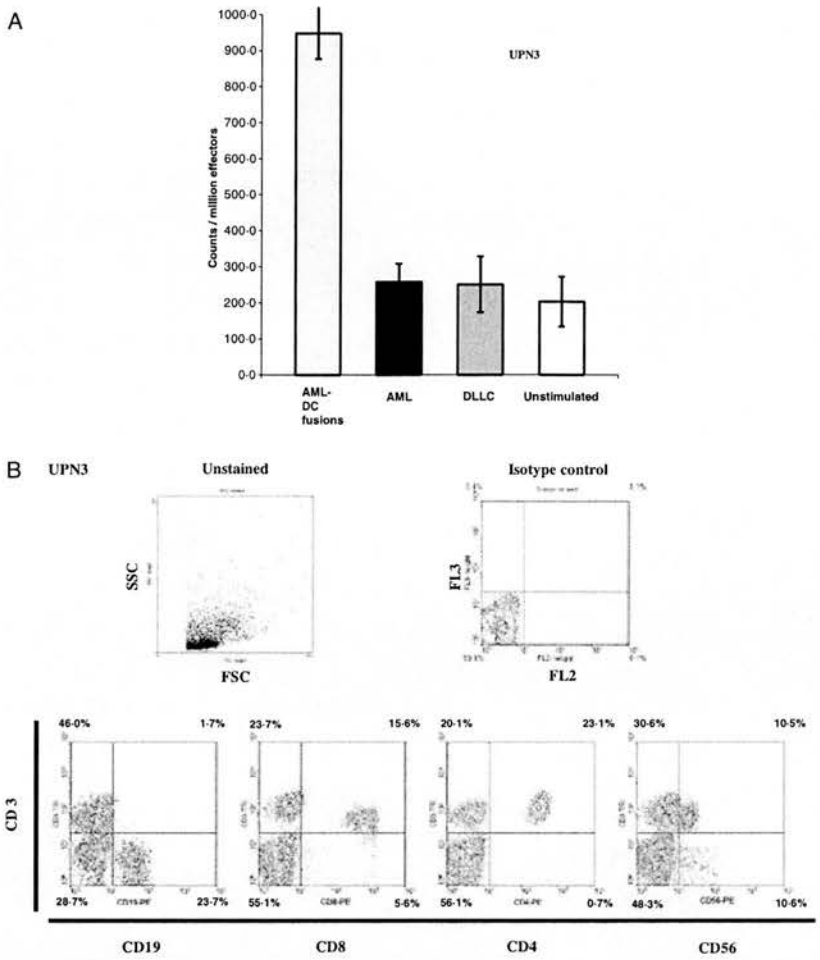


Fig 5. (A) Anti-leukaemic T-cell responses induced by AML–DC fusion hybrids (UPN3) exceed those of other autologous stimulators in an IFN-γ ELISPOT assay. Results of triplicate wells at the effector : stimulator ratio of 20 : 1 are shown. Co-culture with AML–DC fusions (▨) induces a more than 3-fold increase in T-cell IFN-γ release, compared with T cells stimulated by AML (■), DLLC (▩) and unstimulated (□) T cells. (B) Flow-cytometric characteristics of PBMC responders used in ELISPOT IFN-γ release immuno-assay (UPN3). A lymphocyte gate was applied, cells were incubated with monoclonal antibodies against CD3 (tricolour-conjugated) and additionally with either anti-CD19, CD8, CD4 or CD56 (PE-conjugated) to characterize the remission lymphocytes used.

all cases and remain stable over the course of the disease and chemotherapy regimens. Strategies focussing on the generation of CTL responses against single, specific tumour antigens are therefore fraught with the risk of possible tumour immune escape because of clonal evolution and tolerance to known and also unknown tumour antigens, resulting in relapse originating from MRD.

Whole tumour-based vaccine strategies attempt to circumvent this problem by inducing polyvalent immune responses against a large number of antigens expressed by leukaemic blasts. DLLC derived from AML blasts by sequential cytokine incubation have been demonstrated to acquire co-stimulatory molecules that are mostly lacking in native AML blasts and are necessary to induce CTL responses. This approach to generation of a cellular vaccine is, however limited by the resistance to cytokine maturation in a significant number of cases (Choudhury *et al*, 1997; Harrison *et al*, 2001; Roddie *et al*, 2002).

In this study we have taken the approach of fusing whole tumour cells to autologous DC. Thus hybridomas were constructed that expressed both tumour-derived antigens and DC-derived co-stimulatory molecules. We showed that AML–mDC fusion hybrids and, to a lesser degree, mDC co-cultured with irradiated AML blasts were capable of inducing leukaemia-directed *in vitro* cytotoxic responses in autologous remission PBMC. Both of these vaccine constructs can be readily generated with little technical difficulty and are therefore attractive alternatives to DLLC.

Spisek *et al* (2002) have previously described the induction of leukaemia-specific response by cross-presentation of late-apoptotic leukaemic blasts by autologous DC of non-leukaemic origin. Tumour-mDC fusion hybrids have

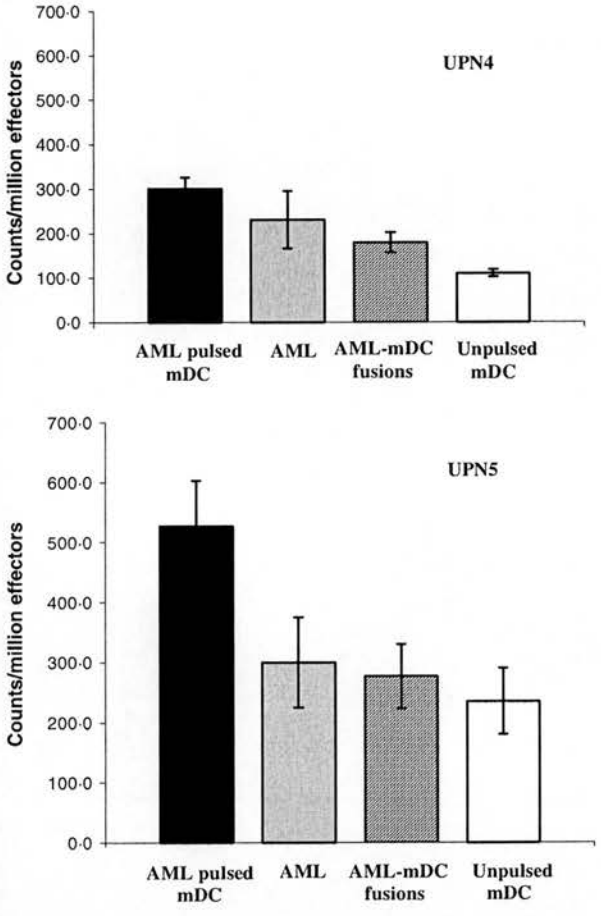
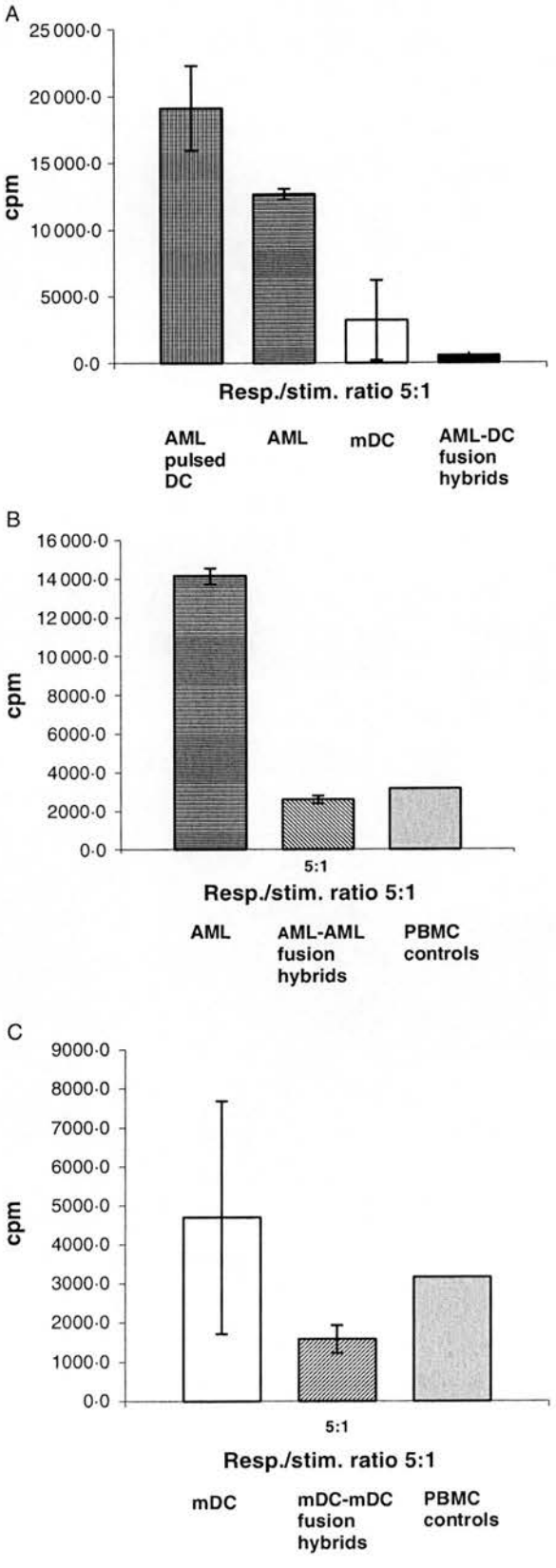


Fig 6. In two patients (UPN4, UPN5) AML-pulsed mDC (■) were better stimulators of anti-leukaemic T-cell responses than AML–DC fusion hybrids (▨), autologous AML blasts (▩) and unpulsed mDC (□). ELISPOT IFN-γ release assay counts at an E : T ratio of 20 : 1 are shown.



been generated and successfully used as vaccines in the prevention and treatment of murine models of breast carcinoma (Gong *et al*, 1997; Gong *et al*, 1998; Gong *et al*, 2000; Chen *et al*, 2003) and induction of *in vitro* cytotoxicity has been demonstrated in B-cell chronic lymphocytic leukaemia (Kokhaei *et al*, 2003) and multiple myeloma (Raje *et al*, 2004). Evidence for their effectiveness as vaccine constructs in AML, however, is limited. Galea-Lauri *et al* (2002) have previously reported one case of successful *in vitro* induction of CTL responses to AML blasts by

Fig 7. (A) AML-pulsed mDC (▥), but not AML–mDC fusion hybrids (■) stimulate proliferation of T cells autologous to the mDC and allogeneic to the AML blasts. (B) A similar antiproliferative effect is induced by allogeneic AML–AML fusion (▧) and (C) autologous mDC–mDC hybrids (▨), suggesting a mechanism not specific to individual fusion partners. PBMC controls (▩) were cultured without stimulators.

autologous fusion hybrids and support this finding by assessment of DC loading strategies with antigens derived from the U937 leukaemic cell line, including U937–DC fusion hybrids (Galea-Lauri *et al*, 2002).

We have been able to elicit leukaemia-directed T-cell responses in three of six patients whose remission PBMC were co-cultured with autologous AML–mDC fusion hybrids. In two cases, anti-leukaemic cytotoxicity generated by fusion hybrids was demonstrated by ^{51}Cr-release assay (Fig 4), in a third case T-cell responses to fusion hybrids exceeded those of other autologous stimulators in an IFN-γ ELISPOT assay (Fig 5A).

In further two of three patients, where AML–mDC fusion hybrids proved ineffective in stimulating leukaemia-directed cytotoxicity above the level induced by native AML blasts, however, mDC cocultured with irradiated AML blasts did induce anti-leukaemic T-cell responses (Fig 6).

The determinants of successful CTL generation by either strategy require further investigation. We have shown that PEG-generated fusion hybrids exert anti-proliferative effects on co-cultured lymphocytes. This appeared to be independent of the fusion partners and was not induced by titrated addition of PEG to cultured PBMC alone. In spite of this anti-proliferative property AML–mDC fusion hybrids remain capable of generating potent anti-leukaemic cytotoxic responses, further emphasizing the potential of these hetero-karyons as cellular vaccines. Their efficacy might be further enhanced by exclusion of non-viable hybrids by means of immunomagnetic selection prior to co-culture, while positive selection of fused from unfused cells based on their characteristic forward and side-scatter profile (Galea-Lauri *et al*, 2002) could be achieved using a cell-sorting facility. Purified hybrid cells from DC and tumour cell fusions have previously been shown to be more effective in inducing an immune response (IFN-γ) when compared with fusion mixture (Li *et al*, 2001). Furthermore, residual unhybridized AML blasts may anergize T cells.

All these parameters could affect the proliferation response when using DC fusion hybrids to stimulate autologous anti-leukaemic T cells.

In conclusion this study provides preclinical data on developing a DC-based vaccination strategy for AML. AML–mDC fusion hybrids can be generated with relative ease and hold promise as a novel potential cellular vaccine, providing an alternative to whole tumour cell pulsed mDC or DLLC.

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References

Banchereau, J. & Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature*, **392,** 245–252.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B. & Palucka, K. (2000) Immunobiology of dendritic cells. *Annual Review of Immunology*, **18,** 767–811.

Brouwer, R.E., van der Hoom, H.M., Kluin-Nelemans, H.C., Zelderen-Bhola, S., Willemze, R. & Falkenburg, J.H. (2000) The generation of dendritic-like cells with increased allostimulatory function from acute myeloid leukemia cells of various FAB subclasses. *Human Immunology*, **61,** 565–574.

Buggins, A.G., Lea, N., Gaken, J., Darling, D., Farzaneh, F., Mufti, G.J. & Hirst, W.J. (1999) Effect of costimulation and the micro-environment on antigen presentation by leukemic cells. *Blood*, **94,** 3479–3490.

Buzyn, A., Petit, F., Ostankovitch, M., Figueiredo, S., Varet, B., Guillet, J.G., Ameisen, J.C. & Estaquier, J. (1999) Membrane-bound Fas (Apo-1/CD95) ligand on leukemic cells: a mechanism of tumor immune escape in leukemia patients. *Blood*, **94,** 3135–3140.

Charbonnier, A., Gaugler, B., Sainty, D., Lafage-Pochitaloff, M. & Olive, D. (1999) Human acute myeloblastic leukemia cells differentiate in vitro into mature dendritic cells and induce the differentiation of cytotoxic T cells against autologous leukemias. *European Journal of Immunology*, **29,** 2567–2578.

Chen, D., Xia, J., Tanaka, Y., Chen, H., Koido, S., Wernet, O., Mukherjee, P., Gendler, S.J., Kufe, D. & Gong, J. (2003) Immunotherapy of spontaneous mammary carcinoma with fusions of dendritic cells and mucin 1-positive carcinoma cells. *Immunology*, **109,** 300–307.

Choudhury, A., Gajewski, J.L., Liang, J.C., Popat, U., Claxton, D.F., Kliche, K.O., Andreeff, M. & Champlin, R.E. (1997) Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood*, **89,** 1133–1142.

Choudhury, B.A., Liang, J.C., Thomas, E.K., Flores-Romo, L., Xie, Q.S., Agusala, K., Sutaria, S., Sinha, I., Champlin, R.E. & Claxton, D.F. (1999) Dendritic cells derived in vitro from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. *Blood*, **93,** 780–786.

Galea-Lauri, J., Darling, D., Mufti, G., Harrison, P. & Farzaneh, F. (2002) Eliciting cytotoxic T lymphocytes against acute myeloid leukemia-derived antigens: evaluation of dendritic cell-leukemia cell hybrids and other antigen-loading strategies for dendritic cell-based vaccination. *Cancer Immunology, Immunotherapy*, **51,** 299–310.

Gong, J., Chen, D., Kashiwaba, M. & Kufe, D. (1997) Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nature Medicine*, **3,** 558–561.

Gong, J., Chen, D., Kashiwaba, M., Li, Y., Chen, L., Takeuchi, H., Qu, H., Rowse, G.J., Gendler, S.J. & Kufe, D. (1998) Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, **95,** 6279–6283.

Gong, J., Apostolopoulos, V., Chen, D., Chen, H., Koido, S., Gendler, S.J., McKenzie, I.F. & Kufe, D. (2000) Selection and characterization of MUC1-specific CD8+ T cells from MUC1 transgenic mice immunized with dendritic-carcinoma fusion cells. *Immunology*, **101,** 316–324.

Harrison, B.D., Adams, J.A., Briggs, M., Brereton, M.L. & Yin, J.A. (2001) Stimulation of autologous proliferative and cytotoxic T-cell responses by "leukemic dendritic cells" derived from blast cells in acute myeloid leukemia. *Blood*, **97,** 2764–2771.

Hirano, N., Takahashi, T., Takahashi, T., Ohtake, S., Hirashima, K., Emi, N., Saito, K., Hirano, M., Shinohara, K., Takeuchi, M., Taketazu, F., Tsunoda, S., Ogura, M., Omine, M., Saito, T., Yazaki, Y., Ueda, R. & Hirai, H. (1996) Expression of costimulatory molecules in human leukemias. *Leukemia*, **10,** 1168–1176.

Kokhaei, P., Rezvany, M.R., Virving, L., Choudhury, A., Rabbani, H., Osterborg, A. & Mellstedt, H. (2003) Dendritic cells loaded with apoptotic tumour cells induce a stronger T-cell response than dendritic cell-tumour hybrids in B-CLL. *Leukemia*, **17,** 894–899.

Li, J., Holmes, L.M., Franek, K.J., Burgin, K.E., Wagner, T.E. & Wei, Y. (2001) Purified hybrid cells from dendritic cell and tumor cell fusions are superior activators of antitumor immunity. *Cancer Immunology, Immunotherapy*, **50,** 456–462.

Raje, N., Hideshima, T., Davies, F.E., Chauhan, D., Treon, S.P., Young, G., Tai, Y.T., Avigan, D., Gong, J., Schlossman, R.L., Richardson, P., Kufe, D.W. & Anderson, K.C. (2004) Tumour cell/dendritic cell fusions as a vaccination strategy for multiple myeloma. *British Journal of Haematology*, **125,** 343–352.

Robinson, S.P., English, N., Jaju, R., Kearney, L., Knight, S.C. & Reid, C.D. (1998) The in-vitro generation of dendritic cells from blast cells in acute leukaemia. *British Journal of Haematology*, **103,** 763–771.

Roddie, P.H., Horton, Y. & Turner, M.L. (2002) Primary acute myeloid leukaemia blasts resistant to cytokine-induced differentiation to dendritic-like leukaemia cells can be forced to differentiate by the addition of bryostatin-1. *Leukemia*, **16,** 84–93.

Schmittel, A., Keilholz, U. & Scheibenbogen, C. (1997) Evaluation of the interferon-gamma ELISPOT-assay for quantification of peptide specific T lymphocytes from peripheral blood. *Journal of Immunological Methods*, **210,** 167–174.

Spisek, R., Chevallier, P., Morineau, N., Milpied, N., Avet-Loiseau, H., Harousseau, J.L., Meflah, K. & Gregoire, M. (2002) Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of non-leukemic origin. *Cancer Research*, **62,** 2861–2868.

Current progress in the development of a cell-based vaccine for the immunotherapy of acute myeloid leukemia

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Evidence that immunological control contributes to the elimination of residual leukemia has emerged from allogeneic hematopoietic stem cell transplantation. This review assesses the current understanding of immunobiology of acute myeloid leukemia and how dendritic cells and T cells may be harnessed using *in vitro* and *in vivo* priming techniques. Preclinical and clinical dendritic cell vaccine trials reported to date are considered and the prospects for immunotherapy with dendritic cell-based vaccine constructs evaluated.

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Current concepts of immune-mediated treatment modalities for acute myeloid leukemia

Acute myeloid leukemia (AML) is a biologically and clinically heterogeneous disease characterized by a clonal, neoplastic proliferation of hematopoietic progenitor cells. Immunological control of this disease, resulting in spontaneous remission, is exceedingly rare, but has been reported after bacterial infections [1], and the discovery of tumor-related antigens recognized by cytotoxic lymphocytes has further highlighted the role of an immune response seen in patients with leukemia.

Intensive multiagent chemotherapy, however, has been the backbone of AML treatment for decades. Complete remissions are achievable with this approach in 70–80% of patients below 60 years of age. However, disease relapse originating from minimal residual disease (MRD) results in an overall 5-year survival of less than 30% [2]. Superior long-term disease-free survival of 50–60% can be achieved in patients that have undergone allogeneic hematopoietic stem cell transplantation (HSCT) and, in recent years, evidence has emerged that much of the therapeutic benefit of this procedure is derived from an immunologically mediated graft-versus-leukemia (GvL) effect. This has led to the development of reduced intensity, nonmyeloablative transplant conditioning regimes, designed to allow donor engraftment, rather than direct tumor cytotoxicity, and the use of donor-lymphocyte infusion (DLI) as a means to treat relapse, following allogeneic HSCT [3,4], proving that immune surveillance of residual leukemic blasts by donor T lymphocytes is possible.

More up-to-date evidence points towards the role of natural killer (NK) cells in controlling and eradicating leukemia in a situation of haplotype-mismatched, haploidentical transplantation. Mismatch for killer immunoglobulin-like receptors (KIR) between donor and recipient pairs in this particular subgroup is associated with activation of donor-derived NK cells that contribute to immune control of MRD and lower relapse risk compared with KIR-matched donor–recipient pairs [5–7]. Recently, a number of specific peptides have been identified that may represent appropriate targets for immune responses in the context of allogeneic HSCT. The hematopoiesis-specific minor histocompatibility antigens (HA)-1 and -2 may act as malignancy-specific targets in the context of allogeneic HSCT [8–10].

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These treatment modalities provide a model for the success- integration of immunotherapeutic approaches to MRD in rrent clinical practice and have improved long-term clinical tcomes. However, there is a need for novel treatment strate-s, in particular for older patients, owing to the unacceptably gh morbidity and mortality associated with HSCT proaches. In clinical practice, the development of GvL is en associated with significant graft-versus-host disease vHD). Unfortunately, GvHD remains a major cause of nsplantation-related mortality (TRM) and limits the use of SCT to younger patients without significant comorbidity.

In recent years, a better understanding of the mechanisms volved in the immune escape of tumors together with the ntification of tumor-associated antigens (TAAs) in AML, ch as the Wilms' tumor gene product WT1 [11–13], oteinase-3 [14–16] and protein products of abnormal fusion nes, such as B-cell antigen receptor (*BCR*)-*ABL* in chronic eloid leukemia (CML) and *PML-RARα* in AML [17,18], have omoted new immunotherapeutic approaches to leukemia tside the setting of HSCT. Dendritic cells (DCs), as profes-nal antigen-presenting cells (APCs) that are able to elicit a totoxic response from naive T cells, have been at the center of erest in developing a cellular vaccine for AML.

development & T-cell interaction *in vivo*

vivo DCs form a heterogeneous group of subsets, continu-y produced from myeloid and lymphoid hematopoietic ecursors in the bone marrow, and widely distributed as mature DCs into lymphoid and nonlymphoid sues [19–22]. Immature DCs, including epidermal Langer-ns cells, splenic marginal zone DCs and interstitial DCs in nlymphoid tissues, continually sample self-antigen to intain T-cell tolerance. However, immature DCs can also e up foreign antigens. When triggered by pathogens, the ttern-recognition receptors expressed by immature DCs use them to mature in the presence of inflammatory 'dan- signals', such as tumor necrosis factor (TNF), interferon FN)-α or bacterial lipopolysaccharide (LPS) [23]. Mature Cs are able to initiate primary T-cell immune responses by ivating naive T cells [19,24] because they express high ounts of cell-surface major histocompatibility complex HC) and costimulatory molecules [19–22], as well as adhe-n molecules and chemokine receptors (CCRs), such as CR-7 and -8 [25], enabling them to migrate to the second- lymphoid tissues, where they present antigen to T cells. e APCs are thought to take up exogenous antigens, luding apoptotic tumor cells, and present these to lym-ocytes via a classical MHC class II pathway, but 'cross-sentation' of these antigens through MHC class I mole-es has also been demonstrated. Such cross-presentation been associated with the stimulation of $CD4^+$ T-helper n) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), gether providing the best conditions for cell-mediated otoxicity. T-cell activation requires two different signals URE 1). The first signal is provided by the interaction of the MHC molecule peptide with the corresponding T-cell receptor. The second signal consists of the binding of costimulatory molecules on the APC with their respective ligands on the T cell. CD40 and B7 on the APC interact with CD40 ligand and CD28 on the T cell to mediate T-cell activation.

The remarkable functional heterogeneity of DC subsets is due to their maturational state, the microenvironment to which the DCs are exposed in various tissues *in vivo*, and a variety of other factors, such as the strength of the T-cell receptor–MHC class II interaction and antigen density, which are also known to influence the ability of DC to induce:

- An interleukin (IL)-12-driven type 1 T-cell (Th1) response, promoting T-cellular cytotoxicity
- A Th2 T-cell response, polarizing towards antibody-mediated immunity
- To induce anergy of a T cell following a tolerizing interaction with a DC

This review will consider the way in which DC function and interaction with other components of the immune system (T cells, NK cells) may be altered or defective in patients with AML, favoring tumor escape from immunological surveillance, as previously demonstrated in animal models of malignancy and in cancer patients [26–29].

DCs in leukemia: part of the problem or the solution?

The role of DCs in cancer has been described in numerous studies of a variety of tumors [30–32], postulating an association of good clinical prognosis with the presence of DCs in tumor tissues. However, functional defects, mainly lack of expression of costimulatory molecules in tumor-associated DCs, have also been identified in more recent animal model studies and in cancer patients [26–29]. In leukemia, similar to other malignancies, release of immunosuppressive cytokines, such as IL-10 [33,34], transforming growth factor (TGF)-β [35–37], and vascular endothelial growth factor (VEGF), and other known soluble factors, such as soluble IL-2 receptor [38], soluble TNF receptor [39] and soluble Fas ligand [40], as well as other, as yet unspecified factors [41], have been implicated in creating an immunosuppressive microenvironment that may not favor the induction of cell-mediated immunity. Recent investigations have shed more light on the ontogeny of both myeloid and plasmacytoid DCs in the peripheral blood of patients with AML and myelodysplastic syndrome (MDS) [42,43]. Although both DC subsets are present in MDS, albeit in lower numbers compared with those in normal blood, both populations are demonstrably derived from the malignant clone [42]. Likewise, in the peripheral blood of AML patients, both DC subsets have been identified [43]; however, the identification of subsets by immunophenotypic characteristics may be complicated by the fact that DCs and leukemic blasts share many markers, hence leukemic blasts can be misidentified as DCs on the basis of abberant expression of DC-associated markers on the surface of leukemic blasts that are not DCs.

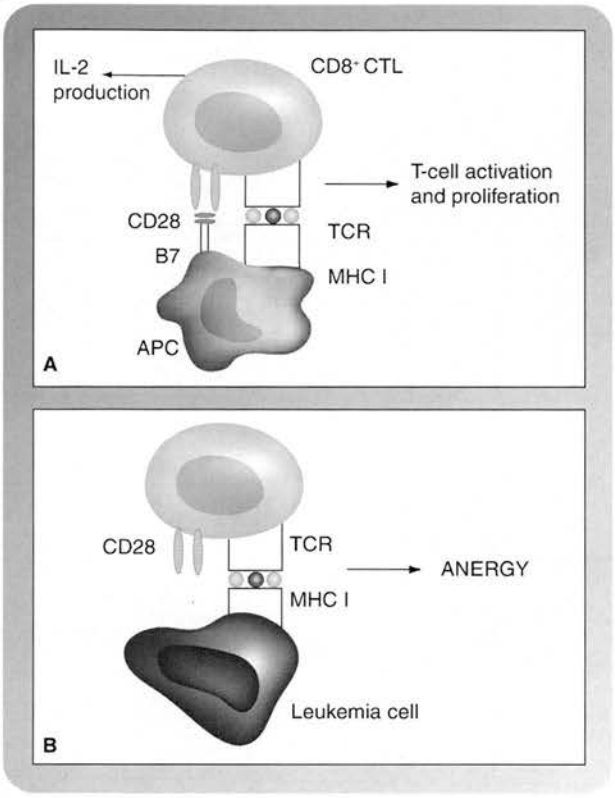

Figure 1. Mechanisms of T-cell activation. (A) Recognition of MHC–peptide complexes on the surface of an APC by a TCR in the presence of a second costimulatory signal mediated by B7 leads to T-cell activation and proliferation. **(B)** Absence of B7 expression by leukemia cells results in an absent costimulatory signal following TCR binding of MHC–peptide complex with consequent T-cell anergy.
APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; IL: Interleukin; MHC: Major histocompatability complex; TCR: T-cell receptor.

In vitro, cytokine-driven differentiation of AML blasts to a dendritic-like phenotype (AML-DC) can be achieved in a proportion of patients [44–48]. As these AML-DCs arise from the leukemic clone, they offer potential as autologous cellular vaccines, expressing both leukemic antigens and DC-characteristic, costimulatory molecules that are mostly lacking in native leukemic blasts. Coculture experiments of AML-DC with autologous T cells have induced antileukemic cytotoxicity [49]. However, as DC populations originating from leukemic blasts *in vivo* are part of the malignancy and may potentially also produce 'tolerogenic' signals, the question arises if AML-DC *in vivo* might be implicated in the disease process and, if so, would their use in therapy be advisable? The fact that the malignant cells are DC precursors, and therefore components of the immune interaction one attempts to study, complicates the characterization of immune responses to AML. Studies of antileukemic T-cell responses in leukemic patients in remission [45,50] and those with fulminant disease [12,51] have been undertaken. CTLs directed against tumor-associated antigens, such as WT1 and proteinase 3, have been demonstrated at some point of the disease process. Such observations have led some investigators to the suggestion that immune responses to tumor might be actively inhibited in advancing disease [52,53], while others have postulated that, in patients with malignancy, immune responses to TAAs are only initiated in disseminated, overwhelming disease and are only detectable at low levels in a situation of low tumor burden [54,55]. In either case, immune control of the tumor ultimately would be ineffective, but the exact immunosuppressive mechanisms remain unclear. Could DCs derived from malignant precursors, one of the unique features of AML, be part of the pathology of AML? If AML-DCs respond to stimuli in their environment with a capacity similar to that of normal marrow precursor-derived DCs, then the pathology in AML may not be at the level of the DC itself, but with the responder T and NK cells. In this case, the use of AML-DCs in therapy of AML may be of benefit. In cases where AML-DCs differ from normal DCs in their responses to stimuli, AML pathology may in part be attributed to the AML-DC itself and their use in therapy of the disease may be less effective. Semimature DCs may trigger regulatory T cells (Tregs), such as IL-10-producing $CD4^+$ $CD25^+$ cells, capable of silencing the immune response, whereas specific manipulation of malignant cells into APCs with a mature phenotype may trigger the immune system to suppress or eradicate leukemia.

Cellular vaccine design strategies in AML

Any successful cellular vaccine needs to overcome the reduced immunogenicity of leukemic cells. This requires the generation of vaccine cells in large numbers to express leukemia-specific antigens in the context of adequate costimulation. Two principal strategies have been pursued in DC-based leukemia vaccine generation:

- Improving the antigen-presenting capacity of leukemia cells themselves: this can be achieved by either transfer of genes that encode the necessary costimulatory molecules into leukemic blasts [56–64] or by cytokine-driven leukemic blast maturation into AML-DC [44–49]
- Utilizing the superior T-cell stimulatory capacity of non-leukemic DCs: either first, by adoptive immunotherapy, using *ex vivo*-generated CTLs specific for TAAs, or second, through vaccination protocols with professional APCs expressing defined TAAs (FIGURE 2) [65–70]

To deliver leukemia-associated antigens, DCs can be generated *ex vivo* from either monocytes [71,72] or $CD34^+$ hematopoietic progenitor cells [73–75]. They can be pulsed as immature DCs *ex vivo* with tumor antigens, matured with cytokines and then injected as cellular vaccines. Reported approaches for this purpose include the following:

- Pulsing DCs with leukemic blast lysates [76,77]
- Pulsing DCs with irradiated or apoptotic blasts [75,78]
- Pulsing DCs with synthetic peptides from tumor-associated antigens (e.g., WT1 in AML, PR1 in AML/CML [11–16,79])

Pulsing DCs with tumor-specific antigens derived from protein products of abnormal fusion genes, (e.g., *BCR-ABL* in CML, *PML-RARα* and *DEK-CAN* in AML [17,18,80])

Loading DCs with tumor-derived RNA or DNA [81]

Fusing DCs with leukemic blasts, generating AML-DC hybridomas [82,83]

AML-DC as cellular vaccine

A strategy explored by several investigators over the last decade [44,47,49,65–70] is the differentiation and maturation of leukemic blasts into dendritic-like cells (AML-DCs). This strategy followed the emerging knowledge of DC development from myeloid progenitors. A variety of different cytokine cocktails, including granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-4, IL-13, TNF-α, ferritin light chain (FLT)-3 ligand and immunomodulators, such as soluble CD40 ligand, have been proposed to induce dendritic-like differentiation and maturation. The 'essential cocktail', identified for generating DCs from blood monocytes, consisting of GM-CSF and IL-4 [84], has been complemented by CD40 ligand or TNF-α to induce differentiation in 18 out of 19 primary AML samples in a seminal study by Choudhury and colleagues [47]. The resulting AML-DC

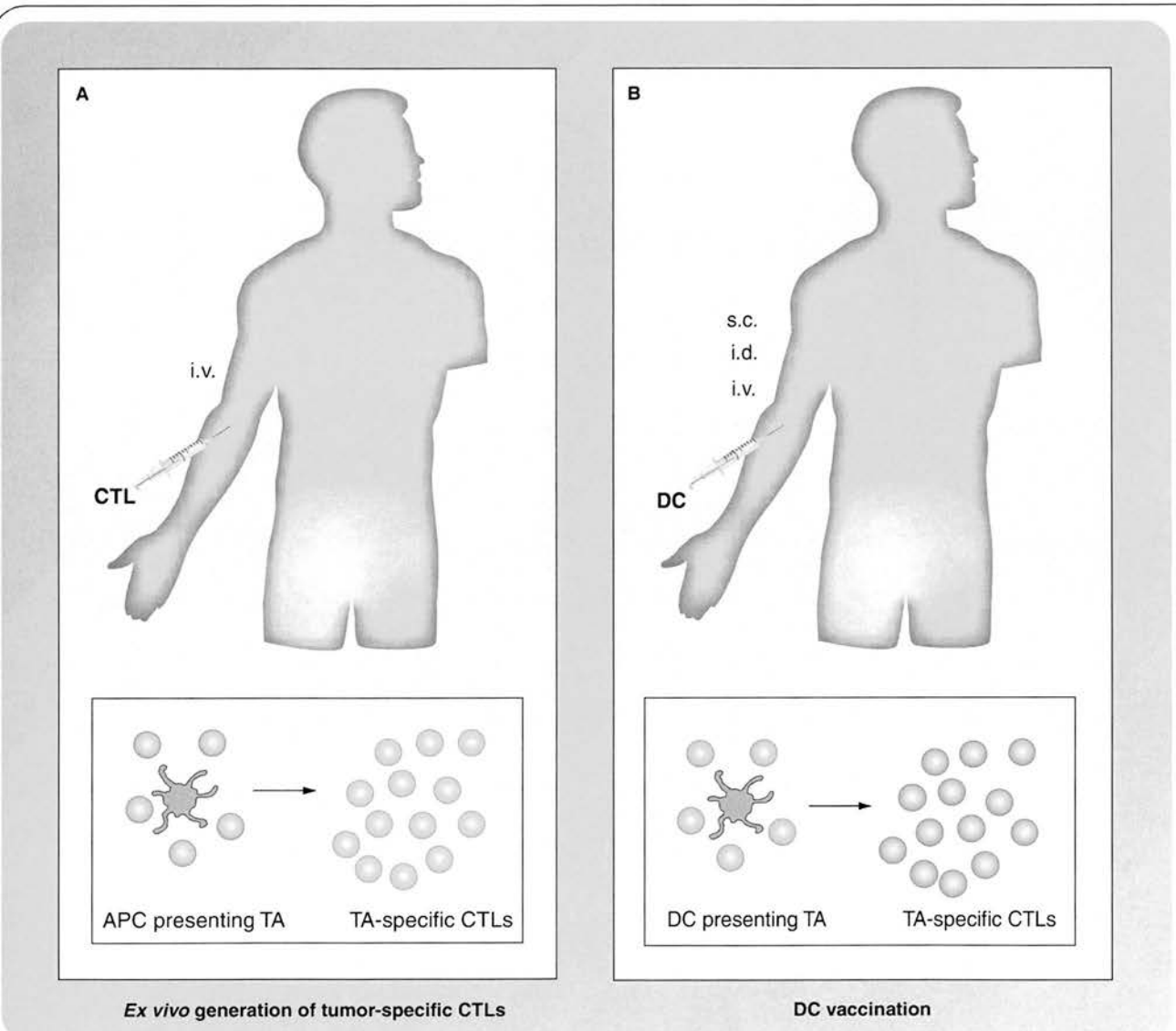

Figure 2. Two strategies for generating antitumor immunity. (A) CTLs are generated *ex vivo* by coculture of autologous or allogeneic T cells with APCs that expressed a defined tumor antigen. Tumor antigen-specific CTLs are then injected intravenously into the patient. **(B)** DCs are generated from the patient and are engineered to express a defined tumor antigen. The DCs are then injected into the patient (either by the s.c., i.d. or i.v. route) and stimulate the *in vivo* development of tumor-specific immunity.
APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; i.d.: Intradermal; i.v.: Intravenous; s.c.: Subcutaneous; TA: Tumor antigen.

showed the characteristic morphology, immunophenotypic properties and T-cell stimulatory properties of mature DCs. Importantly, coculture of autologous T lymphocytes with the AML-DC induced cytotoxic responses directed against the native leukemic blasts, but not against normal autologous cells. Several other studies have confirmed these findings in a proportion of AML blasts investigated [46,85]. The heterogeneity of AML, however, is reflected in the fact that a significant proportion of AML samples prove resistant to cytokine-induced differentiation into fully mature AML-DCs. There are, to date, no published clinical vaccination studies utilizing AML-DCs as cellular vaccines in AML. The authors have recently completed a Phase I/II clinical vaccination study using autologous dendritic-like leukemic cells (AML-DCs) in adult patients with AML in remission, following multiagent chemotherapy, demonstrating the clinical feasibility of this approach and showing antileukemic immune responses following AML-DC vaccination [86]. Using stringent criteria for the generation of AML-DCs from AML blasts, seven out of 22 samples could be differentiated and matured using GM-CSF, IL-4, TNF-α, Poly I:C and IFN-γ. Five patients both achieved complete remission and also had leukemia cells that were permissive to differentiation, and were therefore eligible to proceed to vaccination. Four escalating doses of dendritic-like leukemia cells were administered weekly by subcutaneous injection. An increase in antileukemic T-cell responses was demonstrated in four patients. Of the five patients, two are in continued complete remission of greater than 12 months' duration following vaccination. The remaining patients had relapse of their leukemia.

DC-based vaccination

Clinical Phase I/II study protocols utilizing tumor antigen-loaded DCs have, in principle, documented the feasibility of this approach and demonstrated variable degrees of immunological and clinical *in vivo* responses in patients with melanoma, prostate and ovarian cancer [87–92]. Partial regressions of solid tumors have been demonstrated in a proportion of patients in these studies, with occasional complete responses demonstrated. These early reports are encouraging, but difficult to compare and reproduce, owing to methodological differences in all aspects of study design.

Few clinical vaccination trials in leukemia are published. T-cellular cytotoxic responses to TAA-derived peptides have been studied in the most depth. Molldrem and colleagues have demonstrated that human leukocyte antigen (HLA)-A2-restricted CTLs raised against PR1 peptide, a human leukemia-associated antigen derived from proteinase-3, an aberrantly expressed protein in myeloid leukemia cells, preferentially killed leukemic over normal hematopoietic precursors (79% cytotoxicity against CML cells, 54% lysis of AML blasts) [15]. A later study investigated the correlation between the presence of PR1-specific T cells [50], detected by PR1–HLA-A2 tetrameric complexes in the peripheral blood of leukemic patients following treatment with IFN-α and allogeneic bone marrow transplantation, giving first-time evidence of the role of T-cell immunity in clearing malignant leukemic cells. A subsequent clinical vaccination study with PR1 peptide and adjuvant GM-CSF in 35 patients with AML, CML and MDS elicited both immunologically demonstrable (60% of patients) and clinical responses in patients with refractory and relapsed myeloid leukemia [93].

In AML, there are, to date, no published clinical trials exploiting DC based vaccination strategies as an adjuvant therapeutic approach for MRD following multiagent intensive chemotherapy, where, in the authors' opinion, the chances of immunological control of disease recurrence appear most promising. The only clinical DC vaccination study, undertaken by Lee and colleagues, utilized blast cell lysate-pulsed DCs in two patients in relapse following autologous HSCT [76]. In this setting, immunological responses to vaccination are difficult to differentiate from those directed against advancing leukemic tumor burden, but evidence of delayed hypersensitivity and increased mixed lymphocyte responses (MLRs) were attributed to the subcutaneous vaccination protocol.

A larger number of preclinical studies have been conducted, exploiting various DC-loading protocols.

Schui and colleagues pulsed DCs with tumor lysates and have induced autologous antileukemic cytotoxicity in cocultured T cells in five out of 25 patients only, while in other patients, an inhibitory effect was seen [77]. Spisek and colleagues demonstrated the ability of nonleukemic DCs loaded with late apoptotic leukemic blasts to induce *in vitro* leukemia-specific T-cellular cytotoxicity in three patients [78]; direct comparison with the cytotoxicity induced by AML-DC was only possible in one patient and was found to be similar. An earlier study by Fuji and colleagues showed the induction of antileukemic CTLs by $CD34^+$-derived DC clusters pulsed with autologous, irradiated blasts [75].

In AML and advanced lymphoblastic leukemia, HLA-A2-binding peptides derived from the Wilms' tumor gene-encoded transcription factor WT-1 [94] stimulate a specific antileukemic T-cell response [14,16,95]. Similarly, DCs loaded with mucin (MUC)-1 protein, an epithelial mucin that is overexpressed in AML and multiple myeloma, facilitate the generation of antitumor T-cell responses *in vitro*.

In a subgroup of leukemia patients, chromosomal translocations are demonstrable in the leukemic clone, resulting in gene rearrangements and giving rise to fusion gene protein products, (e.g., *BCR-ABL* in CML and *PML-RARα* in AML) [17,18]. These proteins are potential targets of an immune response. They are attractive for immunotherapy, as they represent true tumor-specific antigens. Furthermore, as some of these fusion proteins are essential for the malignant transformation of the clone, tumor escape through antigen loss in response to selective immunological pressure is unlikely. However, in spite of these attractive features, the potentially immunogenic site of a fusion protein is only located in the amino acid sequences immediately flanking the fusion site. Cleaving by proteases is necessary to arrive at candidate antigenic peptides. Those peptides will have

fit the binding motif of a given patient's HLA molecules in der to elicit CTL responses. This HLA restriction makes it likely that a universal antigenic peptide sequence derived om a fusion protein that would be useful in the majority of tients presenting with a given chromosomal translocation will found.

The authors compared the potency of AML-DC fusion brids to that of DCs pulsed by irradiated tumor bodies to duce *in vitro* leukemia-specific, autologous cytotoxicity in tients with AML [82]. Remission monocytes are matured into Cs and then fused to autologous AML blasts using lyethylenglycol (PEG) as the fusing agent, or by electroporan. The resulting constructs express the full repertoire of ukemic antigens on their surface in conjunction with DC-rived costimulatory molecules. There is no need to establish e HLA type of the patient; in contrast to approaches using lsing of DCs with specific peptides or antigens, no prior entification of TAAs expressed by the individual leukemia sts is required and the fusion technology using PEG is well ablished and simple, making this approach more easily appli-ble. It also avoids the technical difficulties and safety con-rns that surround the use of viral vectors to generate geneti-ly modified leukemic cells for vaccination. Fusion hybrids duced the strongest cytotoxic responses in three out of six tients studied [82]. DCs pulsed by tumor bodies were superior two other patients. Only one patient failed to generate ML-directed cytotoxicity in response to either construct. The thors conclude that AML-DC fusion hybrids hold promise a cellular vaccine for AML.

optive immunotherapy

is approach using *ex vivo*-generated CTLs has been ploited in several clinical and preclinical studies. Mutis and leagues have built on the recognition of minor histocom-tibility antigen (mHAg) mismatch as important factors in e development of GvHD and GvL in allogeneic HSCT [9]. me mHAgs show ubiquitous tissue distribution, others, ch as the mHAgs HA-1 and -2, are only expressed in matopoietic cells [96]. These antigens are therefore attractive As for immunotherapy, as they should selectively direct an ogeneic cytotoxic response against hematopoietic tumor ls. Donor-derived DCs from a HA-1 or -2-mismatched nor are pulsed with HA-1 or -2 peptides corresponding to e recipient's mHAg type. These DCs are cocultured *ex vivo* th donor lymphocytes to generate CTLs specific for these otides. Subsequently, the patients undergo an allogeneic SCT from their HLA-compatible, but HA-1- or -2-mis-tched donor. After complete engraftment of donor-derived matopoiesis, the *ex vivo*-generated mHAg-specific CTLs are used into the patient with antileukemic cytotoxic effect, thout causing GvHD. This approach is limited to a small mber of patients that are undergoing an allogeneic HLA-tched HSCT from a donor with HA-1 or -2 disparity. ore widely applicable strategies have used the characteriza-n of TAAs demonstrated to induce CTL responses specific to tumor cells in hematological malignancies. The transcription factor WT1, which is widely expressed on the leukemic cells from patients with AML and CML, has been demonstrated to be a potential target for *in vitro* CTL generation. Using APCs pulsed with HLA-A2-restricted WT1 peptides, CTLs were generated from peripheral blood mononuclear cells that showed exquisite specificity of action against $CD34^+$ leukemia cells, but spared normal $CD34^+$ progenitors [14]. Some of the limiting factors to successful translation of this type of *ex vivo* CTL generation into clinical practice include the very prolonged period of culture necessary to obtain clinically relevant numbers of CTLs, with all the attendant difficulties that this entails, and the limitations set by MHC class-restricted DC presentation of tumor peptides that may not always be available for every HLA type.

Considerations for delivering a DC-based vaccine for AML

Many facets of DC-based vaccines in AML remain unknown, as the clinical application of this approach is in its infancy. From experiences with earlier DC vaccination studies in solid tumor malignancies, it may be deduced that the state of MRD, achieved following conventional chemotherapy or HSCT, is a desirable setting for the trial of leukemia cell vaccines. Patients with MRD have a low tumor burden. DC-induced CTL responses are likely to be overwhelmed by rapidly advancing leukemia [97] and, importantly, immunological control of MRD has been demonstrated to be associated with superior long-term survival and lower relapse rates originating from residual leukemia in allogeneic HSCT. Preclinical studies have emphasized the importance of vaccinating with fully mature DCs in order to avoid tolerogenic responses associated with immature or semimature forms. Optimal dose, scheduling and route of delivery remain controversial. DCs are frequently administered subcutanuously, intradermally or by direct injection into lymphatics/draining lymph nodes. Recent studies suggest that immature DCs migrate less than mature DCs. De Vries and colleagues demonstrated that, in general, less than 5% of intradermally administered mature DCs reach the draining lymph node [98]. From these data, it can be speculated that increased migration would increase the efficacy of DC vaccines. Attempts to increase migration include the concomitant use of inflammatory cytokines to condition tissues [99]. Matrix metalloproteinases and Toll-like receptor (TLR) ligands might also enhance DC migration [100].

Any *ex vivo* manipulation of human cells must be undertaken conforming to Good Manufacturing Practice (GMP) standards laid down in the USA and the EU. Facilities complying with these standards are regulated in the USA by the Center for Biologics Evaluation and Research. In the UK, the Department of Health, through the Medicines and Healthcare Regulatory Authority, has established an inspectorate whose remit includes cellular therapy. Investigators have to ensure that clinical vaccination studies using cellular products are compliant with standards that are already established in

the commercial pharmaceutical industry. This includes cytokines used in the process that must be of clinical grade standard and cell culture media that should only contain human serum protein or ideally be serum free. The use of fetal calf serum is no longer deemed appropriate.

For any vaccination approach that uses leukemia-derived DCs, prevention of re-engraftment of viable, potentially proliferating cells is of importance. The authors established that 25 Gy of γ-irradiation from a caesium-137 source, routinely used for irradiation of blood products, completely abolished the proliferative potential of AML blasts in tissue culture and have irradiated all AML-DC vaccines with this dose prior to inoculation.

Monitoring responses to DC vaccination in AML

The use of objective criteria to describe clinical responses, as codified by the WHO, and in solid tumor response evaluation schemes (RECIST) [101] would advance the field of DC immunotherapy. Many early studies are compromised by a lack of standardization of clinical responses and no demonstration of specific antineoplastic immune responses.

In a proportion of myeloid leukemias, recurrent chromosomal abnormalities (translocations and inversions) can be detected. Those can be used to quantify the residual tumor burden using highly sensitive, quantitative reverse transcriptase (RT)-PCR assays. In cases without demonstrable cytogenetic defects, stably expressed TAAs, such as WT-1 [94], have been used to quantify MRD from blood and bone marrow. These assays represent minimally invasive, sensitive and validated tools, applicable to the monitoring of treatment responses post-chemotherapy and -HSCT. They can be utilized to objectively quantify treatment responses to DC immunotherapy in a similar way.

Any future attempt to increase the effectiveness of vaccination will also depend on the ability to measure the development of antileukemic T-cell immune responses with a high degree of sensitivity and specificity. In recent years, it has become possible to visualise antigen-specific T-cells under flow cytometry by using soluble, fluorescently labelled multimeric HLA–peptide complexes [102] that bind stably, specifically and avidly to antigen-specific cells. The authors have combined tetramer assays for WT1-recognizing T cells with highly sensitive assays of T-cellular IFN-γ responses to autologous AML blasts (ELISPOT) in combination with RT-PCR for WT-1 assays of tumor burden to monitor the impact of AML-DC vaccination (TABLE 1) [103].

T regs have been investigated in many recent studies owing to their apparent immunomodulatory role in infection, autoimmunity and inhibitory effect on anticancer immune responses [104–107]. Conversely, evidence has emerged that effective anticancer immunity can be restored by suppression of Treg numbers [106,108]. Correlating the number of Tregs before, during and following DC vaccination with the variation of measurable antileukemic cytotoxicity and MRD burden may provide insight into the role of these cells in dampening antileukemic immune responses.

Expert commentary

The clinical application of DC-based vaccination therapy is still in its infancy. We have, in the first clinical study of its kind, demonstrated that leukemia-derived AML-DCs are a feasible, safe cellular vaccine for AML that has induced immunological responses in four out of five vaccinated patients. Differentiation resistance to cytokines in a significant proportion of leukemic blasts, however, limits the broad applicability of AML-DC. Several recent studies attempt to circumvent this problem by transducing an autologous vaccine with genes expressing costimulatory molecules and IL-2 [56,57]. Rousseau and colleagues constructed an autologous vaccine from leukemic blasts admixed with skin fibroblasts transduced with adenoviral vectors encoding human IL-2 and human CD40 ligand [56]. Ten patients (including seven children) with high-risk AML (n = 4) or lymphoblastic leukemia (n = 6) in cytologic remission (after allogeneic stem cell transplantation [n = 9] or chemotherapy alone [n = 1]) received up to six subcutaneous

Table 1. Methods of monitoring *in vitro* and *in vivo* responses to dendritic cell-based vaccination.

Method	Advantages	Disadvantages
Delayed type hypersensitivity reaction (skin test)	Easy to monitor and quantify	Nonspecific, might not reflect responses in other body compartments
In vitro cytotoxicity assay (Cr^{51}-release)	Demonstrates specific T-cellular cytotoxic target cell lysis	Relatively insensitive Involves γ-radiation
In vitro IFN-γ release assay (ELISPOT)	Sensitive assay, automated reading of responses on plate	IFN-γ release is surrogate marker of T-cellular cytotoxicity
HLA-tetramer analysis of tumor antigen-specific circulating CTLs	Very sensitive, specific analysis of antitumor-directed CTLs	Not available for every HLA type
MRD monitoring (quantitative RT-PCR of tumor-associated antigens/chromosomal translocations)	Sensitive, quantifiable assessment of residual tumor burden	Not applicable to every leukemia patient

CTL: Cytotoxic T lymphocyte; ELISPOT: Enzyme-linked immunosorbent spot; HLA: Human leukocyte antigen; IFN: Interferon; MRD: Minimal residual disease; RT-PCR: Reverse transcriptase polymerase chain reaction.

ections of the IL-2/CD40 ligand vaccine. Immunization pro-ced a 10- to 890-fold increase in the frequencies of MHC-tricted T cells reactive against recipient-derived blasts, demon-ting that, even in heavily treated patients, including recipients allogeneic stem cell transplants, recipient-derived antileukemia cines can induce immune responses reactive against leukemic sts. These studies represent the current clinical experience with cellular autologous AML vaccine. The challenge for future proaches will be to translate the antileukemic immune ponses observed into demonstrable clinical benefit. This may achieved by optimizing dosing, timing and sequencing of vac-e administration, as well as potentiating the vaccine through use of adjuvants and addressing the suppressive effect of Tregs. y future vaccination strategy will have to address the possibility various immunological tumor escape mechanisms and the blem of targeting dormant tumor cells [109]. It remains uncer-n whether the antileukemic role of NK cells observed in allogeic stem cell transplantation can be replicated in autologous vac-e constructs, but a recent study in a murine model suggests that an. NK cells that are activated by CXCL10 can kill dormant nor cells that resist CTL-mediated lysis [110].

e-year view

our opinion, 'whole cell'-based DC vaccines (e.g., fusion prids, nonleukemic DCs pulsed with autologous tumor ates or apoptotic tumor cell bodies) provide the most mising therapeutic strategy in the immediate future. These constructs circumvent the obstacle of differentiation resistance encountered when using AML-DCs, and provide 'polyvalent' immune stimulation, making tumor immune escape by antigen drift less likely. They do not require detailed analysis of the individual leukemic antigens presented and are not HLA restricted, thereby representing an easier, albeit individualized, vaccine design. Approaches based on transducing an autologous cellular vaccine with genes encoding costimulatory molecules and IL-2 also appear promising [56,57], providing concerns about safety using a virally transduced vaccine can be addressed. Looking further ahead, the emerging knowledge of important, immunogenic leukemia-associated antigens, in particular those that are integral to the leukemogenic process, might well reveal a number of peptides loadable to a wide range of HLA types, making this vaccine approach more widely applicable. Ultimately, *in vivo* loading of leukemic antigens and achieving activation of the patient's own DCs by targeting specific DC TLRs would seem an ideal mode of vaccine delivery. New, emerging adjuvants might greatly enhance the immune stimulation achieved by *in vivo* DC targeting.

Regardless of the chosen vaccine design, standardization of DC generation, antigen loading and maturation, as well as evaluation of the vaccination response, will be essential to allow observations from different clinical studies to be compared. Only then might an effective cellular vaccine, delivering a sustained clinical benefit to patients with low tumor burden AML post chemotherapy, emerge from the plethora of current hypotheses.

Key issues

Evidence that immunological control contributes to the elimination of residual leukemia has emerged from allogeneic hematopoietic stem cell transplantation.

Current strategies for the generation of a cellular, autologous vaccine are based on either attempts to improve the antigen-presenting capacity of leukemia cells themselves or on adoptive immunotherapy, using *ex vivo*-generated cytotoxic T cells specific for tumor-associated antigens (TAAs) or through vaccination protocols with professional antigen-presenting cells, such as dendritic cells (DCs), expressing defined TAAs.

Clinical experience with antileukemic cellular vaccines is limited at present.

Encouraging antileukemic immune responses have been demonstrated in various preclinical and clinical vaccination studies, but have so far not resulted in lasting clinical benefit, possibly because of tumor immune escape mechanisms.

Standardization of DC generation, antigen loading, maturation and vaccine administration/scheduling is essential to ensure comparability of future clinical studies and increase the efficacy of antileukemia vaccines of the future.

erences

Muller CI, Trepel M, Kunzmann R, Lais A, Engelhardt R, Lubbert M. Hematologic and molecular spontaneous remission following sepsis in acute monoblastic leukemia with translocation (9;11): a case report and review of the literature. *Eur. J. Haematol.* 73(1), 62–66 (2004).

Yin JA, Tobal K. Detection of minimal residual disease in acute myeloid leukaemia: methodologies, clinical and biological significance. *Br. J. Haematol.* 106(3), 578–590 (1999).

3 Kolb HJ, Schattenberg A, Goldman JM *et al.* Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood* 86(5), 2041–2050 (1995).

4 Collins RH Jr, Shpilberg O, Drobyski WR *et al.* Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J. Clin. Oncol.* 15(2), 433–444 (1997).

5 Ruggeri L, Capanni M, Casucci M *et al.* Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. *Blood* 94(1), 333–339 (1999).

6 Ruggeri L, Capanni M, Urbani E *et al.* Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295(5562), 2097–2100 (2002).

- **A seminal paper, suggesting natural killer contribution to graft-versus-leukemia effect in a subgroup of allogeneic transplant patients.**

7 Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* 100(6), 1935–1947 (2002).

8 Mutis T, Gillespie G, Schrama E, Falkenburg JH, Moss P, Goulmy E. Tetrameric HLA class I-minor histocompatibility antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nature Med.* 5(7), 839–842 (1999).

9 Mutis T, Verdijk R, Schrama E, Esendam B, Brand A, Goulmy E. Feasibility of immunotherapy of relapsed leukemia with ex vivo-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood* 93(7), 2336–2341 (1999).

10 Marijt WA, Heemskerk MH, Kloosterboer FM *et al.* Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc. Natl Acad. Sci. USA* 100(5), 2742–2747 (2003).

11 Mailander V, Scheibenbogen C, Thiel E, Letsch A, Blau IW, Keilholz U. Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT1 peptide in the absence of hematological or renal toxicity. *Leukemia* 18(1), 165–166 (2004).

12 Elisseeva OA, Oka Y, Tsuboi A *et al.* Humoral immune responses against Wilms tumor gene WT1 product in patients with hematopoietic malignancies. *Blood* 99(9), 3272–3279 (2002).

13 Oka Y, Tsuboi A, Taguchi T *et al.* Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc. Natl Acad. Sci. USA* 101(38), 13885–13890 (2004).

14 Gao L, Bellantuono I, Elsasser A *et al.* Selective elimination of leukemic CD34(+) progenitor cells by cytotoxic T lymphocytes specific for WT1. *Blood* 95(7), 2198–2203 (2000).

15 Molldrem J, Dermime S, Parker K *et al.* Targeted T-cell therapy for human leukemia: cytotoxic T lymphocytes specific for a peptide derived from proteinase 3 preferentially lyse human myeloid leukemia cells. *Blood* 88(7), 2450–2457 (1996).

16 Ohminami H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8(+) cytotoxic T-lymphocyte clone specific for WT1 peptide. *Blood* 95(1), 286–293 (2000).

17 Clark RE, Dodi IA, Hill SC *et al.* Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the *BCR-ABL* b3a2 fusion protein. *Blood* 98(10), 2887–2893 (2001).

18 Osman Y, Takahashi M, Zheng Z *et al.* Dendritic cells stimulate the expansion of *PML-RAR* α specific cytotoxic T-lymphocytes: its applicability for antileukemia immunotherapy. *J. Exp. Clin. Cancer Res.* 18(4), 485–492 (1999).

19 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 392(6673), 245–252 (1998).

- **An authoritative review of dendritic cell (DC) biology.**

20 Cella M, Sallusto F, Lanzavecchia A. Origin, maturation and antigen presenting function of dendritic cells. *Curr. Opin. Immunol.* 9(1), 10–16 (1997).

21 Sousa C, Sher A, Kaye P. The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Curr. Opin. Immunol.* 11(4), 392–399 (1999).

22 Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. *Nature Immunol.* 1(3), 199–205 (2000).

23 Matzinger P. An innate sense of danger. *Semin. Immunol.* 10(5), 399–415 (1998).

- **This author has contributed much to current understanding of the flexibility of DC responses depending on antigens, tissue type, presence or absence of inflammatory ('danger') signals.**

24 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9, 271–296 (1991).

25 Qu C, Edwards EW, Tacke F *et al.* Role of CCR8 and other chemokine pathways in the migration of monocyte-derived dendritic cells to lymph nodes. *J. Exp. Med.* 200(10), 1231–1241 (2004).

26 Chaux P, Moutet M, Faivre J, Martin F, Martin M. Inflammatory cells infiltrating human colorectal carcinomas express HLA class II but not B7–1 and B7–2 costimulatory molecules of the T-cell activation. *Lab. Invest.* 74(5), 975–983 (1996).

27 Chaux P, Favre N, Martin M, Martin F. Tumor-infiltrating dendritic cells are defective in their antigen-presenting function and inducible B7 expression in rats. *Int. J. Cancer* 72(4), 619–624 (1997).

28 Nestle FO, Burg G, Fah J, Wrone-Smith T, Nickoloff BJ. Human sunlight-induced basal-cell-carcinoma-associated dendritic cells are deficient in T cell co-stimulatory molecules and are impaired as antigen-presenting cells. *Am. J. Pathol.* 150(2), 641–651 (1997).

29 Thurnher M, Radmayr C, Ramoner R *et al.* Human renal-cell carcinoma tissue contains dendritic cells. *Int. J. Cancer* 68(1), 1–7 (1996).

30 Ambe K, Mori M, Enjoji M. S-100 protein-positive dendritic cells in colorectal adenocarcinomas. Distribution and relation to the clinical prognosis. *Cancer* 63(3), 496–503 (1989).

31 Fox SB, Jones M, Dunnill MS, Gatter KC, Mason DY. Langerhans cells in human lung tumours: an immunohistological study. *Histopathology* 14(3), 269–275 (1989).

32 Schroder S, Schwarz W, Rehpenning W, Loning T, Bocker W. Dendritic/Langerhans cells and prognosis in patients with papillary thyroid carcinomas. Immunocytochemical study of 106 thyroid neoplasms correlated to follow-up data. *Am. J. Clin. Pathol.* 89(3), 295–300 (1988).

33 Gastl GA, Abrams JS, Nanus DM *et al.* Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. *Int. J. Cancer* 55(1), 96–101 (1993).

34 Maeda H, Shiraishi A. TGF-β contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J. Immunol.* 156(1), 73–78 (1996).

35 Nagy N, Vanky F. Transforming growth factor β, (TGFβ) secreted by immunogenic *ex vivo* human carcinoma cells, counteracts the activation and inhibits the function of autologous cytotoxic lymphocytes. Pretreatment with interferon γ and tumor necrosis factor α reduces the production of active TGFβ. *Cancer Immunol. Immunother.* 45(6), 306–312 (1998).

36 Espinoza-Delgado I, Bosco MC *et al.* Inhibitory cytokine circuits involving transforming growth factor-β, interferon-γ, and interleukin-2 in human monocyte activation. *Blood* 83(11), 3332–3338 (1994).

Luscher U, Filgueira L, Juretic A *et al.* The pattern of cytokine gene expression in freshly excised human metastatic melanoma suggests a state of reversible anergy of tumor-infiltrating lymphocytes. *Int. J. Cancer* 57(4), 612–619 (1994).

Nakase K, Kita K, Otsuji A *et al.* Diagnostic and clinical importance of interleukin-2 receptor α-chain expression on non-T-cell acute leukaemia cells. *Br. J. Haematol.* 80(3), 317–326 (1992).

Vinante F, Rigo A, Tecchio C *et al.* Serum levels of p55 and p75 soluble TNF receptors in adult acute leukaemia at diagnosis: correlation with clinical and biological features and outcome. *Br. J. Haematol.* 102(4), 1025–1034 (1998).

Tanaka M, Suda T, Haze K *et al.* Fas ligand in human serum. *Nature Med.* 2(3), 317–322 (1996).

Buggins AG, Lea N, Gaken J *et al.* Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood* 94(10), 3479–3490 (1999).
Elucidates the importance of the tumor microenvironment for the design of autologous cellular vaccine strategies

Ma L, Delforge M, van Duppen V, Verhoef G *et al.* Circulating myeloid and lymphoid precursor dendritic cells are clonally involved in myelodysplastic syndromes. *Leukemia* 18(9), 1451–1456 (2004).

Mohty M, Jarrossay D, Lafage-Pochitaloff M *et al.* Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. *Blood* 98(13), 3750–3756 (2001).

Robinson SP, English N, Jaju R, Kearney L, Knight SC, Reid CD. The *in-vitro* generation of dendritic cells from blast cells in acute leukaemia. *Br. J. Haematol.* 103(3), 763–771 (1998).

Panoskaltsis N, Belanger TJ, Liesveld JL, Abboud CN. Optimal cytokine stimulation for the enhanced generation of leukemic dendritic cells in short-term culture. *Leuk. Res.* 26(2), 191–201 (2002).

Harrison BD, Adams JA, Briggs M, Brereton ML, Yin JA. Stimulation of autologous proliferative and cytotoxic T-cell responses by 'leukemic dendritic cells' derived from blast cells in acute myeloid leukemia. *Blood* 97(9), 2764–2771 (2001).

Choudhury BA, Liang JC, Thomas EK *et al.* Dendritic cells derived *in vitro* from acute myelogenous leukemia cells stimulate autologous, antileukemic T-cell responses. *Blood* 93(3), 780–786 (1999).

- **A seminal paper demonstrating the ability of acute myeloid leukemia blasts to be matured into dendritic-like cells using a cytokine cocktail.**

48 Roddie PH, Horton Y, Turner ML. Primary acute myeloid leukaemia blasts resistant to cytokine-induced differentiation to dendritic-like leukaemia cells can be forced to differentiate by the addition of bryostatin-1. *Leukemia* 16(1), 84–93 (2002).

49 Charbonnier A, Gaugler B, Sainty D, Lafage-Pochitaloff M, Olive D. Human acute myeloblastic leukemia cells differentiate *in vitro* into mature dendritic cells and induce the differentiation of cytotoxic T cells against autologous leukemias. *Eur. J. Immunol.* 29(8), 2567–2578 (1999).

50 Molldrem JJ, Lee PP, Wang C *et al.* Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nature Med.* 6(9), 1018–1023 (2000).

- **Presenting evidence, that CTLs directed against peptides derived from tumor-associated antigens (TAAs) (PR1) contribute to leukemia elimination in chronic myelocytic leukemia (CML).**

51 Molldrem JJ, Lee PP, Kant S *et al.* Chronic myelogenous leukemia shapes host immunity by selective deletion of high-avidity leukemia-specific T cells. *J. Clin. Invest.* 111(5), 639–647 (2003).

52 Staveley-O'Carroll K, Sotomayor E, Montgomery J *et al.* Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc. Natl Acad. Sci. USA* 95(3), 1178–1183 (1998).

53 Takasugi M, Ramseyer A, Takasugi J. Decline of natural nonselective cell-mediated cytotoxicity in patients with tumor progression. *Cancer Res.* 37(2), 413–418 (1977).

54 Mintz PJ, Kim J, Do KA *et al.* Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nature Biotechnol.* 21(1), 57–63 (2003).

55 Valmori D, Dutoit V, Lienard D *et al.* Tetramer-guided analysis of TCR β-chain usage reveals a large repertoire of melan-A-specific $CD8^+$ T cells in melanoma patients. *J. Immunol.* 165(1), 533–538 (2000).

56 Rousseau RF, Biagi E, Dutour A *et al.* Immunotherapy of high-risk acute leukemia with a recipient (autologous) vaccine expressing transgenic human CD40L and IL-2 after chemotherapy and allogeneic stem cell transplantation. *Blood* 107(4), 1332–1341 (2006).

57 Chan L, Hardwick N, Darling D *et al.* IL-2/B7.1 (CD80) fusagene transduction of AML blasts by a self-inactivating lentiviral vector stimulates T cell responses *in vitro*: a strategy to generate whole cell vaccines for AML. *Mol. Ther.* 11(1), 120–131 (2005).

58 Nakazaki Y, Tani K, Lin ZT *et al.* Vaccine effect of granulocyte-macrophage colony-stimulating factor or CD80 gene-transduced murine hematopoietic tumor cells and their cooperative enhancement of antitumor immunity. *Gene Ther.* 5(10), 1355–1362 (1998).

59 Stripecke R, Cardoso AA, Pepper KA *et al.* Lentiviral vectors for efficient delivery of CD80 and granulocyte-macrophage- colony-stimulating factor in human acute lymphoblastic leukemia and acute myeloid leukemia cells to induce antileukemic immune responses. *Blood* 96(4), 1317–1326 (2000).

60 Dilloo D, Bacon K, Holden W *et al.* Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nature Med.* 2(10), 1090–1095 (1996).

61 Hirst WJ, Buggins A, Darling D, Gaken J, Farzaneh F, Mufti GJ. Enhanced immune costimulatory activity of primary acute myeloid leukaemia blasts after retrovirus-mediated gene transfer of B7.1. *Gene Ther.* 4(7), 691–699 (1997).

62 Boyer MW, Vallera DA, Taylor PA *et al.* The role of B7 costimulation by murine acute myeloid leukemia in the generation and function of a $CD8^+$ T-cell line with potent *in vivo* graft-versus-leukemia properties. *Blood* 89(9), 3477–3485 (1997).

63 Mutis T, Schrama E, Melief CJ, Goulmy E. CD80-transfected acute myeloid leukemia cells induce primary allogeneic T-cell responses directed at patient specific minor histocompatibility antigens and leukemia-associated antigens. *Blood* 92(5), 1677–1684 (1998).

64 Saudemont A, Buffenoir G, Denys A *et al.* Gene transfer of CD154 and IL12 cDNA induces an anti-leukemic immunity in a murine model of acute leukemia. *Leukemia* 16(9), 1637–1644 (2002).

65 Buhmann R, Nolte A, Westhaus D, Emmerich B, Hallek M. CD40-activated B-cell chronic lymphocytic leukemia cells for tumor immunotherapy: stimulation of allogeneic versus autologous T cells generates different types of effector cells. *Blood* 93(6), 1992–2002 (1999).

66 Cardoso AA, Seamon MJ, Afonso HM *et al.* *Ex vivo* generation of human anti-pre-B leukemia-specific autologous cytolytic T cells. *Blood* 90(2), 549–561 (1997).

67 Choudhury A, Gajewski JL, Liang JC, *et al.* Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. *Blood* 89(4), 1133–1142 (1997).

68 Eibl B, Ebner S, Duba C *et al.* Dendritic cells generated from blood precursors of chronic myelogenous leukemia patients carry the Philadelphia translocation and can induce a CML-specific primary cytotoxic T-cell response. *Genes Chromosomes Cancer* 20(3), 215–223 (1997).

69 Kohler T, Plettig R, Wetzstein W *et al.* Cytokine-driven differentiation of blasts from patients with acute myelogenous and lymphoblastic leukemia into dendritic cells. *Stem Cells* 18(2), 139–147 (2000).

70 Mohty M, Isnardon D, Charbonnier A *et al.* Generation of potent T(h)1 responses from patients with lymphoid malignancies after differentiation of B lymphocytes into dendritic-like cells. *Int. Immunol.* 14(7), 741–750 (2002).

71 Thurner B, Roder C, Dieckmann D *et al.* Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J. Immunol. Methods* 223(1), 1–15 (1999).

72 Bender A, Sapp M, Schuler G, Steinman RM, Bhardwaj N. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* 196(2), 121–135 (1996).

73 Ferlazzo G, Klein J, Paliard X, Wei WZ, Galy A. Dendritic cells generated from $CD34^+$ progenitor cells with flt3 ligand, c-kit ligand, GM-CSF, IL-4, and TNF-α are functional antigen-presenting cells resembling mature monocyte-derived dendritic cells. *J. Immunother.* 23(1), 48–58 (2000).

74 Ye Z, Gee AP, Bowers WE, Lamb LS, Turner MW, Henslee-Downey PJ. *In vitro* expansion and characterization of dendritic cells derived from human bone marrow $CD34^+$ cells. *Bone Marrow Transplant.* 18(5), 997–1008 (1996).

75 Fujii S, Fujimoto K, Shimizu K *et al.* Presentation of tumor antigens by phagocytic dendritic cell clusters generated from human $CD34^+$ hematopoietic progenitor cells: induction of autologous cytotoxic T lymphocytes against leukemic cells in acute myelogenous leukemia patients. *Cancer Res.* 59(9), 2150–2158 (1999).

76 Lee JJ, Kook H, Park MS *et al.* Immunotherapy using autologous monocyte-derived dendritic cells pulsed with leukemic cell lysates for acute myeloid leukemia relapse after autologous peripheral blood stem cell transplantation. *J. Clin. Apher.* 19(2), 66–70 (2004).

77 Schui DK, Singh L, Schneider B, Knau A, Hoelzer D, Weidmann E. Inhibiting effects on the induction of cytotoxic T lymphocytes by dendritic cells pulsed with lysates from acute myeloid leukemia blasts. *Leuk. Res.* 26(4), 383–389 (2002).

78 Spisek R, Chevallier P, Morineau N *et al.* Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. *Cancer Res.* 62(10), 2861–2868 (2002).

79 Menssen HD, Renkl HJ, Rodeck U *et al.* Presence of Wilms' tumor gene (*wt1*) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. *Leukemia* 9(6), 1060–1067 (1995).

80 Ohminami H, Yasukawa M, Kaneko S *et al.* Fas-independent and nonapoptotic cytotoxicity mediated by a human CD4(+) T-cell clone directed against an acute myelogenous leukemia-associated DEK–CAN fusion peptide. *Blood* 93(3), 925–935 (1999).

81 Zeis M, Siegel S, Wagner A *et al.* Generation of cytotoxic responses in mice and human individuals against hematological malignancies using survivin-RNA-transfected dendritic cells. *J. Immunol.* 170(11), 5391–5397 (2003).

82 Klammer M, Waterfall M, Samuel K, Turner ML, Roddie PH. Fusion hybrids of dendritic cells and autologous myeloid blasts as a potential cellular vaccine for acute myeloid leukaemia. *Br. J. Haematol.* 129(3), 340–349 (2005).

83 Galea-Lauri J, Darling D, Mufti G, Harrison P, Farzaneh F. Eliciting cytotoxic T lymphocytes against acute myeloid leukemia-derived antigens: evaluation of dendritic cell-leukemia cell hybrids and other antigen-loading strategies for dendritic cell-based vaccination. *Cancer Immunol. Immunother.* 51(6), 299–310 (2002).

84 Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* 179(4), 1109–1118 (1994).

85 Brouwer RE, van der HM, Kluin-Nelemans HC, Zelderen-Bhola S, Willemze R, Falkenburg JH. The generation of dendritic-like cells with increased allostimulatory function from acute myeloid leukemia cells of various FAB subclasses. *Hum. Immunol.* 61(6), 565–574 (2000).

86 Roddie H, Klammer M, Thomas C *et al.* Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia. *Br. J. Haematol.* (Epub) (2006).

87 Nestle FO, Alijagic S, Gilliet M *et al.* Vaccination of melanoma patients with pep. *Nature Med.* 4(3), 328–332 (1998).

88 Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W. Induction of cytotoxic T-lymphocyte responses *in vivo* after vaccinations with peptide-pulsed dendritic cells. *Blood* 96(9), 3102–3108 (2000).

89 Banchereau J, Palucka AK, Dhodapkar M *et al.* Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res.* 61(17), 6451–6458 (2001).

90 Marchand M, van Baren N, Weynants P *et al.* Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. *Int. J. Cancer* 80(2), 219–230 (1999).

91 Murphy G, Tjoa B, Ragde H. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *Prostate* 29(6), 371–380 (1996).

92 Hernando JJ, Park TW, Kubler K, Offergeld R, Schlebusch H, Bauknecht T. Vaccination with autologous tumour antigen-pulsed dendritic cells in advanced gynaecological malignancies: clinical and immunological evaluation of a Phase I trial. *Cancer Immunol. Immunother.* 51(1), 45–52 (2002).

93 Qazilbash MH, Wieder E, Rios R *et al.* Vaccination with the PR1 leukemia-associated antigen can induce complete remission in patients with myeloid leukemia. *ASH Annual Meeting Abstracts* 104(11), 259 (2004).

94 Inoue K, Ogawa H, Sonoda Y *et al.* Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. Blood 89(4), 1405–1412 (1997).

95 Scheibenbogen C, Letsch A, Thiel E *et al.* CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. *Blood* 100(6), 2132–2137 (2002).

de Bueger M, Bakker A, van Rood JJ, Van der WF, Goulmy E. Tissue distribution of human minor histocompatibility antigens. Ubiquitous versus restricted tissue distribution indicates heterogeneity among human cytotoxic T lymphocyte-defined non-MHC antigens. *J. Immunol.* 149(5), 1788–1794 (1992).

Haining WN, Cardoso AA, Keczkemethy HL *et al.* Failure to define window of time for autologous tumor vaccination in patients with newly diagnosed or relapsed acute lymphoblastic leukemia. *Exp. Hematol.* 33(3), 286–294 (2005).

De Vries IJ, Krooshoop DJ, Scharenborg NM *et al.* Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res.* 63(1), 12–17 (2003).

MartIn-Fontecha A, Sebastiani S, Hopken UE *et al.* Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J. Exp. Med.* 198(4), 615–621 (2003).

Ratzinger G, Stoitzner P, Ebner S *et al.* Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J. Immunol.* 168(9), 4361–4371 (2002).

Therasse P, Arbuck SG, Eisenhauer EA *et al.* New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J. Natl Cancer Inst.* 92(3), 205–216 (2000).

102 Altman JD, Moss PA, Goulder PJ *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274(5284), 94–96 (1996).

103 Klammer M, Waterfall M, Samuel K *et al.* Monitoring immune responses following vaccination with autologous dendritic like leukaemia cells. *ASH Annual Meeting Abstracts* 104(11), 1804 (2004).

104 Casares N, Arribillaga L, Sarobe P *et al.* $CD4^+/CD25^+$ regulatory cells inhibit activation of tumor-primed $CD4^+$ T cells with IFN-γ-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J. Immunol.* 171(11), 5931–5939 (2003).

105 Mesel-Lemoine M, Cherai M, Le Gouvello S *et al.* Initial depletion of regulatory T-cells: the missing solution to preserve the immune functions of T lymphocytes designed for cell-therapy. *Blood* 107(1), 381–388 (2005).

106 Morse MA, Clay TM, Mosca P, Lyerly HK. Immunoregulatory T cells in cancer immunotherapy. *Expert Opin. Biol. Ther.* 2(8), 827–834 (2002).

- **Reviews the role of CD4/CD25 positive regulatory T cells and strategies to circumvent their immunosuppressive effects.**

107 Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin. Cancer Res.* 9(2), 606–612 (2003).

108 Ghiringhelli F, Larmonier N, Schmitt E *et al.* $CD4^+CD25^+$ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur. J. Immunol.* 34(2), 336–344 (2004).

109 Saudemont A, Quesnel B. In a model of tumor dormancy, long-term persistent leukemic cells have increased B7-H1 and B7.1 expression and resist CTL-mediated lysis. *Blood* 104(7), 2124–2133 (2004).

110 Saudemont A, Jouy N, Hetuin D, Quesnel B. NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells. *Blood* 105(6), 2428–2435 (2005).

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