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ORIGINAL ARTICLE

Experimental production of clinical-grade dendritic cell vaccine for acute myeloid leukemia

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Abstract

Dendritic cells (DC) are professional antigen presenting cells of the immune system. Through the use of DC vaccines (DC after exposure to tumour antigens), cryopreserved in single-use aliquots, an attractive and novel immunotherapeutic strategy is available as an option for treatment. In this paper we describe an *in vitro* attempt to scale-up production of clinical-grade DC vaccines from leukemic cells. Blast cells of two relapsed AML patients were harvested for DC generation in serum-free culture medium containing clinical-grade cytokines GM-CSF, IL-4 and TNF-alpha. Cells from patient 1 were cultured in a bag and those from patient 2 were cultured in a flask. The numbers of seeding cells were 2.24 x 10^8 and 0.8 x 10^8, respectively. DC yields were 10 x 10^6 and 29.8 x 10^6 cells, giving a conversion rate of 4.7% and 37%, respectively. These DC vaccines were then cryopreserved in approximately one million cells per vial with 20% fresh frozen group AB plasma and 10% DMSO. At 12 months and 21 months post cryopreservation, these DC vaccines were thawed, and their sterility, viability, phenotype and functionality were studied. DC vaccines remained sterile up to 21 months of storage. Viability of the cryopreserved DC in the culture bag and flask was found to be 50% and 70% at 12 months post cryopreservation respectively; and 48% and 67% at 21 months post cryopreservation respectively. These DC vaccines exhibited mature DC surface phenotypic markers of CD83, CD86 and HLA-DR, and negative for haemopoietic markers. Mixed lymphocyte reaction (MLR) study showed functional DC vaccines. These experiments demonstrated that it is possible to produce clinical-grade DC vaccines *in vitro* from blast cells of leukemic patients, which could be cryopreserved up to 21 months for use if repeated vaccinations are required in the course of therapy.

Keywords: Cellular immunotherapy, dendritic cells, leukemia vaccine, AML

INTRODUCTION

Dendritic cells (DC) constitute a system of cells crucial to the immune response, especially T-cell mediated immunity. DC exist in various part of the human body and are mobile, wandering in the blood and lymph from peripheral organs to the lymphoid organs, especially to T-cells areas such as those in the lymph nodes.[1] Their specific ability in antigen presentation plays a vital role in the induction of anti-tumour responses.[2]

DC can be directly isolated from blood or generated *in vitro* from peripheral blood monocytes or CD34+ bone marrow cells by culturing them in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, or GM-CSF, tumor necrosis factor (TNF)-α, stem cell factor, and FLT3 ligand.[3-5] Upon antigenic stimulation under inflammatory conditions, DC change their chemokine receptor expression patterns and migrate to the secondary lymphoid tissues.[6-8] When DC become fully mature, they secrete an array of chemokines to recruit B cells, T cells, and other DC to efficiently induce an immune response.[9-10] Immunotherapy using DC has been implemented in many diseases, including

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cancer. DC loaded with tumor antigens are potent inducers of T-cell response *in vitro*. Large quantity culture of DC from monocytes *in vitro* pulsed with the tumor-associated antigen presents an opportunity for cancer treatment. For example, DC pulsed with tumor antigens have been successfully used *in vivo* for the induction of anti-tumor T-cell reactivity in patients with melanoma.[11-12] At present, more than 150 DC-based clinical studies for the treatment of solid or hematological malignancies have been reported.[13] According to a review from The Mater Medical Research Institute (MMRI), a centre known for its pioneering works in DC vaccines, melanoma is the most frequent type of cancer using this treatment strategy with 40 published clinical studies. This is followed by prostate cancer (20), renal cell carcinoma (16), breast cancer (12), multiple myeloma, leukemia, colorectal cancer and gliomas (9).[14]

Repeated DC vaccinations are critical to generate potent anti-tumor responses to eradicate tumor cells and induce long-lasting anti-tumor T-cell memory. Most of the studies have used weekly, biweekly or monthly injections with at least two vaccine administrations.[15-17] Besides, the production of DC vaccine is time consuming and required fresh viable cells. Therefore, a large-scale production of sufficient number of functional DC vaccines, cryopreserved in multiple single-use aliquots, is an attractive and cost effective immunotherapeutic strategy to enable multiple injections at different time points.[18] The objective of this study is to assess the possibility of a scaled-up production of clinical grade DC-based vaccines from leukemic cells *in vitro*.

MATERIALS AND METHODS

Patients and sample collection

Two patients with acute myeloid leukemia in relapse were recruited in this study with informed consent. 100mL of each patient's bone marrow aspirate was collected into vacutainer tubes (Beckton Dickinson, UK) containing EDTA under strict aseptic technique.

DC generation and cryopreservation

Bone marrow aspirate was diluted with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (CliniMACs®, Miltenyi Biotec, Germany) in 1:1 ratio and layered on Ficoll-paque Plus (GE Healthcare, UK, formerly Amersham Bioscience) for density-gradient centrifugation at 2500 rpm for 20 minutes as recommended by the manufacture. Mononuclear Cells (MNC) were then harvested from the interface. The obtained MNC were washed with two parts of PBS.

The isolated MNC were used as a source for DC vaccine generation in clinical-grade CellGro FREE™ (Cell-Gro, Mediatech) culture medium following good manufacturing practice guidelines at a cell concentration of 2×10^6 cells/ml. MNC from patient 1 and patient 2 were cultured in culture bag (Cell-Gro, Mediatech, US) and in tissue-factory flask (Nunclon™, Nunc, Denmark) respectively in 5% CO_2 at 37ºC. Each culture was supplemented with clinical-grade cytokines including 1000U/ml of GM-CSF, IL-4 and 100ng/ml TNF-alpha (Cell-Gro, Mediatech, US) and 10% autologous plasma. Half medium exchange was performed every other day with fresh cytokine-supplemented medium. After seven days in culture, the DC vaccines were harvested. These DC vaccines were washed with PBS and cryopreserved in approximate one million cells per ml per vial with 20% fresh frozen human group AB plasma and 10% dimethyl sulfoxide (DMSO). Prior to cryopreservation, sterility, viability, phenotype and functionality were evaluated.

Thawing of DC vaccines

At 12 months and 21 months post cryopreservation, a sample vial of the DC vaccines was thawed, and their sterility, viability, phenotype and functionality were re-evaluated.

Sterility testing

Sterility testing consisted of testing these DC vaccine aliquots for both aerobic and anaerobic bacterial contamination using BACTEC™ culture bottles (BACTEC™ Plus Aerobic/F* and Plus Anaerobic/F* Becton Dickinson, USA) and Mycoplasma (Venor®Gem, Minerva Biolabs, Germany).

DC morphology, yield, recovery and viability

The DC vaccines were checked for viability using trypan blue cell exclusion method. DC morphology was assessed by light microscopy on Giemsa-stained cytospin specimens. DC yield was calculated as the number of viable DC, as determined by morphology and trypan blue cell exclusion, at harvest and thawing. Percentage DC conversion from blasts was calculated as the DC yield divided by the starting number of blasts x 100. Percentage viability was calculated by dividing the number of viable DC by the total number of DC upon cryopreservation.

Immunophenotypic analysis of DC
DC immunophenotypes were determined with standard staining methods, using PE- and FITC-labeled antibodies for the surface molecule of interest, and analysed with a FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA). The FITC-labeled antibodies used were CD 1a, CD14, CD 83 and HLA-DR; and PE-labeled antibodies used were CD11c, CD 40, CD80 and CD86. Isotype controls were used (Becton Dickinson, San Diego, CA, USA) for each flow cytometric run.

Allogenic-mixed lymphocyte reaction (allo-MLR)
DC vaccines were tested for their ability to stimulate allogenic T lymphocytes to proliferate. Three 10 fold dilutions DC vaccine (1 x 10^3 to 1 x 10^5/well) in triplicates were used as stimulator cells and co-cultured with 10^5 pooled-allogenic lymphocytes (effector cells) in 96-well flat-bottomed plates for 5 days in 5% CO_2 incubator at 37ºC. T-cell proliferation was assessed by anti-BrdU ELISA assay (Roche Diagnostic, Basel) incorporated for 24 h. It is a non-radioactive alternative using 5-bromo-2'-deoxyuridine (BrdU) to replace traditional [^{3}H]-thymidine DNA incorporation assay. This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by immunoassay reflected in the absorbance measurement. Absorbance correlates to the number of viable cells.[19-24] Results were expressed as the mean of the triplicate. Un-stimulated lymphocytes were used as a control for the allo-MLR.

RESULTS

Sterility testing
Aliquots of the vaccines were found negative for mycoplasma test, anaerobic and aerobic bacterial cultures, up to 21 months of cryopreservation.

DC yield and viability
DC were generated from blast cells by culturing them with GM-CSF, IL-4 and TNF-α in both the bag and flask containers. Bag culture yielded 10.5 x 10^6 mature DC from 2.2 x 10^8 seeding blast cells; the conversion rate was 4.7%. Flask culture had a higher conversion rate, 37%, from 0.8 x 10^8 seeding blast cells to 29.8 x 10^6 mature DC. Viability of the bag-cultured and flask-cultured cryopreserved DC showed changes from initial viability study of 100%. The results were found to be 50% and 70% at 12 months post cryopreservation and 48% and 67% at 21 months post cryopreservation, respectively. The results of the two clinical-grade cultures are shown in Table 1.

Phenotypes of DC
The thawed DC retained typical morphology of DC possessing dendrites (Figure 1). In the phenotypic studies prior to cryopreservation, these DC were shown to have phenotypic markers of DC by flow cytometry as reported previously.[25] Figure 2a and 2b show representative histograms from flow cytometry analysis of the bag-cultured and flask-cultured DC. Both the thawed bag-cultured and flask-cultured DC retained the expression of HLA-DR, CD83 and CD86.

Allo-MLR stimulatory capacity of DC
Allo-MLR showed that the thawed DC vaccines were able to stimulate the proliferation of pooled-

TABLE 1. Results of DC yield, conversion rate and viability studies.

Values expressed as mean of triplicate		Bag-cultured DC	Flask-cultured DC
Seeding blast cells		2.2 x 10^8	0.8 x 10^8
DC yield		10.5 x 10^6	29.8 x 10^6
Conversion rate (leukemic blasts to mature DC)		4.7%	37%
Viability	Pre-cryopreservation	100%	100%
	12 month post cryopreservation	50 %	70%
	21 month post cryopreservation	48 %	67 %

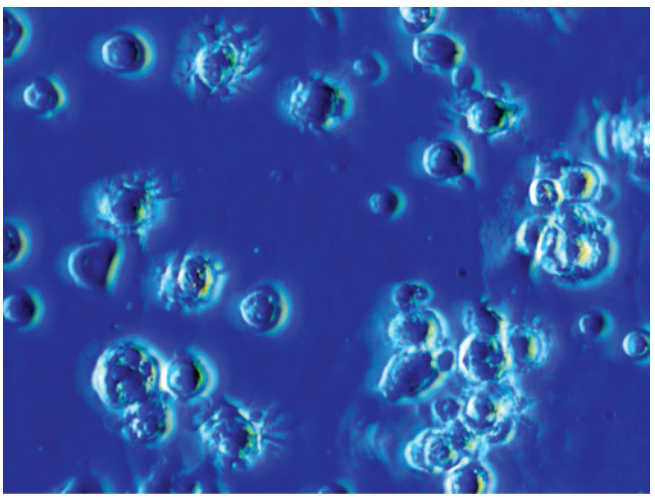


FIG. 1: Thawed mature DC show substantial dendritic projections

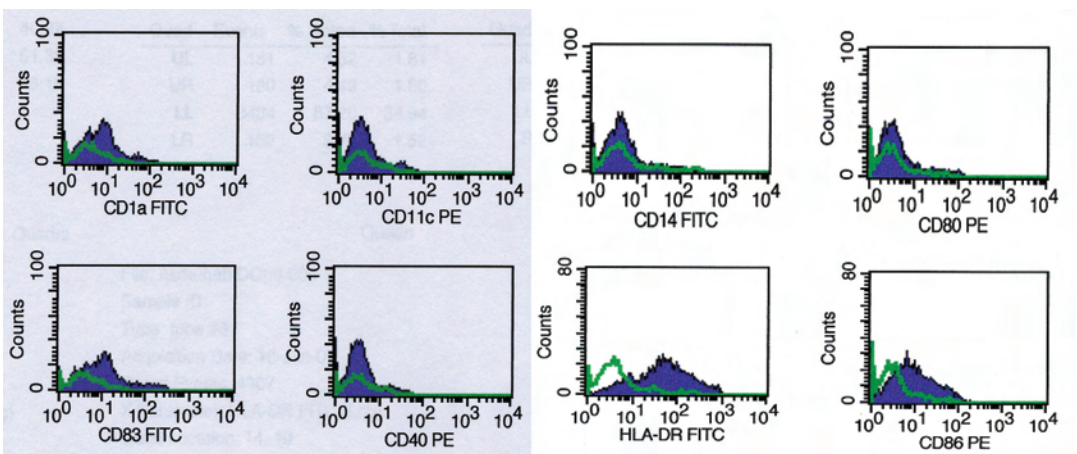


FIG. 2 (a): Flow cytometric analysis showed that the thawed bag-cultured DC vaccines retain expression of CD1a, CD83, HLA-DR and CD86 but are negative for CD11c, CD14, CD40 and CD80

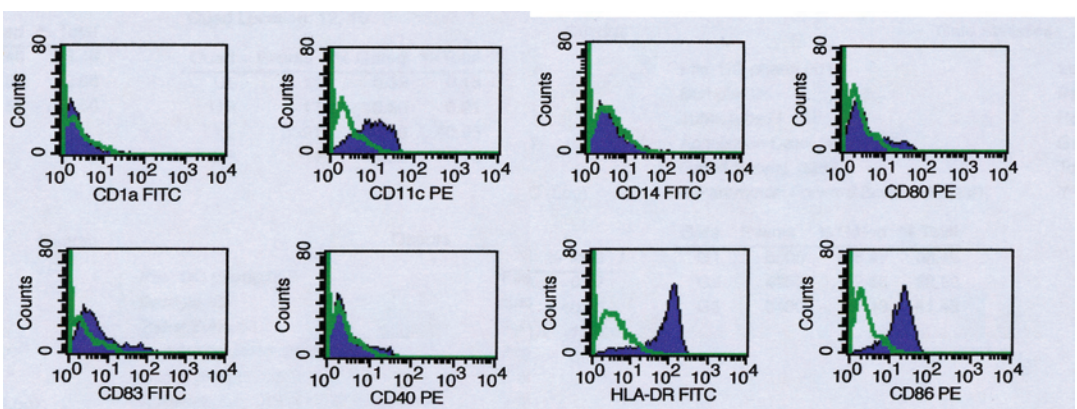


FIG. 2 (b): Flow cytometric analysis showed that the thawed flask-cultured DC retain expression of CD11c, CD83, HLA-DR and CD86 but negative for CD1a, CD14, CD40 and CD80.

allogenic lymphocytes in the 5-day co-cultures [Figure 3(a) and (b)]. Higher lymphocyte proliferation was observed at stimulator cell to effector cell ratio of 1:1. The rate of cellular proliferation in the two culture systems was not measured.

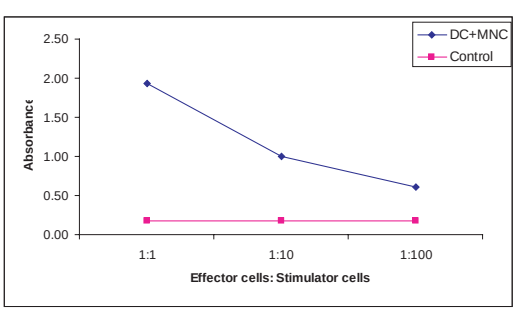
DISCUSSION

Leukemic blasts are poor immunogens and fail to induce a sustained anti-leukemic response.[26] It has been shown that the blasts inhibit T-cell proliferation and cytokine production through secretion of soluble factors.[27] However, DC derived from myeloid lineage are potent antigen-presenting cells responsible for anti-leukemic immunoreactivity.[28-29] In this study, we have successfully differentiated the blasts from patients with acute myeloid leukemia to DC using clinical grade cytokines and these DC vaccines were shown to retain the mature DC phenotypes and capable of stimulating lympocytes proliferation up to 21 months of cryopreservation.

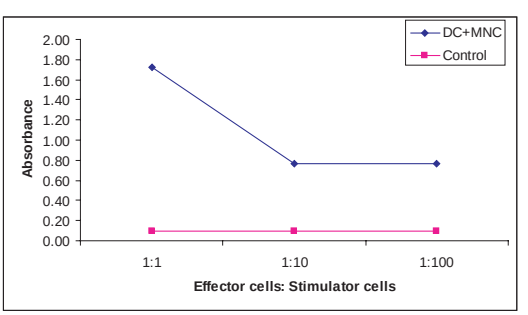
The conversion rates of blasts to DC in bag-culture and flask culture system were 3.7% and 40% respectively. This observation is consistent with findings by Maffei *et al*,[28] whose group subsequently introduced styrene copolymer beads into culture bags to increase available surface area which is similar to that found in the flasks and thus yielded better conversion rates. However, other research groups reported that DC generated in cell culture bags are equivalent[29-31] or superior[32] to flask-or well-cultured DC. Thus, surface area difference alone may not explain the difference in conversion rates. An alternative explanation could be the seeding population. The lower seeding population found in the flask could have favoured the conversion rate in our study.

Prior to cryopreservation, these DC were found to be 100% viable and shown to have characteristic phenotypic markers of DC by flow cytometry as reported previously.[25] Negative anaerobic and aerobic bacteria culture and mycoplasma test indicate no contamination of the culture. 10% dimethyl sulphoxide (DMSO), a polar aprotic solvent is commonly added into cryoprotectant freezing solution.[33] DMSO suppresses ice crystal formation and hence protects cells from freeze injury.[34] However, intermediate temperature during cooling and thawing of cells are particular lethal to cell survival as DMSO is toxic at higher temperature.[35] The freezing process and the addition of DMSO may explain the decrease in viability after thawing at 12 months post cryopreservation observed in our study. Our findings are consistent with the observation by *Udoh et al* where they reported the viability at 1 year post cryopreservation as 61.6%.[34]

The thawed DC vaccines showed mature DC phenotypes ie expression of HLA-DR, CD83 and CD86. The use of mature DC is crucial as immature DC are less immunogenic and more incline to induce peripheral tolerance.[36-39] Also, the high expression of stimulatory and co-stimulatory molecules promotes the DC vaccine capacity to induce T-cell response. CD1a expression was in the bag-cultured (but not in the flask-cultured) DC vaccine. CD1a belongs to type 1 CD1 membrane proteins generally used as human DC markers expressed early in their development.[5] It is considered to be a specific marker of immature DC.[40-41] However, the membrane expression of CD1a does not depend on DC maturation[42] but little is known on the transcriptional and/or ligand-dependent regulation of this process. This coincides with finding of Thurner *et al* that fully matured DC



(a) Bag-cultured DC vaccine



(b) Flask-cultured DC vaccine

FIG 3 (a) and (b): Allogenic-mixed lymphocyte culture assay showed that the thawed DC retain their ability to induce proliferative responses of allogenic lymphocytes.

still expressed CD1a molecules.[42] Similarly, Zheng *et al* demonstrated that CD1a was further up-regulated in mature DC.[43] These observations may explain why CD1a expression was found in the bag-cultured DC vaccine.

DC are known to secrete a variety of chemokines allowing them to attract and interact with lymphocytes to induce an immune response.[6,44] We have shown that the thawed DC vaccines were able to stimulate allogenic lymphocytes and generated proliferative responses in the mixed lymphocyte reaction. This demonstrates that DC vaccines retained their functionality up to 21 months of cryopreservation.

Standardization and quality control of DC-based vaccines are important aspects of good manufacturing practice to ensure high quality products for patient care.[45-46] Generating high purity (>80%) of DC based vaccines[46] is highly desirable. Our study shows that the qualities desired of these products could be fulfilled as we scaled-up our production from bench to clinics. However, the efficacy of these generated vaccines remains to be tested in future clinical trial.

In conclusion, DC vaccines were successfully generated from blast cells of two relapsed AML patients in serum-free culture media following good manufacturing practice guidelines. Prior to cryopreservation, these generated DC vaccines are of quality showing typical DC morphology, viability of 100%, free of contamination of pathogens and capable of inducing proliferation of lymphocytes. In clinical trials reported so far, DC are freshly prepared for each vaccination or cryopreserved for not more than 6 months.[13,47] We have demonstrated that it is possible to produce clinical-grade DC vaccines from patients' blast cells *in vitro*, which could be cryopreserved up to 21 months for use in clinical trial.

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