

Comparing the Ability of Freshly Generated and Cryopreserved Dendritic Cell Vaccines to Inhibit Growth of Breast Cancer in a Mouse Model

Ammu Kutty Radhakrishnan,¹ Geok Choo Sim,¹ and Soon Keng Cheong²

Abstract

Repetitive vaccinations with dendritic cell (DC)-based vaccines over long periods of time can break pre-existing tolerance to tumors and achieve clinically relevant immune response. This requires a large number of DCs to be generated under good manufacturing protocol, which is time- and cost intensive. Thus, producing a large number of DCs at one time point and cryopreserving these cells in ready-for-use aliquots for clinical application may overcome this constraint. This could also reduce batch-to-batch variations. In this study, we generated DCs from bone marrow obtained from BALB/c mice. Some of the generated DCs were cryopreserved before conducting various tests. There were no significant differences in the morphology and phenotype between cryopreserved and freshly generated DCs. Both types of DCs pulsed with tumor lysate (TL) from 4T1 murine mammary cancer cells (DC + TL) possessed a similar capacity to stimulate the proliferation of T-cells. In addition, cryopreserved and fresh DC pulsed with TL showed similar tumor growth inhibition patterns. Both DCs induced initial retardation of tumor growth ($p < 0.05$) and prolonged the survival ($p < 0.05$) of tumor-bearing mice treated with DC + TL as compared with nontreated control mice. Cryopreserved DCs shared similar therapeutic efficacy to fresh DCs, and this finding lends supports the routine use of cryopreserved DCs in future clinical trials.

Key words: cryopreservation; dendritic cells; DNDC; tumor immunotherapy

Introduction

DENDRITIC CELLS (DCs) were first described by Steinman and Cohn in 1973.¹ Since their discovery, there have been an increasing number of studies conducted on DCs to understand their immunobiology and the role played by these cells in the immune system.²⁻⁴ DCs are the most powerful professional antigen-presenting cells (APC) and possess the ability to initiate both arms of the immune responses.² Several *in vivo* and *in vitro* studies have demonstrated that tumor antigen-loaded DCs (DC vaccines) can elicit anti-tumor T-cell responses.⁵⁻⁹ These studies demonstrated that tumor-specific cytotoxic T-lymphocytes can be activated by the DC vaccines, and this property of DCs has enabled these cells to be the most suitable candidates for cancer immunotherapy. There are some promising results obtained with studies using DC vaccines in the treatment of melanoma,¹⁰⁻¹² and non-Hodgkin's lymphoma¹³ has opened a new field for testing the therapeutic efficacy of the DC vaccine in other clinical trials. Furthermore, several animal studies^{6,7,14}

and clinical data^{10-12,15} suggest that repetitive vaccinations with DC-based vaccines over a longer period of time might be necessary to break pre-existing tolerance and achieve clinically relevant anti-tumor immune responses. The prerequisite for performing a DC vaccine clinical trial is the ability to generate large amounts of DCs, a process that would usually entail repeated leucopheresis and venesections to generate sufficient numbers of DCs.¹⁶ Leucopheresis is not a procedure that cancer patients undergo very frequently, as most of these patients are already debilitated from their disease. In addition, the protocols for the production of DCs for human clinical trials should be good manufacturing protocol compliant. This would mean that these procedures are generally time- and cost-intensive, and this significantly limits the feasibility of repeated vaccinations with DC vaccines. One possible approach to overcome this constraint is by producing sufficient numbers of DCs at one time point and subsequently cryopreserving the generated DCs in ready-for-use aliquots for clinical application. This approach would significantly facilitate and improve the practicality of

¹Pathology Division, Faculty of Medicine and Health, International Medical University, Kuala Lumpur, Malaysia.

²Faculty of Medicine and Health Sciences, University Tunku Abdul Rahman, Kajang, Malaysia.

using DC-based vaccination. In addition, this approach can also minimize batch-to-batch variations of the DCs generated, that is, it allows identical DCs from a single venesection to be used and tested in a clinical study. There are some concerns that cryopreservation may result in changes of DC morphology, immunophenotype, and their ability to elicit an appropriate T-cell response. If any of these changes take place, then this would pose a setback for the use of this approach in preparing large-scale, clinical-grade, DC-based cancer vaccines for clinical trials. To date, attempts to freeze and thaw DCs for this purpose have shown apparently no significant alterations in phenotype, viability, ability to resist pro-apoptotic stimuli, and functional properties of human DCs *in vitro*.^{16,17} Similar observations in some *in vitro* studies were also reported with antigen-loaded DCs cryopreserved in liquid nitrogen for 7 months.¹⁸ This implies that successful cryopreservation methods can reduce the laboriousness of the DC generation protocols and variability in the quality of DCs due to the repetitive generation of DCs before each vaccination step. In this study, we generated murine DCs (MDC) from bone marrow (BM) obtained from BALB/c mice. We compared the morphology, phenotype, and stimulatory properties between cryopreserved and fresh MDC using both *in vitro* and *in vivo* experimental systems. Our *in vitro* studies findings confirm previous findings^{17,18} that cryopreserved DCs can retain their functional properties. Schuler-Thurner et al.¹⁹ have shown that an injection of cryopreserved DCs derived from monocytes that were loaded with antigens could induce Thq 1 immune responses in melanoma patients. However, to date, the ability of cryopreserved DCs to retain their functional properties and therapeutic efficacy as their fresh counterpart has not yet been addressed in an *in vivo* experimental model of breast cancer, which can provide a better insight on the therapeutic efficacy between cryopreserved DCs compared with freshly generated DCs. The findings from this study show that cryopreserved DCs function in a similar manner to freshly generated DCs, which could support the feasibility of using cryopreserved DCs to produce DC vaccines in clinical studies.

Materials and Methods

Animals

Inbred 6-week-old female BALB/c female mice were obtained from the Institute for Medical Research (IMR), Kuala Lumpur, and housed at its animal maintenance facility. The animals were maintained on a commercially available pellet diet and water *ad libitum*. Soda bedding was changed every 4-days. All experiments with animals were performed in accordance with the guidelines approved by the ethics committee of the IMR.

Tumor cell lines

Murine 4T1 mammary gland tumor cells, which are spontaneously metastatic tumor cells derived from BALB/c mice, were purchased from the American Type Culture Collection (ATCC). The 4T1 mammary gland tumor cells are comparable to human stage IV breast cancer.²⁰ The tumor cells were cultured as recommended by the ATCC in a complete RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 2% HEPES (Gibco), 2% sodium pyruvate

(Gibco), and 1% penicillin–streptomycin antibiotics (Gibco) in 25-mL cell culture flasks (Nunc). The culture medium was regularly changed when the cells reached 70% confluence or every 2–3-days.

Generation of DCs from mouse BM

Murine BM cells were harvested by flushing the marrow cavities of the femur and tibia bones of the BALB/c mice with a medium under aseptic conditions. The harvested marrow was depleted of erythrocytes and cultured in either complete RPMI 1640 (Sigma) medium alone or RPMI medium supplemented with either 100 ng/mL Flt-3 ligand (Stem Cell Technologies), 100 ng/mL stem cell factor (SCF), 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 100 ng/mL IL-4, and 100 ng/mL TNF- α (Chemicon) or a medium containing 100 ng/mL GM-CSF, 100 ng/mL IL-4, and 100 ng/mL TNF- α (Chemicon). These growth factors (Flt-3 ligand, GM-CSF, IL-4, and TNF- α) are used in cultures, as they have been reported to have the ability to generate large numbers of DCs from BM.²¹ All cultures were maintained at 37°C in a 5% CO₂ humidified incubator. Half of the medium was replenished with a fresh complete medium containing the same cytokines cocktail every 2–3-days. Cultured cells were observed by phase-contrast and inverted microscopy as evidence of increasing size and cytoplasmic projections. On days 7–9, nonadherent fractions of DCs were harvested, and cell numbers were stained with trypan-blue and counted using a hemocytometer. The harvested cells were enriched by using anti-mouse CD11c⁺ antibody-bound magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. The isolated CD11c⁺DCs were collected and were either cultured as previously described in a complete medium supplemented with the cytokine cocktail or cryopreserved for further studies. For the assessment of DCs morphology, cyto-centrifuged preparations of isolated CD11c⁺ cells were stained with the May–Grunwald Giemsa (MGG) stain.

Cryopreservation of DCs

The generated DCs were cryopreserved by adjusting the cell number to 5×10^6 cells/mL in a freezing medium that consists of 10% (V/V) DMSO (Edward Lifescience) and 90% (V/V) chilled FBS (GibcoBRL). Freezing medium was added drop-wise to the DCs with careful shaking. The cell suspension was then transferred into 1.8 mL cryovials (Nal-gene), and the cryovials were placed in freezing containers containing 2-isopropanol. The vials in the container were slowly frozen to -80°C by using a cryo-freezing container where the rate of cooling was controlled at $-1^\circ\text{C}/\text{min}$ (Nunc). After 24 h, the cryovials were transferred and stored in liquid nitrogen at -196°C . To thaw the frozen DCs, cryovials were placed in a 37°C water bath for 2 min and washed once with RPMI medium (Sigma), and the cells were recovered by centrifugation (200 g for 10 min). The resultant pellet was resuspended in complete RPMI 1640 culture medium.

Assessment of DCs phenotype by FACS analysis

DCs generated from murine cultures were subjected to direct immunostaining using appropriately conjugated monoclonal antibodies (Pharmingen) against murine CD4, CD11c, CD11b, CD40, CD54, CD80, CD86, H-2D^d, and I-A^d

and analyzed using FACScan flow cytometry (Becton Dickinson) with the CellQuest software (Becton Dickinson). For flow cytometry data analysis, all phenotypic markers were analyzed using the dot plot. Dot plots of forward scatter (FSC) versus side-scatter (SSC), FL1 (FITC) versus FL2 (PE) and FL2 versus FL3 (Percp) were drawn for each sample. The gain in the FSC and SSC channel was adjusted so that the cell populations were centralized on the dot plot. The desired cell population was included in the analysis by gating, while debris with low FSC and SSC properties were excluded by adjusting the threshold and through the gating approach. Data of all the parameters were saved, and all subsequent acquisition and analysis on the flow cytometer was performed at the gated region and saved parameters. In each acquisition, 10,000 events were acquired for a later data analysis. For the dot plot, the percentage of gated cells that expressed certain cell surface marker phenotypes were obtained from the quadrant statistics of dot plots. The cryopreserved DCs were also similarly analyzed by flow cytometry by following one freeze-and-thaw cycle. Appropriate isotype control monoclonal antibodies were used in this analysis. FSC and SSC gates were used to exclude cell debris and clumps before data analysis.

Preparation of tumor lysates

Mouse 4T1 breast tumor cells were harvested from culture flasks by trypsinization, and cell numbers were adjusted to 2×10^7 cells/mL. The cells were then frozen in liquid nitrogen until further use. For the preparation of tumor lysate (TL) from 4T1 cells, cryopreserved cells were thawed at 37°C and re-frozen in liquid nitrogen. These freeze-thaw procedures were repeated for four-cycles. At the end of the fourth freeze-thaw cycle, the cells were centrifuged (100g for 10 min), and the supernatant was collected. The supernatant, which contains the TL, was passed through a 30 μ m nylon filter column (Miltenyi Biotec). The TL was then aliquoted into cryovials and stored at -80°C.

Preparation of murine responder cells

Mouse T-cells were isolated from the spleens of 6-week-old female BALB/c mice using paramagnetic beads (Miltenyi Biotec) as recommended by the manufacturer. Briefly, the mice were sacrificed by cervical dislocation after they had been anesthetized with diethyl ether (Merck), and their spleens were aseptically removed and placed in a sterile petri dish containing complete RPMI medium. The splenocytes were released from the splenic capsule using aseptic techniques. The splenocyte suspension was transferred to a 15 mL centrifuge tube, and the cells were recovered by centrifugation (200g for 10 min at room temperature). After the supernatant had been discarded, the cells were resuspended in 5 mL of RBC lysing buffer. After 5 min at room temperature, complete RPMI medium was added to fill the tube, and the cells were recovered by centrifugation (200g for 10 min). The pellet was resuspended in complete RPMI medium.

Lymphocyte proliferation assay

The isolated CD11c⁺ MDC were pulsed with TL from 4T1 cells at a ratio of three-tumor cells equivalent to one DCs (3:1)

in complete medium. After 18 h of incubation, the DCs were collected and irradiated at 3000 rad (⁶⁰Co). The irradiated DCs, which served as stimulator cells, were washed twice in Hank's balanced salt solution (HBSS; Gibco BRL). Then, the stimulator cells were resuspended in complete RPMI medium for the lymphocyte proliferation assay. Freshly prepared splenocytes served as responder cells. The concentration of the irradiated stimulator DCs was adjusted to 1×10^4 cells/well. After this, a serial dilution was carried out to generate four different stimulator-to-responder ratios of 1:5, 1:10, 1:20, and 1:40 when added with freshly prepared splenocytes that served as responder cells (5×10^4 cells) in a 96-well U-bottomed tissue culture plate (Nunc). The T-responder cells stimulated with Concanavalin A (5 μ g/mL) (Sigma Aldrich) served as a positive control, while unstimulated splenocytes were used as the negative control (T-responder cells alone). All samples were prepared in triplicate wells. The 96-well plate was incubated at 37°C in a humidified 5% CO₂ incubator for 5 days. Proliferative responses were measured using a BrdU ELISA according to the manufacturer's recommended protocol (Roche). Proliferative responses are reported as mean absorbance (OD) \pm SD from triplicate wells.

Induction of breast tumor in mice

About 0.05 mL of 4T1 cells ($\sim 1 \times 10^4$ 4T1 cells) were injected into the right flank mammary pad of 6-week-old female BALB/c mice. The animals were palpated every 2–3 days after inoculation of tumor cells to monitor the development of the tumor. Animals that developed tumors after 14 days were divided into three groups of six mice each. Tumor-laden mice in the control group (group 1) did not receive any treatment, while mice in the second and third groups were treated with either 1×10^6 freshly generated (group 2) or cryopreserved (group 3) MDC, thrice, at intervals of 7 days. The tumor volume was assessed in a blinded, coded fashion every 2–3 days by measuring the length and perpendicular diameter of the tumors.⁷ Mice were observed for survival and were monitored for symptoms of pain and distress. Any mice that became moribund or were found to be in distress were promptly sacrificed.

Statistical analysis

The difference in the average time for tumor volumes to reach ~ 500 mm³ as well as the survival times of control and experimental mice were analyzed using the Mann-Whitney-U test from the SPSS software. The differences between the mean tumor volumes on day 30 from experimental and control mice were also analyzed using the same test. In this analysis, *p*-values of less than 0.05 were considered significant.

Results

Morphology and yield of cryopreserved and fresh DCs derived from BM

We compared the morphology of cryopreserved and freshly generated DC in a culture medium supplemented with combinations of Flt-3 ligand, SCF, GM-CSF, IL-4, and TNF-alpha for 7–9 days.²¹ The addition of the Flt 3-ligand in the cytokine cocktail induced the generation of large numbers of mouse CD11c⁺ DCs from BM (data not shown). Morphological observation on day 7–9 of the freshly cultured DCs

showed the formation of multicellular clusters and the cytoplasmic projection or veils that are typical of DCs (Fig. 1). These typical DC neurite-like cytoplasmic projections were also observed in the DCs generated from BM cultures after staining with MGG (Fig. 2). The morphology of cryopreserved mouse bone marrow (mBM)-derived cells was compared with freshly generated DCs to study whether DMSO and one cycle of the freeze-thaw process induced any changes on the DC morphology. No significant morphological differences were observed between the cryopreserved DCs and freshly generated DCs after one freeze-and-thaw cycle (Figs. 1 and 2). The cryopreserved mBM-DCs still maintained a typical DC morphology, that is, they displayed thin cytoplasmic projections and veils, which were similar to those observed in freshly generated DCs.

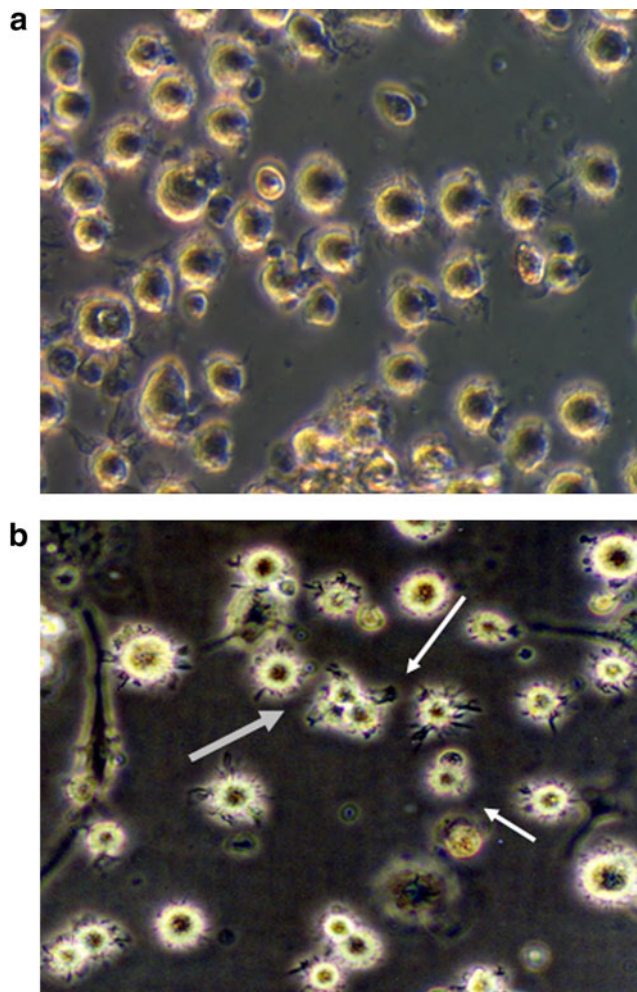


FIG. 1. Morphology of freshly generated and cryopreserved mouse bone marrow (BM)-derived dendritic cells (DCs) analyzed by inverted microscopy. Mouse BM was cultured in a medium containing DC differentiation cytokine cocktail as described in the materials and methods. Some of the generated DC were cryopreserved. The (a) cryopreserved and (b) freshly generated mouse BM-derived DC were analyzed with inverted microscopy. (a) cryopreserved (magnification of 400 \times) and (b) freshly generated (magnification 200 \times) DC from mouse BM cells display typical DC cytoplasmic processes. Arrows show the dendrites present on the DCs.

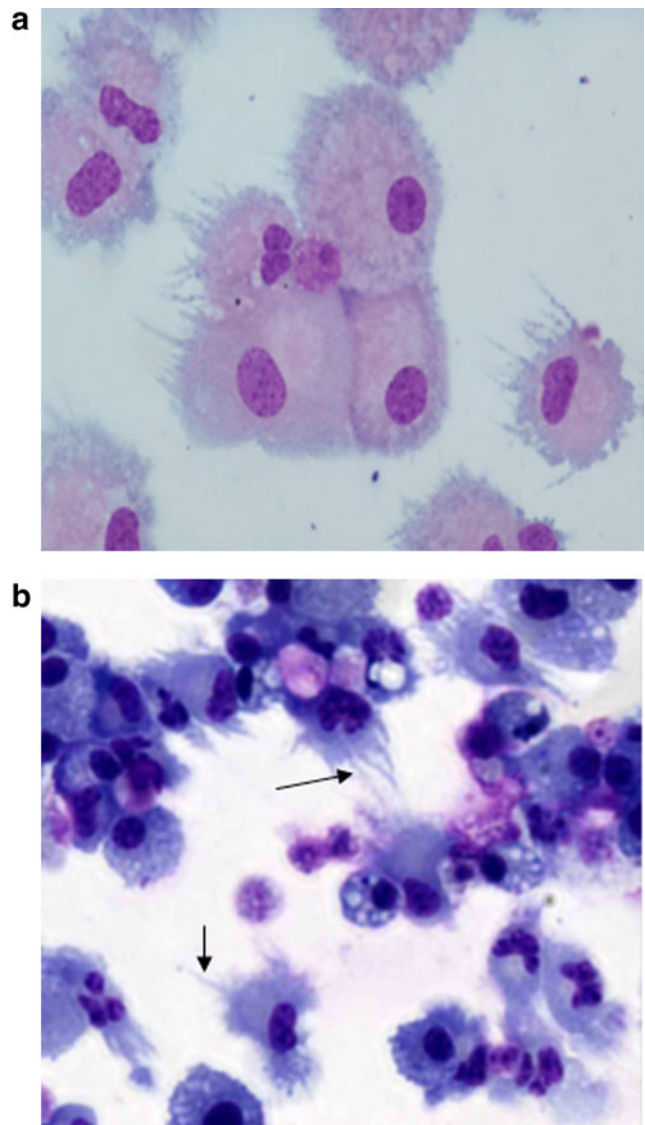


FIG. 2. Morphology of mouse BM-derived DCs as analyzed using May-Grünwald Giemsa (MGG) staining. Mouse BM was cultured in a medium containing DC differentiation cytokine cocktail, and some of the generated DC were cryopreserved as described in the materials and methods. The (a) freshly generated and (b) cryopreserved mouse BM-derived DC were stained with (a) MGG staining of freshly generated DC from CD11c⁺ mouse BM cells showing a typical DC morphology, that is, cytoplasmic projections (magnification 400 \times); (b) MGG staining of cryopreserved DC generated from CD11c⁺ mouse BM derived-DC also showing a typical DC morphology (magnification 200 \times). Arrows show the dendrites present on the DCs.

Immune phenotype of cryopreserved and fresh DCs

Mouse BM-DCs harvested on days 7–9 represent a population of cells that express CD11c, CD80, CD86, CD40, CD1d, and CD11b (Table 1). As shown in Table 1, the majority of the BM-derived DCs also expressed MHC class I and II molecules, that is, H-2D^d and I-A^d (94.1% and 74.17% respectively) and CD54 (ICAM-1) molecules (88.12%). The mouse BM-derived DCs population did not express any of the lineage markers for B-cells (e.g., CD19), T-cells (e.g., CD3), and

TABLE 1. COMPARISON OF THE EXPRESSION OF VARIOUS PHENOTYPIC CELL SURFACE ANTIGENS ON FRESHLY GENERATED DENDRITIC CELLS AND CRYOPRESERVED MURINE DENDRITIC CELLS DERIVED FROM BONE MARROW CELLS USING FLOW CYTOMETRY

Phenotype	Expression (%)	
	Fresh	Cryopreserved
CD11c ⁺	77.48	81.66
CD80 ⁺ /CD11c ⁺	67.14	60.18
CD86 ⁺ /CD11c ⁺	65.75	53.47
CD40 ⁺ /CD11c ⁺	52.04	51.57
CD11b ⁺ /CD11c ⁺	52.59	55.08
CD4 ⁺	2.9	5.34
CD8 α ⁺	1.89	3.08
CD54 ⁺	88.12	77.62
H-2D ^{d+}	94.11	74.6
I-A ^{d+}	74.17	76.81

plasmacytoid DCs (anti-PDCA) (data not shown). The majority (77%) of the mouse BM-derived DCs harvested in this study were CD11c⁺, suggesting that mouse BM cells cultured in a medium containing DCs differentiation cytokine cocktail for 7–9 days could successfully generate DCs that displayed typical DCs cell surface antigens. The cultured mouse BM-derived DCs generated in this study were mostly CD4⁺CD8[−], which is phenotypically similar to the double-negative DCs (DNDC) subset previously reported in the murine spleen.⁴

Mixed leucocyte reaction

A mixed leucocyte reaction (MLR) was performed to assess the ability of MDC pulsed with TL from 4T1 cells to prime splenic T-cells isolated from BALB/c mouse. The 4T1 TL pulsed DCs were co-cultured with isolated syngeneic splenic T-cells for 5-days, and proliferation was measured by the BrdU ELISA assay according to the manufacturer's instructions. An enhanced level of T-cell proliferation was observed when T-cells were co-cultured with DNDC pulsed with TL from 4T1 cells (Fig. 3). The DC pulsed with TL from 4T1 cells (DC vaccines) at a stimulator:responder (S:R) ratio of 1:40 triggered a substantial T-cell proliferation response (Fig. 3). When the stimulatory ability of freshly generated and cryopreserved DCs derived from mouse BM were compared, results show that both types of DCs triggered similar levels of T-cell proliferation (Fig. 3). In both cases, the enhanced proliferation observed was higher than the baseline levels observed in the negative control. The stimulatory ability of both cryopreserved and freshly generated DCs were proportional to the S:R ratio.

Effects of therapy with fresh and cryopreserved DCs

We next compared the therapeutic potential of freshly generated and cryopreserved murine BM-derived, double-negative DCs (BM derived-DNDC) pulsed with TL from 4T1 cells in treating breast cancer using a mouse mammary carcinoma model.²⁰ The mean time for tumor volume to reach 500 mm³ in the mice treated with freshly generated or cryopreserved BM-derived DCs pulsed with TL from 4T1 tumor cells was compared with the mice in the untreated/control group. The mean time taken for tumor volume in the control

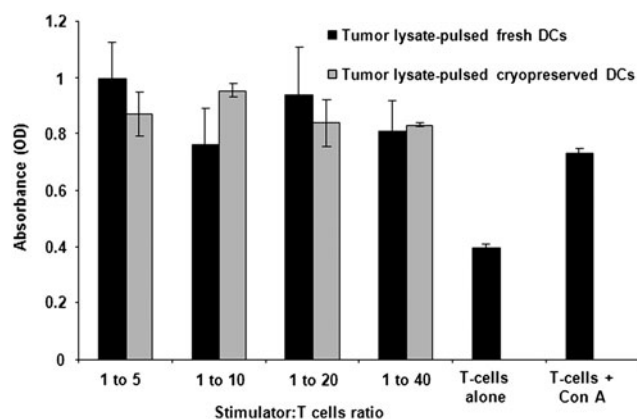


FIG. 3. Results of mixed leucocyte reaction performed using murine T-cells co-cultured with either freshly generated or cryopreserved double-negative DCs (DNDC) and were incubated with tumor lysate (TL) from 4T1 cells. Murine T-cells were isolated from the spleens of BALB/c mice. The isolated murine T-cells were co-cultured with either (i) freshly generated CD11c⁺DC pulsed with TL from 4T1 cells; (ii) cryopreserved CD11c⁺DC pulsed with TL from 4T1 cells; (iii) T-cells alone; or (iv) T-cells cultured in the presence of Concanavalin A (T + Con A). The T-cells alone served as a negative control, whereas T + Con A functioned as the positive control. Proliferation was measured by BrdU ELISA assay according to the manufacturer's recommended protocol.

group to reach 500 mm³ was 25.7 days, while for the groups treated with either freshly generated or cryopreserved BM-DC, they were 33.9 and 35 days, respectively (Fig. 4). This means that there was a significant ($p < 0.05$) delay in the mean time required for the tumor in the animals treated with DCs vaccines made using fresh or cryopreserved DCs to reach the volume of 500 mm³ when compared with the control group. However, there were no significant differences ($p > 0.05$) in the mean time for tumor volume to reach 500 mm³ between the mice treated with either freshly generated or cryopreserved DCs (see Fig. 4). Despite the initial retardation of tumor growth in the DC-treated groups, the tumors in all the experimental mice progressively developed after day 24. However, in mice treated with either freshly generated or cryopreserved DC, vaccines still had a significantly ($p < 0.05$) smaller tumor volume on day 30 as compared with the untreated control group. All the mice in the control groups survived for at least 29 days, while the mice in the DNDC-treated groups were culled after day 36 (Fig. 5). It should be noted that when all the untreated mice died from tumor growth on day 33, 100% of the mice treated with freshly generated DCs pulsed with TL from 4T1 cells and 83% of the mice treated with cryopreserved DCs were still alive (Fig. 5). This meant that treatment with cryopreserved DCs had a similar potential in prolonging the survival time and reducing tumor load as compared to treatment with freshly generated DCs. The mice treated with the DC vaccines survived significantly ($p < 0.05$) longer than the control group, with a mean survival time (MST) of 37.5 and 35.3 days, respectively, while the MST for control mice was only 28 days. Again, there were no significant differences of MST observed between the mice treated with freshly generated

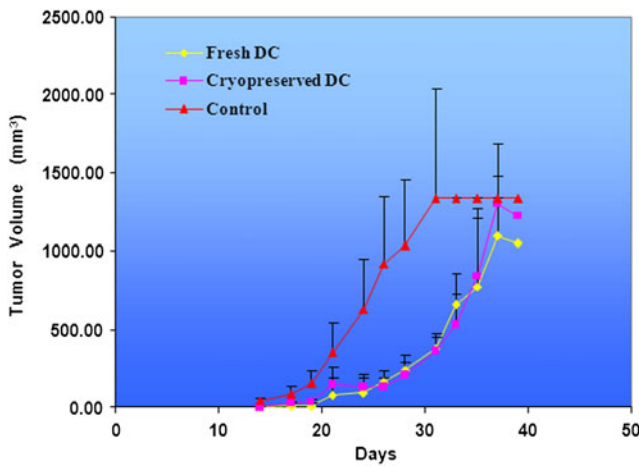


FIG. 4. The growth of 4T1 tumor in mice treated with mouse BM-derived DCs. Mice were inoculated with 1×10^4 cells of 4T1 tumor cells at the mammary pad of BALB/c mice as described in materials and methods. Freshly generated or cryopreserved murine BM-derived DC after being pulsed with TL from 4T1 tumor cells. These DC were subcutaneously injected thrice in the left flank of mice as described in the Materials and Methods section. Mice in the control groups are those that received no DC treatment. The tumor size was assessed in a coded, blinded fashion; was recorded as tumor volume (mm^3) by measuring the largest perpendicular diameters; and was calculated as described in materials and methods. The data represent the average tumor volume \pm SD in each group of mice. The end point of the line represents the death or termination of the mice.

and cryopreserved DCs. Both cryopreserved and freshly generated DCs demonstrated higher survival time as compared with untreated mice. Our data indicate that DC-based treatment was effective in inhibiting the growth of the mammary tumor *in vivo* to some extent.

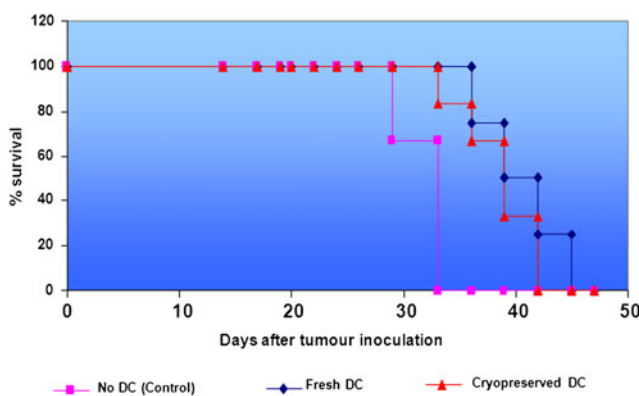


FIG. 5. Survival of mice treated with mouse BM-derived DNDC pulsed with TL from 4T1 cells. Mice were inoculated with 1×10^4 cells of 4T1 tumor cells at the mammary pad of BALB/c mice. Freshly generated or cryopreserved DC pulsed with TL from 4T1 tumor cells were subcutaneously injected thrice in the left flank of mice. Mice in the control groups received no DC treatment. The survival of mice from each group was monitored over time after DC treatment, and the mean survival time in days was determined.

Discussion

Many clinical trials and animal studies have demonstrated the critical role of DCs as well as the feasibility of the repetitive administration of DCs in cancer immunotherapy.^{6,7,14} However, a consistent and reliable supply of DCs to provide sequential administration of treatment remains one of the constraints that needs to be overcome before the potential of DC immunotherapy can be fully exploited. The use of cryopreserved DCs in cancer immunotherapy could be considered the most feasible approach, as it can allow us to get around the problems associated with repetitive generated DCs from time to time such as batch-to-batch variations, issues on safety and cost. If successful, this will be an important step forward in DCs immunotherapy. In the current study, we compared the immunobiology of freshly generated murine BM-derived DCs with cryopreserved MDC using both *in vitro* and *in vivo* test systems. Our data demonstrate that cryopreserved murine DNDC showed no adverse alteration in morphology when examined by inverted microscopy and MGG staining. Similar observations have been reported in human monocyte-derived DCs that were recovered from cryopreservation.¹⁶ In addition, the thawed cryopreserved murine and freshly generated DCs showed a similar expression of all the DC-specific cell surface markers tested. This finding is consistent with the studies performed on human monocyte-derived DCs by others, who reported that the expression of most phenotypic markers observed in fresh and cryopreserved DCs derived from normal human monocytes were similar.^{16,17,22} The expression stability of these cell surface antigens on DCs was also demonstrated in monocyte-derived DCs obtained from chronic myeloid leukemia patients in whom the expression levels were found to be comparable to those seen on DCs isolated from healthy donors even after cryopreservation.¹⁷ Thus, our findings show that the expression stability of DC-specific cell surface antigens on cryopreserved MDC was similar to that observed in the human system. In the mixed lymphocytes reaction, the stimulatory properties of freshly generated and cryopreserved BM-derived DCs in inducing a proliferative response on the T-lymphocytes were compared. The cytokine-differentiated fresh MDC vaccines generated a significant T-cell proliferative response *in vitro*. Comparable results were obtained when cryopreserved DCs were used instead of freshly generated DCs in the MLR assay. These findings further support the hypothesis that the stimulatory ability of DCs are well preserved even after these cells were subjected to one cycle of freeze-thaw process. Similar observations have been reported in human monocyte-derived DCs that were recovered from cryopreservation.^{16,17,19} The ability to stimulate strong T-cell proliferation responses by the cryopreserved DCs pulsed with TL shows that their function as APC is not impaired due to the cryopreservation or freeze-thaw procedures. This is an important finding, as any reduction in the function of the cryopreserved DCs in the activation of the host immune response might affect the efficacy of DCs when used in cancer immunotherapy.

As discussed in this article, there have not been any *in vivo* studies conducted to compare the therapeutic efficacy of cryopreserved and freshly generated DCs. Hence, it was important to test whether the cryopreserved and freshly generated DCs, which had well-preserved functional properties in the

in vitro studies, can exert a similar therapeutic efficacy *in vivo*. We developed a murine breast cancer model to compare the ability of freshly generated and cryopreserved DCs in generating an anti-tumor immune response. We observed that treatment with either freshly generated or cryopreserved DCs vaccines resulted in similar initial growth retardation of the 4T1 cell-induced tumor in the mice, and there was no significant difference seen between both forms of treatments. Although none of the treatment regimens used in this study was capable of completely eradicating the tumor mass, tumor growth was, nevertheless, suppressed initially, and a significant ($p < 0.05$) and a smaller tumor volume was observed in mice from the experimental group compared with those from the control group. The slower tumor growth as indicated by mean tumor volume in mice that received either fresh or cryopreserved DC treatment compared with control mice suggests that DC treatment was successful in activating the murine immune system to a certain extent. The findings from our *in vitro* and *in vivo* studies show that MDC recovered from cryopreservation have similar functional properties and therapeutic efficacy as freshly generated DCs. Our findings also suggest that the application of cryopreserved DCs for cancer immunotherapy in a clinical setting is feasible. In addition, the tumor-laden BALB/c mice treated with either fresh or cryopreserved DC survived significantly longer than control mice. However, there was no significant difference in terms of survival between the mice treated with cryopreserved and/or fresh DCs. The ability to produce a large number of cryopreserved DCs that retained their normal morphology and function allows the repeated use of DCs in cancer immunotherapy and DC vaccinations. In this study, the administration of three repeated doses of freshly generated or cryopreserved DCs injections at a 7-day interval displayed similar initial tumor retardation but were, however, insufficient to sustain the immune response produced. It is also possible that the ability of DCs treatment to retard tumor growth may be counteracted by the large tumor burden and possibly due to the induction of inhibitory factors by tumor cells which can result in the "tumor escape" phenomenon.²³ So, we postulate that the ability of these DCs to exert long-lasting anti-tumor responses may be achieved with more increased and more frequent treatment of DCs. In conclusion, our findings show that cryopreserved DCs are able to retain their morphology, phenotype, and functions in activating the proliferation of T-cells. In addition, cryopreserved and fresh DCs pulsed with tumor lysate showed similar tumor growth inhibition patterns and are able to induce the initial retardation of mammary tumor growth.

Acknowledgments

This work was supported by a grant from the Ministry of Science, Technology, and Innovation (MOSTI), Malaysia, grant number IRPA-06-02-02-PR0001/06-01. The authors are grateful to all the staff from the Department of Animal Care, IMR, and the National University of Malaysia (UKM), Malaysia, for their assistance in establishing the animal model in this study. They would also like to thank all the staff from the Department of Radiology, Makna-Hospital UKM (IKM-HUKM), Malaysia, for their assistance in this research.

Author Disclosure Statement

No competing financial interests exist.

Reference

- Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med.* 1973;137:1142-1162.
- Banchereau J, Briere F, Caux C, et al. Immunobiology of dendritic cells. *Annu Rev Immunol.* 2000;18:767-811.
- Feng B, Inaba M, Lain ZX, et al. Development of mouse dendritic cells from lineage-negative c-kit^{low} pluripotent hemopoietic stem cells *in vitro*. *Stem Cells.* 2000;18:53-60.
- Hochrein H, Shortman K, Vremec D, et al. Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. *J Immunol.* 2001;166:5448-5455.
- Lanzavecchia A. Mechanisms of antigen uptake for presentation. *Curr Opin Immunol.* 1996;8:348-354.
- Fields RC, Shimizu K, Mule JJ. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses *in vitro* and *in vivo*. *Proc Natl Acad Sci USA.* 1998;95:9482-9487.
- Shimizu K, Fields R, Giedlin M, Mule JJ. Systemic administration of interleukin 2 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines. *Proc Natl Acad Sci USA.* 1999;96:2268-2273.
- Timmerman JM, Czerwinski DK, Davis TA, et al. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: Clinical and immune responses in 35 patients. *Blood.* 2002;99:1517-1526.
- Brody JD, Engleman EG. DC-based cancer vaccines: Lessons from clinical trials. *Cytotherapy.* 2004;6:122-127.
- Banchereau J, Ueno H, Dhodapkar M, et al. Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-pulsed dendritic cells derived from CD34+ progenitors and activated with type I interferon. *J Immunother.* 2005;28:505-516.
- Panelli MC, Wunderlich J, Jeffries J, et al. Phase 1 study in patients with metastatic melanoma of immunization with dendritic cells presenting epitopes derived from the melanoma-associated antigens MART-1 and gp100. *J Immunother.* 2000;23:487-498.
- O'Rourke MG, Johnson M, Lanagan C, et al. Durable complete clinical responses in a phase I/II trial using an autologous melanoma cell/dendritic cell vaccine. *Cancer Immunol Immunother.* 2003;52:387-395.
- Lopez-Guillermo A, Cabanillas F, McLaughlin P, et al. Molecular response assessed by PCR is the most important factor predicting failure-free survival in indolent follicular lymphoma: Update of the MDACC series. *Ann Oncol.* 2000;11:137-140.
- Tjoa BA, Simmons SJ, Elgamal A, et al. Follow-up evaluation of a phase II prostate cancer vaccine trial. *Prostate.* 1999;40:125-129.
- Candido KA, Shimizu K, McLaughlin JC, et al. Local administration of dendritic cells inhibits established breast tumor growth: Implications for apoptosis-inducing agents. *Cancer Res.* 2001;61:228-236.
- John J, Hutchinson J, Dalglish A, Pandha H. Cryopreservation of immature monocyte-derived dendritic cells results in enhanced cell maturation but reduced endocytic activity and efficacy of adenoviral transduction. *J Immunol Methods.* 2003;272:35-48.

17. Westermann J, Korner IJ, Kopp J, et al. Cryopreservation of mature monocyte-derived human dendritic cells for vaccination: Influence on phenotype and functional properties. *Cancer Immunol Immunother.* 2003;52:194–198.
18. Feuerstein B, Berger TG, Maczek C, et al. A method of the production of cryopreserved aliquots of antigen-preloaded mature dendritic cells ready for clinical use. *J Immunol Methods.* 2000;245:15–29.
19. Schuler-Thurner B, Schultz ES, Berger TG, et al. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med.* 2002;195:1279–1288. Erratum in: *J Exp Med.* 2003; 197:395.
20. Pulaski BA, Terman DS, Khan S, et al. Cooperativity of Staphylococcal aureus Enterotoxin B Superantigen, major histocompatibility complex Class II, and CD80 for Immunotherapy of Advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model. *Cancer Res.* 2000;60:710–715.
21. Inaba K, Inaba M, Romani N, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1992;176:1693–1702.
22. Hori S, Heike Y, Takei M, et al. Freeze–thawing procedures have no influence on the phenotypic and functional development of dendritic cells generated from peripheral blood CD14+ monocytes. *J Immunother.* 2004;27:27–35.
23. Dunn GP, Bruce AP, Ikeda H, et al. Cancer immunoeediting from immune surveillance to tumour escape. *Nat Immunol.* 2002;4:1206–1212.

Address correspondence to:

*Ammu Kutty Radhakrishnan, BSc(Hons), MSc, PhD
Pathology Division
Faculty of Medicine and Health
International Medical University
126, Jalan Jalil Perkasa 19
Bukit Jalil,
57000 Kuala Lumpur
Malaysia*

E-mail: ammu_radhakrishnan@imu.edu.my