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ORIGINAL ARTICLE

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Cryopreservation of mature monocyte-derived human dendritic cells for vaccination: influence on phenotype and functional properties

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Abstract In the past decade there has been increasing evidence that tumor antigen-loaded dendritic cells (DC) are able to elicit anti-tumor T-cell responses. Initial clinical data for different tumor entities are encouraging, with objective tumor regressions being observed in some patients. Since GMP production of DC for clinical vaccination protocols is a time- and cost-intensive procedure, cryopreservation of DC in aliquots ready for clinical use would significantly facilitate DC-based vaccination in the clinic. We asked whether freezing and thawing alters the phenotype or functional properties of DC. DC from healthy volunteers and from patients with chronic myeloid leukemia (CML) were analyzed after freezing and thawing for their viability, morphology, immunophenotype (FACS profile), T-cell stimulatory capacity (mixed lymphocyte reaction) and mobility (time-lapse cinemicroscopy). Our results demonstrate that cryopreservation does not cause significant changes in the phenotype or function of DC, neither in DC from healthy volunteers nor in those from CML patients. Our data indicate that cryopreserved aliquots of DC are suitable for clinical application in DC-based immunotherapy protocols.

Keywords Dendritic cells · Cryopreservation · Vaccination

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Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) and probably the only ones able to prime naive T cells (reviewed in [1] and [13]). In recent years there has been increasing evidence that tumor antigen-loaded DC can elicit tumor-specific T-cell responses both in animal models and in humans (reviewed in [3]). In healthy volunteers, a single subcutaneous injection with antigen-loaded DC was able to induce an antigen-specific T-cell response [4]. First clinical studies using DC-based vaccines are encouraging in non-Hodgkin's lymphoma [6], advanced melanoma [11], prostate cancer [10] and bladder cancer [12]. DC-based vaccination strategies have recently been reviewed by Banchereau et al. [2]. Nevertheless, for clinical vaccination with DC many open questions remain to be answered: i.e., the cell number per vaccination, the route of administration and the optimal time and way of antigen loading (protein/peptide pulsing versus genetic modification, addition of adjuvans). Furthermore, animal studies and first clinical data suggest that repetitive vaccinations over a longer period might be necessary in order to break pre-existing tolerance and to achieve clinically relevant immune responses.

Generation of DC according to GMP (good manufacturing practice) criteria is a very time- and cost-intensive procedure, which limits significantly the feasibility of repeated vaccinations. Production of sufficient numbers of DC at one time point with subsequent cryopreservation in aliquots ready for clinical application would dramatically improve practicability of DC-based vaccination.

In the current study we evaluated the influence of freezing and thawing on the function of monocyte-derived human DC from healthy donors and from CML patients. Phenotypic and functional properties of DC were analyzed in fresh, cultured cells and after one cycle of freezing and thawing.

Material and methods

Generation of DC from peripheral blood mononuclear cells

DC were generated from healthy donors and from patients with chronic phase bcr/abl+ CML. The rationale for using DC vaccination in CML has been described [15]. Peripheral blood mononuclear cells (PBMC) were collected by standard leukapheresis using an AS 104 cell separator (Fresenius, Bad Homburg, Germany). PBMC were further enriched by density gradient centrifugation on Lymphoprep (Life Technologies, Eggenstein-Leopoldshafen, Germany) and then stored on liquid nitrogen in 80% human serum albumin (HSA, 20% solution, Octapharma, Langenfeld, Germany) and 20% DMSO (v/v) (Sigma, St.Louis, Mo., USA). Thawing of PBMC was performed by the addition of HBSS w/o Ca++/Mg++ (Biowhittaker, Walkersville, USA) containing 5% HSA. PBMC were washed twice and then exposed to plastic adherence for monocyte enrichment. Briefly, PBMC were seeded at a density of 1.4×10^6 cells/cm^2 in 185-cm^2 culture flasks (Nunc, Roskilde, Denmark) and allowed to adhere for 2 h at 37°C in RPMI 1640 containing 5% HSA and 1% sodium pyruvate (Biowhittaker). Non-adherent cells were removed by washing four times. Adherent cells were cultured in RPMI 1640 supplemented with 100 ng GM-CSF (Novartis, Basel, Switzerland), 1,000 U/ml clinical grade interleukin-4 (kindly provided by Shering-Plough Research Institute, Kenilworth, N.J., USA), 10% fetal calf serum (FCS) (Summit, USA) and 1% sodium pyruvate for 5 days. On day 5, 50 ng/ml clinical grade TNF-α (Bender, Vienna, Austria) was added to the culture medium for another 3–4 days. On days 8–9, DC were harvested and washed three times.

Freezing and thawing protocol of DC

The cell number was adjusted to 2×10^6 cells/ml in RPMI/10% FCS, and cells were cooled down to 4–6°C. Then, the same volume of cold freezing solution consisting of 80% (v/v) HSA 20% and 20% (v/v) DMSO was added. Freezing medium was added drop-wise under careful shaking. The cell suspension was then transferred into 1.8-ml cryo tube vials (Nunc). Vials were immediately frozen by using a computer-assisted freezing device (Consartic, Stockstadt, Germany; standard freezing program for PBMC) and stored on liquid nitrogen.

For thawing of DC, cryo-tube vials were slightly shaken in a 37°C water bath for 2 min. The cell suspension was then transferred into a 10-ml tube. Ice-cold HBSS w/o Ca++/Mg++ (Biowhittaker) containing 5% HSA (20%) was added under cautious shaking of the tube (initially drop-wise, then addition of 2.0 ml). After centrifugation (10 min, 1,000 rpm, 20°C), the cell pellet was re-suspended in culture medium (RPMI 1640, 10% FCS, 1% sodium pyruvat).

Cryopreserved DC were compared with fresh DC. In order to perform comparative experiments for each DC preparation, cryopreservation was limited to 4–5 h.

Viability testing

Viability of DC was determined before and after freezing and thawing using the trypan blue dye exclusion.

FACS analysis

Phycoerythrin (PE)-conjugated mouse monoclonal antibodies against the following antigens were used: CD1a, CD3, CD4, CD14, CD19, CD80, CD86, HLA-DR, MHC class I, isotype control antibody (all Becton-Dickinson, Mountain View, Calif., USA) and CD83 (Immunotech, Hamburg, Germany).

Mixed lymphocyte reaction (MLR)

Allogeneic MLR was performed as previously described [14]. Briefly, 156 to 10,000 irradiated (30 Gy) autologous DC were used as stimulator cells together with 10^5 allogeneic T cells as responder cells in RPMI 1640 containing 10% FCS in a 5-day standard 3H-thymidine incorporation assay. Responder T cells for MLR were taken from the same donor. Proliferation was analyzed in triplicates; mean values of each triplicate were calculated. The stimulatory capacity of DC before and after cryopreservation was expressed as the stimulation index (S.I.).

Analysis of DC mobility

DC were seeded on tissue culture dishes (Nunc) at a density of 0.5×10^6 cells/ml and analyzed by time-lapse cinemicroscopy. Cells were automatically recorded for 4 h using an Axiovert microscope (Zeiss) equipped with a custom-made incubator (37°C, 5% CO_2) and a CCD camera (Sony). The mobility of individual cells was evaluated by morphology, veil structure and tracking their movement. Actively moving cells with dynamic veil structures were classified as mobile. The proportion of dead cells did not exceed 10% of the cell population seeded.

Results

Phenotype of DC before and after cryopreservation

After freezing and thawing, no significant changes in morphology and viability of the DC preparations could be observed. In DC cultures of four healthy donors, viability was in the range of 90% before and after the freezing/thawing procedure. Similar results were obtained in four DC preparations from CML patients in which a cell viability of approximately 89% was not changed by the freezing/thawing procedure (Table 1).

FACS analysis showed an immunophenotype characteristic of mature DC with high expression levels of costimulatory molecules (CD80, CD86), CD83, MHC class I and MHC class II molecules. CD14 was extremely weak or negative showing efficient suppression of monocytoid differentiation by the chosen culture conditions, which include GM-CSF and IL-4 with the addition of TNF-α as a final maturation stimulus during the last 3–4 days of the cell culture. In healthy donors, T-cell contamination (CD3+) of the DC preparation was between 5 and 15%, B cell contamination (CD19+) was in the range of 9–24%. In DC from CML patients, the proportion of T or B cells in the cell culture was less than 10% in three out of four cases; in one DC culture of a CML patient, T cells were measured in the range of 15%.

Freezing and thawing did not cause significant changes in the immunophenotype of DC (Table 2A and B).

T-cell stimulatory capacity of DC

In order to study the influence of freezing/thawing on T-cell stimulatory capacity, allogeneic MLRs were performed before and after cryopreservation. Considering

Table 1 Viability (%) of DC in healthy donors (*A*) and CML patients (*B*). Viability of DC was assessed before and immediately after cryopreservation by using the trypan blue staining method

	Before cryopreservation	After cryopreservation
A		
No. 1	92	83
No. 2	93	88
No. 3	93	80
No. 4	89	91
Mean ± SD	91.8 ± 1.9	88 ± 3.6
B		
CML no. 1	83	88
CML no. 2	88	85
CML no. 3	99	92
CML no. 4	85	85
Mean ± SD	88.8 ± 7.1	87.5 ± 3.3

the stimulation indices at different stimulator: responder ratios before and after freezing/thawing, no significant decrease in the stimulatory capacity of DC was found. Cryopreserved DC from healthy donors as well as from CML patients still were potent stimulators in MLR (Fig. 1, Fig. 2, Table 3).

T-cell stimulatory capacity in MLR after cryopreservation is expressed as ratio of the stimulation index (S.I.) after cryopreservation: stimulation index before cryopreservation. The ratio is shown for each number of stimulator cells in the MLR separately.

$$\text{Ratio: } \frac{\text{S.I. after cryopreservation}}{\text{S.I. before cryopreservation}} \pm \text{S.D} \qquad (1)$$

Mobility of DC

Since cell mobility is a characteristic feature of DC and a potential marker for viability and functional integrity, time-lapse cinemicroscopy experiments were performed to assess the number of viable and mobile DC before and after cryopreservation. For both DC from healthy donors and from CML patients, mobility was not changed by the freezing/thawing procedure, and the percentage of viable mobile cell was consistently found to be $>70\%$ (Fig. 3).

Discussion

Generation of sufficient numbers of functional DC is a prerequisite for DC-based immunotherapy protocols. DC production according to GMP criteria is a time- and cost-intensive procedure. If repeated vaccinations are required, both the time and the costs necessary for a clinical study could be prohibitive. Drawing blood at different time points for direct generation of DC from freshly isolated mononuclear cells cannot be recommended because the quality of DC generated at different time points may undergo considerable variations.

Makino and Baba [9] described a cryopreservation method for human PBMC for the production of DC: they found that freezing/thawing does not impair the ability of PBMC to differentiate into DC under the chosen culture conditions. DC from frozen PBMC did not differ substantially from DC that were generated

Table 2 Immunophenotype of DC before and after cryopreservation. Immunophenotype of DC from healthy donors (*A*) and from CML patients (*B*) was studied by FACS before and after cryopreservation. Percentage of marker-positive cells in the ungated population is shown in *A* and *B* (*lack of staining due to technical fault)

	No. 1 before	No. 1 after	No. 2 before	No. 2 after	No. 3 before	No. 3 after	No. 4 before	No. 4 after
A. Healthy donors								
CD3	15	14	n.d.	9	16	13	7	5
CD14	2	2	1	1	3	3	2	2
CD19	14	13	24	23	21	17	11	9
CD4	74	78	65	63	67	69	65	72
CD1a	62	62	54	53	63	62	66	68
CD80	65	65	66	63	69	68	67	74
CD86	78	87	70	64	81	75	67	78
CD83	68	63	65	54	76	59	67	65
CD11c	70	70	71	64	73	68	73	73
Class-I	80	75	*	74	93	82	74	84
Class-II	80	77	92	90	91	87	78	77
B. CML patients								
CD3	4	5	3	5	14	16	8	7
CD14	1	1	0	2	1	1	1	0
CD19	4	5	0	4	2	2	2	2
CD4	66	60	67	61	60	73	40	41
CD1a	14	9	14	12	64	66	52	57
CD80	68	68	70	68	51	62	38	46
CD86	78	78	79	80	69	72	64	62
CD83	51	52	37	54	59	64	45	49
CD11c	70	71	78	79	71	73	69	66
Class-I	77	73	80	75	67	76	53	59
Class-II	79	79	82	82	74	75	63	70

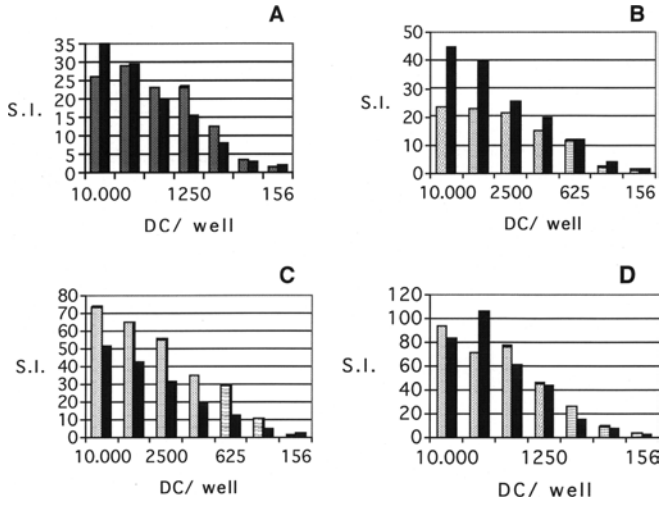

Fig. 1 T-cell stimulation after cryopreservation in healthy donors. T-cell stimulatory capacity in MLR before (*dotted bars*) and after (*black bars*) cryopreservation is compared for each stimulator: responder ratio (from 156 DC/well up to 10,000 DC/well, always 10^5 T cells as responder cells). T-cell stimulation by DC is expressed as the stimulation index (S.I.). Each figure represents data from one healthy donor

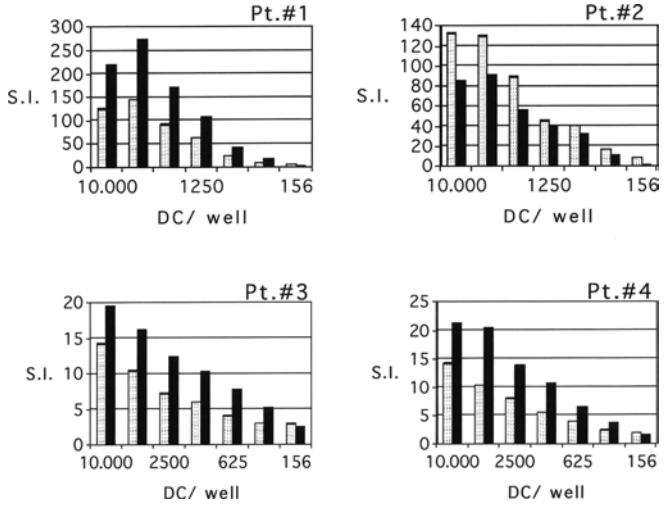

Fig. 2 T-cell stimulation after cryopreservation in CML patients. T-cell stimulatory capacity in MLR before (*dotted bars*) and after (*black bars*) cryopreservation is compared for each stimulator: responder ratio (from 156 DC/well up to 10,000 DC/well, always 10^5 T cells as responder cells). T-cell stimulation by DC is expressed as the stimulation index (S.I.). Each figure represents data from one CML patient

from fresh PBMC in terms of viability, cell yield, phenotypic and functional properties. But using this approach, mononuclear cells still have to be thawed and differentiated for each single vaccination. Since, in a GMP lab, only one single-cell product can be processed in a production line and the generation time of DC is about 9–11 days, considerable limitations in the number of patients that can be treated would be present. From a practicability point of view, it would clearly be of advantage to perform cryopreservation of DC ready for clinical use.

In this study we asked whether terminally differentiated DC can be frozen and thawed without compromising their viability and functional integrity.

Viability of our different DC preparations was not significantly altered by our cryopreservation protocol, showing that the procedure is feasible for large scale production and preservation of DC. The immunophenotype of DC was consistent with mature DC (Lin-, CD80+, CD86+, CD83+, MHC class I+, MHC class II+); final maturation was achieved by the addition of TNF-α as previously described [5]. The higher percentage of T- and B-cell contamination in DC cultures from healthy donors compared with CML patients can be attributed to a lower relative frequency of lymphocytes in peripheral blood of CML patients. In healthy donors, cryopreservation did not change the percentage of cells with a surface marker profile characteristic of DC. In CML patients comparison of FACS profiles before and after cryopreservation gave similar results for all surface markers. With regard to CD83, a maturation marker of DC, it has to be taken into account that the fraction of marker-positive cells can easily be underestimated since histograms of negative and positive samples are overlapping. Nevertheless, FACS profiles before and after cryopreservation (not shown) did not reveal significant changes and the results for other markers such as CD80, CD86 and MHC molecules emphasized a mature phenotype of thawed DC.

MLR experiments confirmed the notion that DC maintain their mature phenotype after cryopreservation: cryopreserved DC still had a good T cell stimulatory capacity, showing that the freezing/thawing procedure does not significantly impair their APC function.

Additionally, results of our cinemicroscopy experiments demonstrate that DC also maintain their mobility, a characteristic functional feature, after cryopreservation.

Table 3 Ratio of stimulation indices before and after cryopreservation in healthy donors (*A*) and CML patients (*B*)

DC/well						
A. Healthy donors						
10,000	5,000	2,500	1,250	625	312	156
1.2 ± 0.54	1.22 ± 0.47	0.86 ± 0.27	0.87 ± 0.32	0.67 ± 0.25	1.0 ± 0.63	1.48 ± 0.45
B. CML patients						
10,000	5,000	2,500	1,250	625	312	156
1.32 ± 0.48	1.54 ± 0.59	1.49 ± 0.57	1.58 ± 0.47	1.53 ± 0.54	1.57 ± 0.68	0.66 ± 0.32

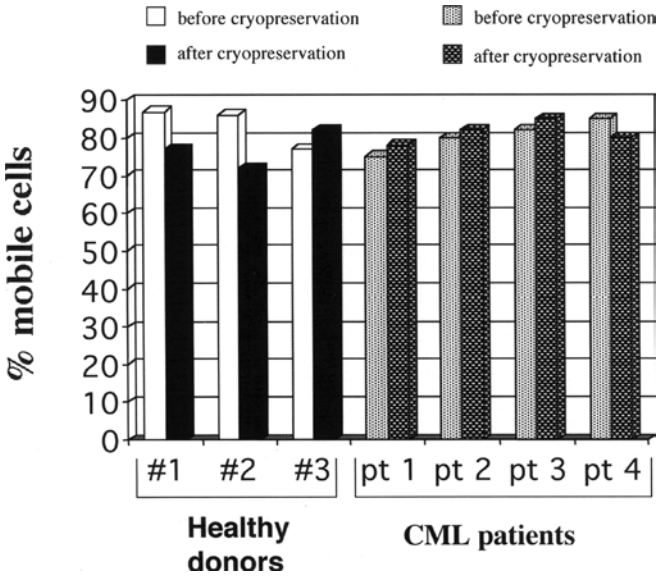

Fig. 3 Mobility of DC after cyropreservation. Mobility of DC before and after cyropreservation was analyzed by time-lapse cinemicroscopy. The proportion of highly mobile DC before and after cyropreservation is shown in percent. Preparations from three healthy patients and four CML patients were analyzed

After s.c. injection in mice it could be demonstrated that peptide-pulsed DC migrate into regional lymph nodes where they are able to interact with peptide-specific T cells. This interaction between DC and T-cell results in T-cell activation [7]. Since these studies suggest that DC mobility plays a role in vivo for migration into lymph nodes and subsequent efficient T-cell priming, our cinemicroscopy experiments further support cryopreservation as a suitable method for DC-based immunotherapy.

Since our results obtained from the different DC preparations show high similarity in all samples tested, we did not extend our studies on a higher number of DC preparations.

Our results are in line with a recent study by Feuerstein et al. [5], which shows that 85–100% of DC, frozen in autologous serum +10% DMSO can be recovered after thawing. Thawed DC maintained their morphology, phenotype and functional properties as measured by allogeneic MLR and induction of peptide- and protein-specific cytotoxic T-cell responses. Lewallel et al. [8] recently reported similar results with freezing of DC, generated from leukapheresis products. Both studies demonstrate that cryopreservation of DC is a feasible procedure for immunotherapy protocols and that cryopreserved DC maintain their ability to induce MHC class-I restricted peptide-specific T-cell responses.

Our study confirms these data. Particulary video cinemicroscopy contributes to the view that cryopreserved DC are still functional and clinically useful for vaccination and suggests that the migration properties of DC are unaffected. Furthermore, it is demonstrated here for the first time that cryopreservation of DC is also feasible in CML patients in whom DC are an attractive tool to induce immunity against leukemia-associated antigens such as bcr/abl [15].

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