Short Communication

Freezing and Thawing of Murine Bone Marrow-Derived Dendritic Cells Does Not Alter Their Immunophenotype and Antigen Presentation Characteristics

(dendritic cells / tumour lysate / DC priming)

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Abstract. The aim of this paper was to assess whether the BMDC after freezing and thawing are capable to retain the immunophenotype and antigen-presenting capacity. BMDC were generated from bone marrow precursor cells as described previously by culturing the cells in medium containing GM-CSF and IL-4. Afterwards, the cells were harvested, counted and used for phenotyping and priming of syngeneic spleen cells. For cryopreservation, the BMDC were frozen in the presence of 10% of dimethylsulphoxide (DMSO) and 90% foetal calf serum. Forty to fifty percent of both samples, frozen/thawed as well as fresh BMDC, exhibited characteristic DC morphology, and the DC obtained from the frozen/thawed samples expressed a similar level of MHC class I-, MHC class II-, CD80-, CD86-, CD11c-, CD11b-, CD54- and CD205-molecule as fresh DC. To examine the *in vitro* priming effect of cryopreserved BMDC on syngeneic non-adherent murine C57BL/6 (B6) spleen cells, the BMDC were thawed, pulsed with the lysate prepared from HPV 16-associated tumour MK16 and used for ^{3}H-thymidine assay. The findings of the experiments indicate that fresh as well as cryopreserved murine BMDC preparations pulsed with tumour lysate were efficient to prime the mitogenic activity of syngeneic non-adherent splenocytes. Taken together, the results suggest that frozen/thawed BMDC are morphologically, phenotypically and functionally comparable with fresh BMDC and can be used for construction of dendritic cell-based tumour vaccines.

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Abbreviations: BMDC – bone marrow-derived dendritic cells, B6 – C57BL/6, DMSO – dimethylsulphoxide, FACS – fluorescence-activated cell sorter, GM-CSF – granulocyte-macrophage colony-stimulating factor, HPV – human papilloma virus, IL – interleukin, mAb – monoclonal antibody, MFI – mean fluorescence intensity, MHC – major histocompatibility complex, MK16 cells – MK16/1/III ABC cells.

Construction of dendritic cell-based tumour vaccines using fresh bone marrow-derived dendritic cells (BMDC) is a laborious procedure, particularly when autologous BMDC-based vaccines have to be prepared and used repeatedly. These technical difficulties can be overcome by using cryopreserved BMDC. The frozen BMDC stored in liquid nitrogen containers represent a standard and reproducible source for vaccine construction. Therefore, we were interested to see whether in our experimental tumour system (Bubeník et al., 1999, 2000, 2002; Mendoza et al., 2000, 2002; Indrová et al., 2001; Šmahel et al., 2001) cryopreservation of BMDC does not change their phenotype and antigen-presenting capability.

Material and Methods

Mice

B6 mice, 2–4 months old, were obtained from Anlab Co., Prague, Czech Republic.

Cell line

The metastatic, major histocompatibility complex (MHC) class I-negative MK16/1/III ABC (MK16) carcinoma cell line of B6 origin was developed by *in vitro* co-transfection of murine kidney cells with a mixture of activated (G12V) *ras* (plasmid pEJ6.6), HPV 16 *E6/E7* (plasmid p16HHMo) and neomycin resistance gene

(plasmid pAG60) DNA (Šmahel et al., 2001). The tumour cell line was maintained at 37°C in a humidified atmosphere with 5% CO_2 and cultured in RPMI 1640 medium supplemented with 10% foetal calf medium (FCS, Bioclot (Pty) LTd., Aidembach, Germany), 30 μg/ml L-glutamine (Sevac, Prague, Czech Republic), penicillin, streptomycin, and 0.5 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO).

Generation of BMDC

The protocol used in this study for generation of BMDC has been described previously (Mendoza et al., 2000) and employed with some modifications. Briefly, bone marrow was flushed from femurs and tibias of B6 mice. The cells were seeded in a concentration of 1×10^6 cells per ml, grown in complete RPMI 1640 medium supplemented with 10 ng/ml GM-CSF (R&D systems, Biomedica, Czech Republic) plus 10 ng/ml interleukin-4 (IL-4), and incubated for 2 h. The IL-4 was derived from culture supernatants of murine myeloma cell line X63-m-IL-4 producing IL-4 and kindly provided by F. Melchers, Basel Institute for Immunology (Karasuyama and Melchers, 1988). The non-adherent cells were placed in a new culture flask, and twice refed with the same amount of fresh medium containing cytokines. After 6 to 7 days of culture, the non-adherent BMDC were harvested and used for the experiments.

Preparation of tumour lysate

The tumour lysate was prepared by repeated (3x) freezing and thawing of the MK16 cells. Prior to the freezing and thawing, the tumour cells were irradiated with a dose of 150 Gy. The BMDC were incubated with the MK16 lysate for 24 h at a ratio of three tumour cell equivalents to one dendritic cell.

Freezing/thawing of BMDC

For freezing, BMDC cultured for 6 to 7 days in complete RPMI 1640 medium containing GM-CSF and IL-4 were used. The samples were suspended in cold FCS with dimethylsulphoxide (9 : 1) at a concentration of 15–20 $\times$ 10^6 BMDC/ml and frozen in CryoTubes (Nunc, Roskilde, Denmark) using a Nalgene Cryo 1°C freezing container (Nunc) at controlled rate of cooling, i.e. –1°C per min until the temperature of –70°C was reached. Then, the frozen BMDC samples were stored at –180°C in liquid nitrogen and left at least for 2 weeks until use. Before use, the BMDC samples were thawed in a water bath at 37°C and washed 3 times in complete RPMI 1640 medium to remove dimethylsulphoxide.

Fluorescence-activated cell sorter (FACS) phenotyping

The cytofluorometric analysis was performed as described previously (Šímová et al., 2000). Briefly, the frozen/thawed and fresh BMDC were washed twice with PBS and incubated with non-antigen-specific Fcγ III/II receptor CD16/CD32 monoclonal antibody (mAb). Then, the samples were mixed with the respective PE- or FITC-conjugated mAb. Expression of MHC class I was detected with anti-mouse H-$2K^b$/H-$2D^b$ mAb (clone 28-8-6) incubated with FITC-conjugated goat anti-mouse Ig antibody. The expression of MHC class II molecules was detected with fluorochrome-labelled anti-mouse I-A^b mAb (clone AF6-120.1). Similarly, CD80 molecules were detected with fluorochrome-labelled anti-CD80 mAb (clone 16-10A1), CD86 with anti-CD86 mAb (clone GL1), CD11b with anti-CD11b mAb (clone M1/70), CD11c with anti-CD11c mAb (clone HL3), CD54 with anti-CD54 mAb (clone 3E2), and the expression of the CD205 molecule was detected with rat anti-mouse CD205 mAb (clone NLDC-145) incubated with FITC-conjugated $F(ab')_2$ goat anti-rat IgG. All the mAbs were purchased from BD PharMingen (Interact s.r.o., Prague, Czech Republic), except for the CD205 that was purchased from Serotec (Biomedica, Prague, Czech Republic).

Proliferative spleen cell responses induced by lysate-pulsed BMDC

For priming the proliferative spleen cell responses, preparations of frozen/thawed or fresh BMDC were used. Before pulsing, the 6-day fresh or frozen/thawed BMDC were washed 3 times with the RPMI 1640 medium, and suspended in complete RPMI 1640 medium containing GM-CSF and IL-4. Then, the MK16 lysate was added for the last 24 h of cultivation. Mitomycin C was added to BMDC preparations for 30 min, washed 3 times, and these BMDC were co-cultured with syngeneic spleen responder cells for 4 days in complete RPMI 1640 medium at 37°C in a responder : stimulator cell ratio of 5 : 1, 10 : 1, 50 : 1, and 100 : 1 using 96-well round-bottom microtiter plates (Nunc). Twenty hours before harvesting, the cells were labelled with 0.04 MBq ^{3}H-TdR/well and the uptake of ^{3}H-TdR was measured in the liquid scintillation system (Indrová et al., 2001).

Results and Discussion

We have examined the expression of MHC class I and II, CD80, CD86, CD11c, CD11b, CD54 and CD205 antigens on fresh and frozen/thawed BMDC cultured for 7 days in complete RPMI 1640 medium containing GM-CSF and IL-4. More than 40% of the cells from the fresh or frozen/thawed samples exhibited the characteristic dendritic cell (DC) morphology (Mendoza et al., 2002). The viability of BMDC after freezing and thawing was higher than 95%. Both types of BMDC preparations were positive for all the markers studied and the frozen/thawed BMDC expressed a similar profile of positive cells and similar levels of MFI as fresh BMDC (Fig. 1).

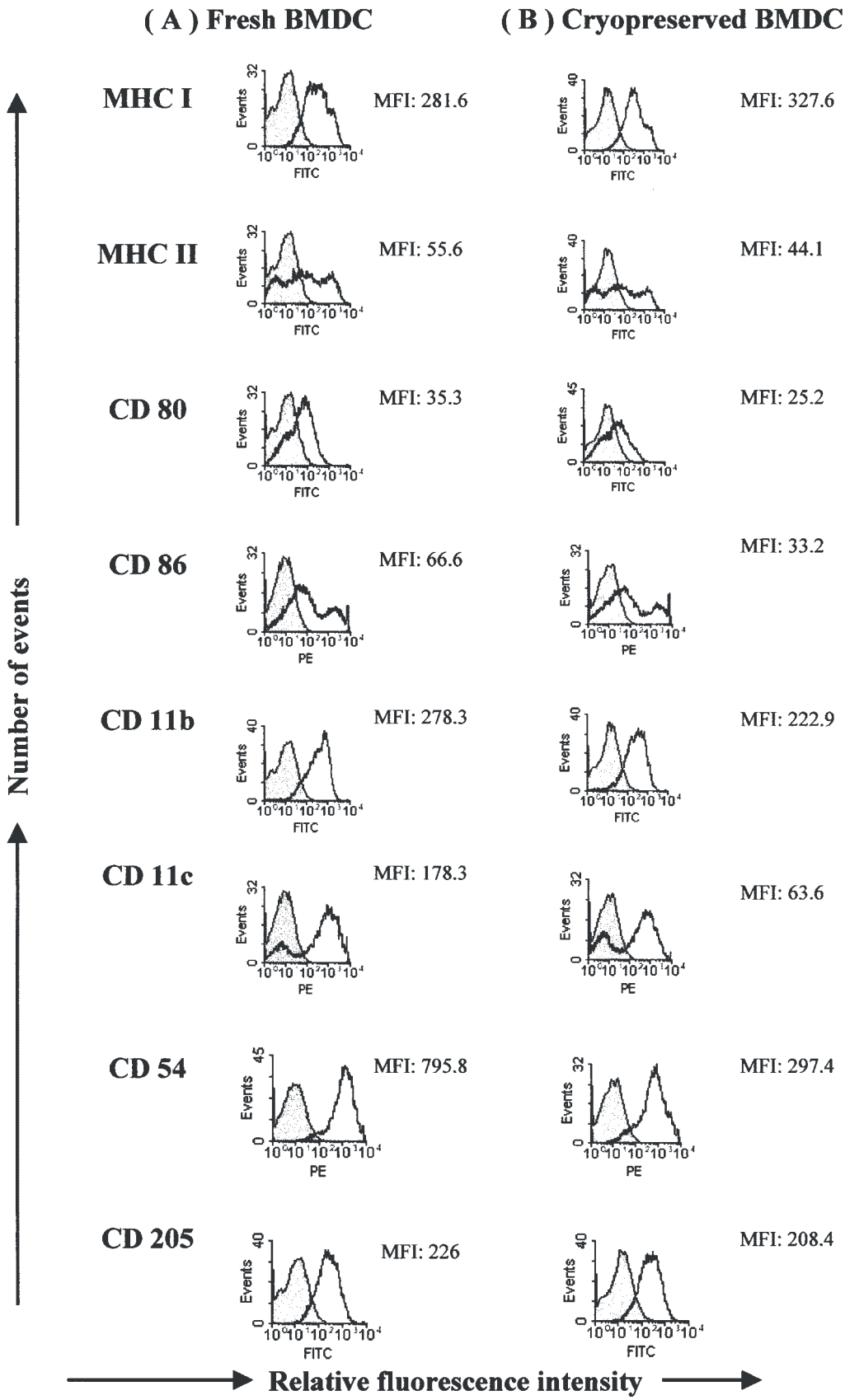

Fig. 1. Cytofluorometric characterization of fresh BMDC (A) and cryopreserved BMDC (B). Fluorochrome-labelled mAbs against MHC class I and II, CD80, CD86, CD11b, CD11c, CD54 and CD205 molecules were utilized for the FACS analysis. The FACS analysis of both samples was repeated several times and the representative results are shown; MFI = mean fluorescence intensity.

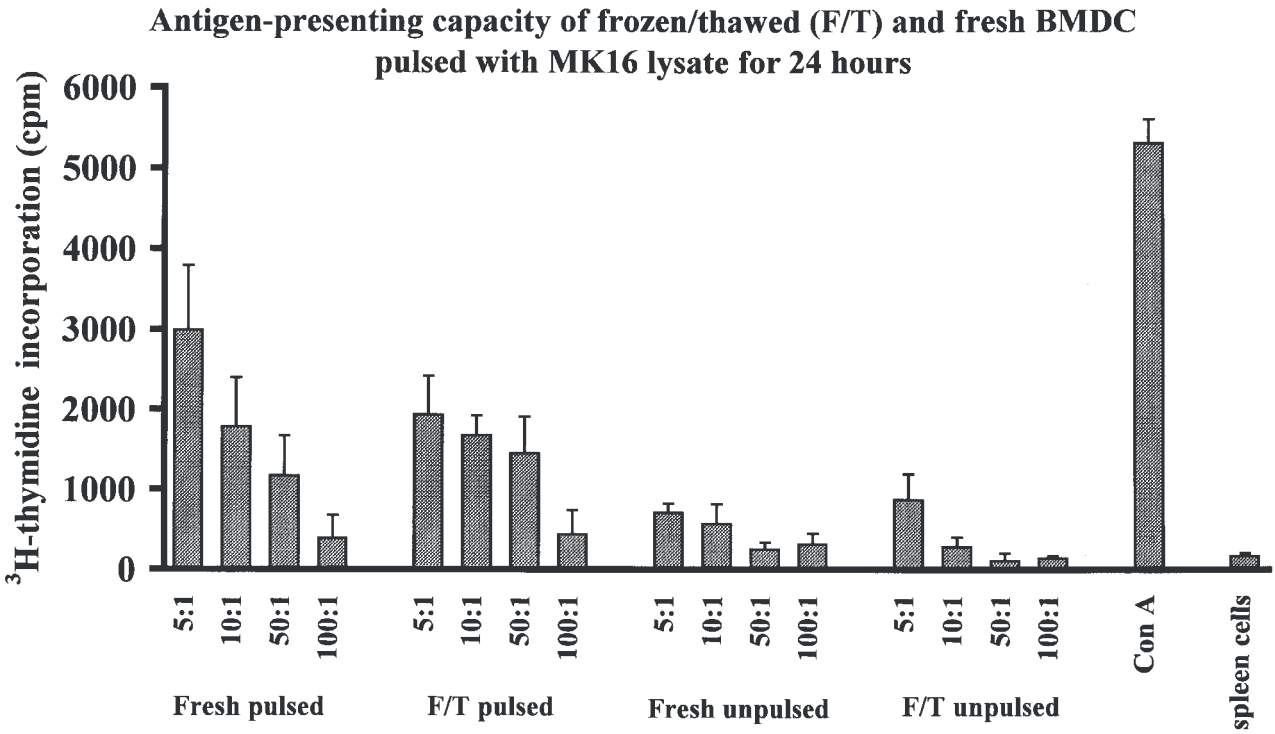

Fig. 2. Proliferative spleen cell responses induced by MK16 tumour lysate-pulsed fresh and frozen/thawed BMDC. The responder : stimulator cell ratio was 5 : 1, 10 : 1, 50 : 1, and 100 : 1. As controls, stimulation with concavalin A (Con A) (5.300 ± 300 cpm) and ^{3}H-thymidine uptake of spleen cells only (164 ± 40 cpm) were utilized. The experiment was repeated three times with similar results.

We have further compared the *in vitro* priming effect of fresh and frozen/thawed BMDC on syngeneic non-adherent murine B6 spleen cells. As can be seen in Fig. 2, fresh and frozen/thawed BMDC pulsed with MK16 lysate were efficient in priming the mitogenic activity of syngeneic lymphocytes. In repeated experiments we have not found any differences between fresh and frozen/thawed BMDC preparations.

These results suggest that the frozen/thawed BMDC are both phenotypically and functionally comparable with fresh BMDC and, therefore, can be used for construction of dendritic cell-based tumour vaccines. Utilization of the frozen/thawed BMDC substantially facilitates the construction and repeated use of the vaccines.

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