# Short Communication

# Tumour-Inhibitory Effects of Dendritic Cells Administered at the Site of HPV 16-Induced Neoplasms

( HPV 16 / dendritic cells / adjuvant therapy )

L. MENDOZA[1], J. BUBENÍK[1], J. ŠÍMOVÁ[1], J. KORB[1], J. BIEBLOVÁ[1], V. VONKA[2], M. INDROVÁ[1], R. MIKYŠKOVÁ[1], T. JANDLOVÁ[1]

[1]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague Czech Republic
[2]Institute of Haematology and Blood Transfusion, Prague, Czech Republic

**Abstract. Experiments were designed to examine whether administration of APC at the site of HPV 16-associated tumours can inhibit tumour growth and whether the efficacy of established dendritic cell lines is comparable to that of fresh BMDC populations. Mice were inoculated s.c. with APC, either bone marrow-derived dendritic cells differentiated in medium supplemented with GM-CSF and IL-4 (BMDC), or with established dendritic cell lines DC2.4 or JAWS II. The pretreated mice, together with untreated controls, were challenged with syngeneic HPV 16-transformed cells MK16 at the site of APC administration. It has been found that both BMDC and dendritic cell lines can inhibit tumour growth and that the efficacy of the established dendritic cell lines DC2.4 and JAWS II was comparable to that of fresh BMDC populations. In vitro induction of proliferative spleen cell responses by co-cultivation with MK16 antigen-pulsed BMDC or MK16 antigen-pulsed dendritic cell lines revealed that both types of APC populations can prime immune reactions directed against syngeneic HPV 16-associated neoplasms. Taken together, the results suggest that local increase in the number of dendritic cells at the site of HPV 16-associated tumours can inhibit progression of the tumours and that the dendritic cell lines which are efficient in this respect can be considered and should be tested in both, preclinical and human systems for delivery of therapeutic vaccines against HPV 16-associated neoplasms.**

Immortalized dendritic cell lines represent cell populations which can provide certain advantages for the development of therapeutic vaccines, as compared to fresh bone marrow-derived dendritic cells (BMDC) (Bubeník 1999a; 2001; 2002). The BMDC selected and differentiated by short-term cultivation of their precursors in media containing granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and other cytokines are heterogenous, dependent on the initial population of bone marrow cells, on the age and immunological anamnesis of the donor, as well as on the cultivation and selection conditions. In contrast, the immortalized permanent dendritic cell lines are usually cloned homogenous cell populations, well characterized with regard to their immunological potency and expression of major histocompatibility complex (MHC) specificities, co-stimulatory molecules, degree of differentiation and growth requirements. Dendritic cell lines can be expanded *in vitro* and their cells can be obtained in the required quantity and quality. The capacity to present the respective antigen of dendritic cell lines can be pretested, and taken together, these cell lines can provide a better defined and a more standard biological material. We were interested in comparing the antigen-presenting and tumour-inhibitory capacity of immortalized dendritic cell lines DC2.4 (Shen et al., 1997) and JAWS II (ATCC Collection) with freshly obtained BMDC populations of C57BL/6 (B6) origin in syngeneic mice carrying tumours formed by human papilloma virus (HPV) 16-transformed MK16 cells (Bubeník, 1999a; Bubeník et al., 1999; Šmahel et al., 2001). It has been shown previously in the MK16 experimental tumour system that peritumoral administration of antigen-stimulated fresh BMDC can inhibit tumour growth and reduce tumour recurrences (Rössner et al., 1999) after MK16 tumour surgery (Mendoza et al., 2000).

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Corresponding author: Luis Mendoza, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 37 Praha 6, Czech Republic. Tel.: +420 (2) 20183461; Fax: +420 (2) 24310955.

Abbreviations: APC – antigen-presenting cells, B6 – C57BL/6, BMDC – bone marrow-derived dendritic cells, GM-CSF – granulocyte-macrophage colony-stimulating factor, FACS – fluorescence-activated cell sorter, HPV – human papilloma virus, IL – interleukin, Mabs – monoclonal antibodies, MHC – major histocompatibility complex, MK16 – MK16/1/III ABC, TEM – transmission electron microscopy.

## Material and Methods

### *Mice*

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

### *Cell lines*

The metastatic, 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 *ras* (plasmid pEJ6.6), HPV 16 *E6/E7* (plasmid p16HHMo) and neomycin resistance gene (plasmid pAG60) DNA. In the MK 16 cells the expression of HPV 16 E7 and mutated (G12V) Ha-ras oncoproteins was demonstrated by immunoblotting with the respective monoclonal antibodies (Mab), and the presence of both unspliced and spliced forms of E6/E7 transcripts was revealed by RT-PCR (Šmahel et al., 2001; unpublished results). The DC2.4 cell line was established from bone marrow cells of B6 mice by infection with a retrovirus encoding v-*myc* and v-*raf* (Shen et al., 1997). The DC2.4 cells were generously provided by Dr. K. L. Rock (Department of Pathology, University of Massachusetts Medical School, MA). The JAWS II cells, a GM-CSF-dependent cell line established from bone marrow cells of B6 origin, were obtained from American Type Culture Collection (CRL-11904, ATCC, Rockville, MD). All cells were maintained in culture at 37°C in the RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS, Bioclot (PTy) Ltd., Aidenbach, Germany), 30 μg/ml L-glutamine (Sevac, Prague, Czech Republic), 0.5 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO) and 40 μg/ml gentamycin (Sigma). The media for cultivation of DC2.4 and JAWS II cells were, in addition, supplemented with 2 x $10^{-5}$ M mercaptoethanol (Calbiochem, La Jolla, CA), and the medium for JAWS II cells also with 3 ng/ml GM-CSF (R & D systems, Minneapolis, MN).

### *MK16 tumour lysate*

The MK16 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 and dendritic cell lines were incubated with the tumour lysate for 24 h at a ratio of three tumour cell equivalents to one dendritic cell.

### *Generation of BMDC*

The protocol used in this study for generation of BMDC has been described previously (Mendoza et al., 2000). Briefly, bone marrow was flushed from femurs and tibias of B6 mice. The cells were seeded in a concentration of 1 x $10^6$ cells per ml, grown in complete RPMI 1640 medium supplemented with 10 ng/ml GM-CSF plus 10 ng/ml IL-4, and incubated for 2 h. The IL-4 was derived from culture supernatants of murine myeloma cell line X63-m-IL-4 (transformed with murine IL-4 cDNA, producing IL-4, and kindly provided by F. Melchers, Basel Institute for Immunology) (Karasuyama and Melchers, 1988). The assessment of IL-4 concentration was performed by the ELISA assay (PharMingen, San Diego, CA). The non-adherent cells were placed in a new culture flask and on day 3 refed with the same amount of fresh medium containing cytokines. After 7 days the non-adherent BMDC were harvested and used for the experiments. In some cases, the BMDC were purified by sorting in fluorescence-activated cell sorter (FACS) with fluorochrome-labelled Mabs against MHC class II and CD86 specifities, and the double-positive cells were characterized by transmission electron microscopy (TEM). Ultrastrustural analysis was performed with ultrathin sections of cells fixed in 3% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.5, postfixed in 1% osmium tetroxide in veronal buffer and embedded in Epon 812 resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined with a JEM 1200EX electron microscope at a 60 kV accelerating voltage. As can be seen in Fig. 1A, the BMDC displayed an irregular villous surface containing projections. The nucleus was rather lobulated and chromatin was found in clusters in perinuclear localization. In the cytoplasm, relatively high amounts of vacuoles of different sizes were observed in addition to lysosomal structures. Besides the Golgi apparatus, well structuralized RER and mitochondria were clearly apparent in many dendritic cells. For comparison, Fig 1B and C shows the morphology of DC2.4 and JAWS II cells.

The cytofluorometric analysis was performed as described previously (Šímová et al. 2000). Briefly, the cells were washed twice with PBS, incubated with anti-mouse H-2K$^b$/H-2D$^b$ monoclonal antibody (clone 28-8-6, PharMingen, San Diego, CA) or with isotype control antibody (Sigma, St. Louis, MO) at 4°C for 30 min, washed, and incubated with FITC-conjugated goat anti-mouse Ig antibody (PharMingen) at 4°C for 30 min. Expression of MHC class II molecules was detected with anti-mouse I-A$^b$ Mab (clone AF6-120.1), that of CD11b with anti-CD11b Mab (clone M1/70), and that of CD80 with anti-CD80 Mab (clone 16-10A1), all labelled with FITC and produced by PharMingen (Beckton Dickinson PharMingen, San Diego, CA). The expression of CD11c was detected with anti-CD11c Mab (clone HL3), that of CD86 with anti-CD86 Mab (clone GL1), and that of CD54 with

anti-CD54 Mab (clone 3E2), all labelled with PE and produced by PharMingen. As can be seen in Fig. 2A and Table 1, the BMDC population was characterized by high expression of MHC class I and II, CD11b, CD11c, CD80, CD86 and CD54 molecules. The DC2.4 cells (Fig. 2B, Table 1) displayed high expression of MHC class I, CD86 and CD54 molecules, as well as low expression of CD80 molecules, negligible expression of CD11b and CD11c molecules and the absence of MHC class II molecules. The JAWS II cells (Fig. 2C, Table 1) were characterized by high expression of MHC class I, CD11b and CD54 molecules, as well as by low expression of CD11c, CD80 and CD86 molecules, and by the absence of MHC class II molecules.

### *Tumour-inhibitory effects of BMDC and bone marrow-derived dendritic cell lines*

Groups of B6 mice were pretreated on days 7 and 14 prior to challenge with 3 x $10^6$ fresh BMDC cultured for 7 days in RPMI 1640 medium containing GM-CSF and IL-4, or with 3 x $10^6$ irradiated (50 Gy) DC2.4 cells, or with 3 x $10^6$ irradiated (50 Gy) JAWS II cells. The pretreated mice, together with untreated controls, were challenged with 1 x $10^5$ MK16 tumour cells into the site of vaccination. During the experiments, the s.c. MK16 tumours were measured twice a week, growth curves were constructed and compared by two-way analysis of variance as described earlier (Hájková et al., 1999).

### *Proliferative spleen cell responses induced by MK16 tumour lysate-loaded dendritic cells*

For priming the proliferative spleen cell responses, mitomycin C-treated and MK16 lysate-loaded or unloaded stimulator dendritic cells were cultured for 4 days in complete RPMI 1640 medium at 37°C with syngeneic nylon-wool non-adherent spleen responder cells at a stimulator/splenocyte responder cell ratios 1 : 5, 1 : 10 and 1 : 50 using 96-well round-bottom microtiter plates (Nunc, A/S, Roskilde, Denmark). 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.

*Table 1. Summary of surface phenotypes of the fresh BMDC, DC2.4, and JAWS II populations*[a]

| Marker | Dendritic cell population | | |
|---|---|---|---|
| | BMDC | DC2.4 | JAWS II |
| MHC class I | ++ | ++ | ++ |
| MHC class II | ++ | - | - |
| CD11b | ++ | +/- | ++ |
| CD11c | ++ | +/- | + |
| CD80 | ++ | + | + |
| CD86 | ++ | ++ | + |
| CD54 | ++ | ++ | ++ |

[a]Relative fluorescence intensity is indicated with the plus and minus signs

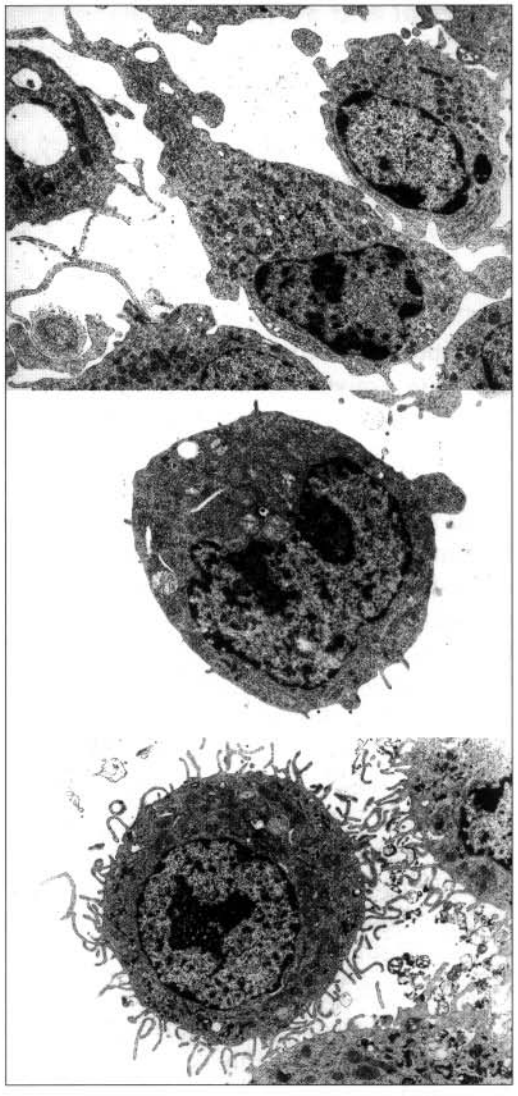


*Fig. 1.* TEM of murine dendritic cells generated from bone marrow precursors. (A) TEM of fresh BMDC population (magnification 5500x), (B) TEM of DC2.4 cells (magnification 8000x), (C) TEM of JAWS II cells. Magnification 6000x.

### *Statistical analyses*

For statistical analyses, analyses of variance and Student's t-test from the Number Cruncher Statistical System (NCSS, Kaysville, UT) statistical package were used.

## Results and Discussion

Groups of mice were pretreated on days 7 and 14 prior to challenge with BMDC, DC2.4, or JAWS

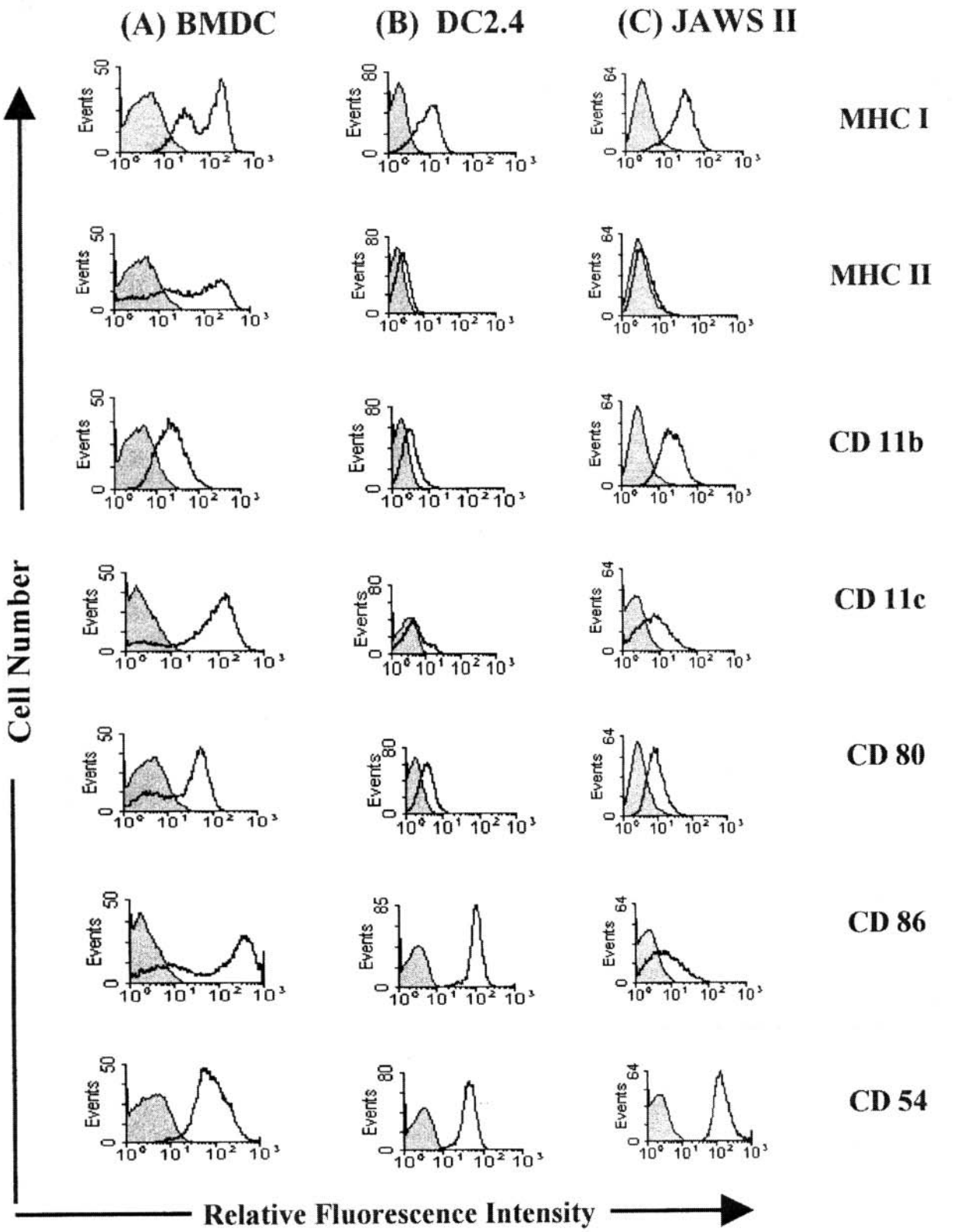


*Fig. 2.* Cytofluorometric characterization of BMDC (A), DC2.4 (B) and JAWS II (C) cells. Fluorochrome-labelled Mabs against MHC class I and II, CD11b, CD11c, CD80, CD86, and CD54 were utilized for the FACS analysis. The analysis was repeated 3–10 times and the representative results are shown in this figure.

II cells (3 x $10^6$ cells/mouse, s.c.) and then challenged at the site of the pretreatment with 1 x $10^5$ MK16 carcinoma cells. As can be seen in Fig. 3, administration of APC populations, leading to their higher concentration at the site of the subsequent challenge, substantially ($P < 0.05$) inhibited MK16 tumour growth. The

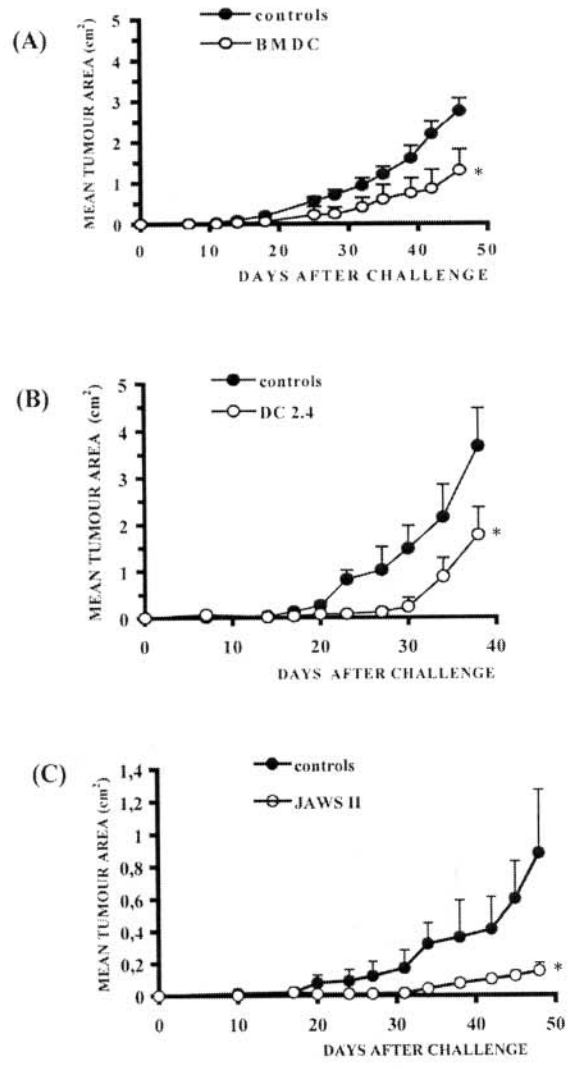


*Fig. 3.* Tumour-inhibitory effects of BMDC (A), DC2.4 (B) and JAWS II (C) cells administered s.c. at the site of the subsequent MK16 tumour inoculation. *P < 0.05. The experiment was twice repeated with similar results.

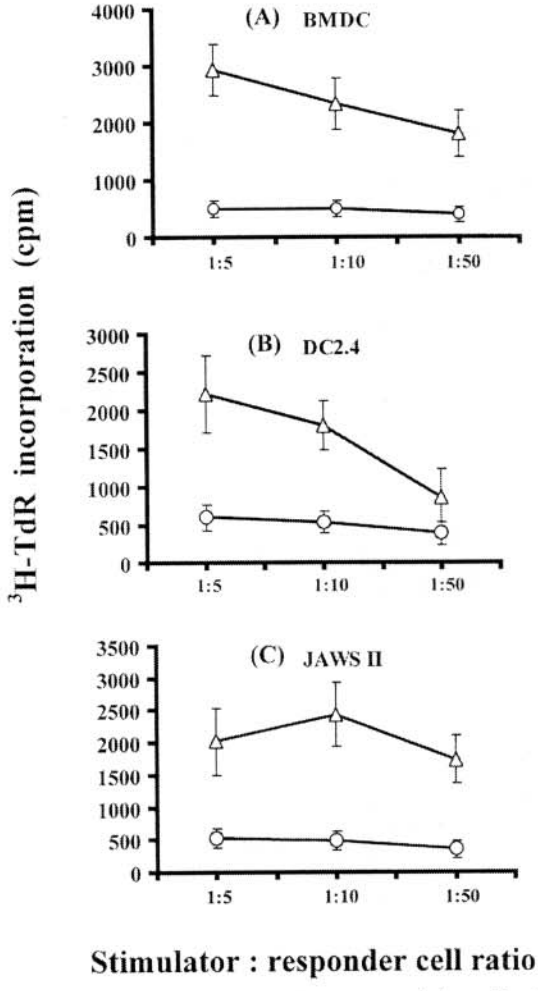


*Fig. 4.* Proliferative spleen cell responses induced by MK16 tumour lysate-loaded BMDC (A), MK16 tumour lysate-loaded DC2.4 cells (B), or MK16 tumour lysate-loaded JAWS II cells (C). The stimulator/responder cell ratio was 1 : 5, 1 : 10, 1 : 50, respectively. As controls, stimulation with Con A (4.500 ± 500 cpm) and $^3$H-thymidine uptake of spleen cells only (108 ± 100 cpm) were utilized.

tumour-inhibitory efficacy of BMDC (Fig. 3A) was similar to that of the established cell lines DC2.4 (Fig. 3B) and JAWS II (Fig. 3C) derived from dendritic cell precursors. It can be hypothesized that these APC have captured the MK16 tumour cells, processed their antigens, and expressed the processed MK16 oncoproteins in the context of MHC class I and II molecules on their cell surface to stimulate T-cell responses. The first step in testing this hypothesis was to demonstrate the ability of the three APC populations to prime *in vitro* immune reactions directed against the MK16 tumour cells. For priming the proliferative spleen cell responses, mitomycin C-treated and MK16 lysate-pulsed BMDC, DC 2.4 and JAWS II cells were co-cultured *in vitro* with syngeneic, non-adherent spleen cells for 4 days. Twenty-four hours before harvesting, the cells were labelled with $^3$H-TdR and the uptake of the $^3$H-TdR was recorded. It has been found that the MK16 oncoprotein-pulsed BMDC (Fig. 4A) as well as MK16 oncoprotein-pulsed dendritic cell lines DC 2.4 and JAWS II (Fig. 4B, C) were capable of priming the proliferative spleen cell responses, whereas the activity of the MK16 oncoprotein-unloaded APC was negligible.

Taken collectively, these results indicate that the established dendritic cell lines are capable of priming spleen cell responses against HPV 16-associated neoplasms in a similar way as the fresh BMDC populations, and can exert a similar tumour-inhibitory effect. Therefore, the dendritic cell lines, which represent a more standard and technically more easily utilizable populations as compared to BMDC, can be considered as an attractive vehicle for the delivery of therapeutic vaccines against HPV 16-associated tumours and should be tested for these purposes in preclinical model systems, as well as in clinical trials.

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