Table 1. Comparison of two strategies for in vitro differentiation of DC from their BM precursors

	Modified method #1 (Fields et al., 1998)	Modified method #2 (Lutz et al., 1999)
BM cell concentration (cells/ml)	1.0×10^6	2.0×10^5
GM-CSF (ng/ml)	10	20
IL-4 (ng/ml)	10	0
Culture (days)	10	10
Total cell yield (per mouse)	$2.5 \pm 5.6 \times 10^8$	$8.0 \pm 9.3 \times 10^8$
DC yield[a] (per mouse)	5.0×10^6	5.35×10^7

[a]DC were identified according to the morphological criteria.

Table 2. Comparison of the adherent and non-adherent BM cell yield after 8 and 10 days of culture[a]

	Modified method #1 (Fields et al., 1998)		Modified method #2 (Lutz et al., 1999)	
	Day 8	Day 10	Day 8	Day 10
Adherent cells ($\times 10^7$)	2.90 ± 0.16	0.78 ± 0.07	14.90 ± 0.59	2.38 ± 0.43
Non-adherent cells ($\times 10^7$)	1.65 ± 0.20	0.93 ± 0.16	4.58 ± 0.51	5.56 ± 1.20

[a]Results of independent experiments

laboratory from supernatants of murine myeloma cell line X63-Ag8.653 of BALB/c origin transformed by murine IL-4 cDNA; this X63-m-IL-4 cell line was kindly provided by F. Melchers, Basel Institute for Immunology (Karasuyama and Melchers, 1988). The concentration of IL-4 in the supernatants was determined by ELISA.

In the method 2 (Lutz et al., 1999), the BM-derived cells were seeded in a concentration of 2.0×10^5 per ml and grown in the complete RPMI 1640 medium supplemented with 20 ng/ml GM-CSF.

On day 3, in both protocols the cultures were refed with the same amount of fresh medium containing cytokines. On days 6 and 8, 50% of the culture supernatant was collected, centrifuged and resuspended in complete RPMI 1640 medium containing the respective concentrations of cytokines and given back into the original culture flask. On day 10, the supernatants containing cells were admixed to the adherent cells that were harvested by gentle scraping in Versene and counted. At the end of cultivation, the total yield of DC was higher in BM cell cultures prepared according to method 2 (Table 1). The morphology of BM-derived DC was similar in both methods. The cultures displayed formation of adherent and non-adherent clusters with differing number of cells (from 5 to 50 cells) during the first 6 days. Round cells with increasing dendritic morphology were released from the clusters of non-adherent cells. After this period, many mature DC were detectable in the suspension (Fig. 1C). On day 8, both methods of cultivation showed the maximum number of cells with a decrease on day 10 (Table 2). Phenotypic analysis of the resulting cultures obtained by both methods revealed upregulation of the expression of MHC class II, CD80, CD86 and CD11c molecules (Fig. 2). Cells from 10-day BM culture obtained by both methods were compared in *in vitro* antigen-presentation experiments. In pilot experiments, higher syngeneic spleen cell (SC) proliferative responses were obtained after priming with DC prepared according to the method 1. Throughout this paper, the *in vivo* experiments were performed with the whole population of BM-derived DC (BMDC) cultures prepared using the method 1, and such cells obtained after 10-day cultivation in RPMI 1640 medium supplemented with GM-CSF and IL-4 were designated as BMDC.

Cell line

MKl6/1/III ABC (MKl6) 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 pl6HHMo) and neomycin resistance gene (plasmid pAG60) DNA (Šmahel et al., unpublished). The culture medium used for *in vitro* culture of MKl6 cells was RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, antibiotics, and 250 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO). It has previously been found that the MKl6 carcinoma is sensitive to immunotherapy with MKl6 lysate-pulsed BMDC vaccine (Bubeník, 1999).

Pulsing of DC with MK 16 tumour lysate

The tumour lysate was prepared by repeated (3-fold) freezing and thawing of MKl6 cells. Prior to the freezing and thawing, the tumour cells were irradiated with a dose of 150 Gy. BMDC were incubated with the tumour lysate at a ratio of three tumour cell equivalents to one BMDC for 24 h.

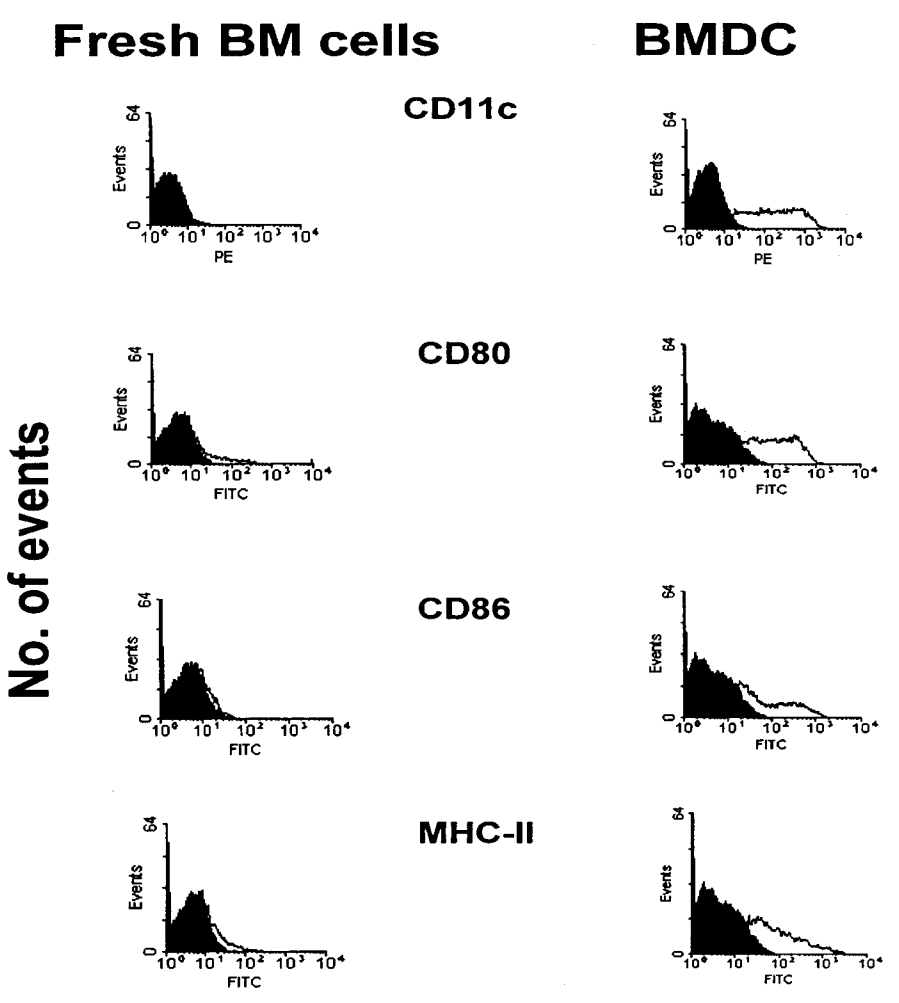

Fig. 2. Differentiation of BMDC from their precursors by 10 days of culture in the medium supplemented with GM-CSF and IL-4. Phenotyping of the differentiated cells using cytofluorometric analysis and FITC- or PE-labelled monoclonal antibodies.

Flow cytometry

Surface expression of CD11c, CD80, CD86 and MHC class II on BMDC was determined by staining with specific fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAbs. The cells were washed, resuspended in PBS containing 1% bovine serum albumin and 0.02% NaN_3 and directly labelled with the following monoclonal antibodies: PE-anti-CD11c monoclonal antibody (mAb) clone HL3, FITC-anti-CD80 mAb clone 16-10A1, FITC-anti-CD86 mAb clone GL1 and FITC-anti-MHC II Ia mAb clone AF6-120.1. All antibodies (Pharmingen, SanDiego, CA) were used in a concentration 1 μg per 500,000 cells (45 min incubation at 4°C). Unstained cells served as a negative control. FACS analysis was performed using an ELITE flow cytometer (Coulter, Miami, FL); 10,000 cells were employed for each measurement.

Mixed leukocyte reaction

Mitomycin C-treated, MKl6 lysate-pulsed and unpulsed BMDC were co-cultured for 4 days with syngeneic nylon-wool non-adherent SC responder cells (Julius et al., 1973) in various target-to-effector cell ratios 1 : 5, 1 : 10 and 1 : 50 in 96-well round-bottom microtiter plates (Nunc, A/S, Roskilde, Denmark). The cells were cultured in complete RPMI 1640 medium at 37°C in a humidified atmosphere with 5% $C0_2$. 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.

In vivo experiments

In the DC pretreatment experiments, the B6 mice were injected s.c. twice, on days 0 and 14, with 3.0×10^6 BMDC. On day 21, the pretreated mice, together with untreated controls, were challenged with 5.0×10^5 MKl6 tumour cells into the site of vaccination.

The therapeutic experiments were performed with mice carrying small MKl6 tumours, approximately 2 mm in diameter. The tumour-bearing mice were randomized into two groups: the experimental mice were injected with 3.0×10^6 BMDC peritumorally and the control mice were untreated.

Treatment of surgically induced minimal residual MKl6 disease was performed as described previously (Vlk et al., 1998; Bubeník et al., 1999). Briefly, groups of mice were inoculated s.c. with 1.0×10^6 MKl6 cells. Mice with tumours of approximately 8 to 12 mm were used for surgery. Tumours were excised under i.p. anaesthesia, leaving no visible tumour residue. Operated mice were injected with 3.0×10^6 BMDC at the site of the excised tumour on day 3 after surgery.

During the experiments, mice were inspected twice a week. The number of tumour-bearing mice, size of the tumours, and survival of tumour-bearing mice were