Immunogenicity of Dendritic Cell-Based HPV16 E6/E7 Peptide Vaccines: CTL Activation and Protective Effects

(HPV16 E6/E7 peptides / dendritic cells / cytotoxic lymphocytes)

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Abstract. We have investigated the capacity of cellular vaccines based on dendritic cells loaded with human HPV16 E6/E7 oncoprotein-derived peptides to induce immune responses *in vitro* and to elicit protective immunity in a murine experimental model mimicking human HPV16-associated carcinomas. Dendritic cells loaded with the HPV16 E6/E7 peptides exhibiting CTL or Th epitopes ($E6_{41-50}$, $E6_{81-90}$, $E6_{98-107}$, $E6_{130-137}$, $E7_{49-57}$, and $E7_{44-62}$) were able to stimulate *in vitro* DNA synthesis in syngeneic H-2^b spleen cells. The priming capacity of peptide-loaded BMDC and peptide-loaded dendritic cell lines DC2.4 and JAWS II was compared. It has been found that both peptide-loaded BMDC and established dendritic cell lines can activate the syngeneic responder cells, but the priming capacity of BMDC was substantially higher. In the second set of experiments, we have examined the cytolytic activity of syngeneic spleen cells after repeated activation *in vitro* with BMDC loaded with HPV16 synthetic peptides containing CTL epitopes. Significant cytotoxic responses against HPV16 E6/E7 antigen-expressing TC-1 targets have been found after repeated *in vitro* activation with all peptides containing the CTL epitopes. In contrast, peptide $E7_{44–62}$ harbouring both Th and CTL epitopes induced significant cytotoxic responses already after single *in vitro* activation. This cytotoxic effect could be enhanced with admixture of the $E7_{49-57}$ peptide. Experiments with MHC class I proficient (TC-1, MK16-IFNγ) and deficient (MK16) target cells revealed that the dendritic cells loaded with the E6/E7 HPV16 peptides activated CTLs *in vitro*, but not the other cytolytic effector mechanisms. The effectiveness of the peptide-loaded BMDC-based cellular vaccines was also investigated *in vivo*. C57BL/6 (H-2^b) mice were immunized with various peptide-loaded BMDC and subsequently challenged with TC-1 cells. The strongest protective effect was achieved with the BMDC loaded with the peptide $E7_{44–62}$ harbouring both CTL and Th epitopes. Mice immunized with the $E7_{44–62}$ peptide remained tumour-free after s.c. transplantation of the TC-1 cells and exhibited long-lasting protective immunity, whereas the mice immunized with $E6_{81-90}$ and $E7_{49-57}$ peptides did not remain tumour-free and exhibited only partial inhibition of tumour growth detectable as depression of the tumour growth curves.

Cervical neoplasms are associated with the human papilloma virus (HPV) infection, mostly with the "high-risk" types HPV16 and HPV18. The high-risk HPV types encode two oncoproteins, E6 and E7, which are necessary for the maintenance of the malignant phenotype. They are expressed in all tumour cells and therefore have been used as targets for specific immunotherapy promoting the immune response against HPV-associated tumours. Immunization with peptides presenting a cytotoxic T lymphocyte (CTL) E6/E7 protein epitope was shown to be an effective strategy inducing a specific CTL response that prevents the outgrowth of HPV16-associated tumours (Feltkamp et al., 1993; Okada et al., 1998).

Dendritic cells (DC), the professional antigen-presenting cells, are good candidates for presentation of peptide antigens. Indeed, the peptide-pulsed DC are significantly more efficient in the development of anti-tumour protection than immunization with the peptide alone (Paglia et al., 1996) or with the peptide emulsified in incomplete

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Abbreviations: B6 – C57BL/6, BMDC – bone marrow dendritic cells, CTL – cytotoxic T lymphocyte(s), DC – dendritic cells, HPV – human papilloma virus, MK16 – MK16/1/IIIABC, rGM-CSF – recombinant granulocyte-macrophage colony-stimulating factor, SC – spleen cells, Th – T helper.

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Freund's adjuvant (Mayordomo et al., 1995). Cellular vaccines based on the DC pulsed with synthetic peptides have previously been studied for HPV-associated tumour growth protection or immunotherapy (Ossevoort et al., 1995). Interestingly, HPV16 E6/E7 peptide presentation by dendritic cells has been shown to eliminate tolerization that can occasionally be seen after peptide immunization (Toes et al., 1998).

Several peptides have been used in a number of immunization protocols in preclinical models, although the number of functional CTL and T helper (Th) epitopes on the E6/E7 proteins seems to be limited (Khammanivong et al., 2003). Vaccination with peptides exhibiting multiple CTL and Th epitopes might be beneficial as compared to the use of 9–11 amino acid long peptides exhibiting only one CTL epitope and optimally binding the MHC class I molecule (Zwaveling et al., 2002). It is plausible that the longer peptides can also be effectively endocytosed by antigen-presenting cells, intracellularly processed and correctly expressed on the cell surface in the context of the MHC class I molecule. Another important question is whether immunization with peptides exhibiting both CTL and Th epitopes, either physically linked on the same peptide (Shirai et al., 1994), or delivered separately (Fernando et al., 2002), is of particular importance for the development of long-lasting protective immunity.

In the current study, we addressed the problem of using bone marrow dendritic cells (BMDC) pulsed with HPV16 E6/E7-derived peptides containing CTL or Th epitopes as cellular vaccines. The capacity of BMDC pulsed with a single peptide as well as with mixtures of two peptides to induce the immune response was compared both *in vitro* and *in vivo* in the C57BL/6 (H-2^b) murine model. The second goal was to evaluate the importance of the length of the peptides and the presence of the Th epitopes in BMDC-based vaccines.

Material and Methods

Mice

C57BL/6 (B6) mice, 2–4 months old, were obtained from Anlab, Prague, Czech Republic. All animals were housed in the animal facility of the Institute of Molecular Genetics. Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague, and by the Institutional Animal Care and Use Committees at SUNY Health Science Center at Brooklyn, NY, and Wadsworth Center, Albany, NY.

Tumour cell lines

The murine MHC I^+ cell line TC-1, immunogenic in the syngeneic B6 mice, and murine MHC class I^- cell line MK16/1/IIIABC (MK16), moderately immunogenic in syngeneic B6 mice were previously described in detail elsewhere (Lin et al., 1996, Šmahel et al., 2001). Both cell lines were obtained after *in vitro* co-transfection of murine B6 cells with HPV16 *E6/E7* and activated human *Ha-ras* (G12V) oncogene DNA. The cell lines were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum, L-glutamin and antibiotics (complete medium). For induction of MHC class I molecules on the MHC class I^- MK16 cells, the cells were cultivated for 48 h in the RPMI 1640 medium in the presence of 1 μg/ml IFNγ (R&D Systems, Minneapolis, MN) and designated as MK16-IFNγ (Indrová et al., 2003; Mikyšková et al., 2003).

Dendritic cells

Dendritic cells of bone marrow origin and two types of dendritic cell lines were used. The DC2.4 cell line was established from bone marrow cells of B6 mice after infection with retrovirus encoding v-*myc* and v-*raf* (Shen et al., 1997). These cells were generously provided by Dr. K.L. Rock, Department of Pathology, University of Massachusetts Medical School, MA, USA. The JAWS II cells, a granulocyte-macrophage colony stimulating factor (GM-CSF)-dependent cell line established from bone marrow cells of B6 origin, were obtained from the American Type Culture Collection (CRL-11904, ATCC, Rockville, MD). These cell lines were cultivated in complete RPMI medium supplemented with mercaptoethanol (2×10^{-5} M; Calbiochem, La Jolla, CA); for cultivation of JAWS II cells, 5 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) were added. BMDC-derived dendritic cells were obtained by cultivation of non-adherent B6 bone marrow-derived DC precursors for 7 days in the complete RPMI 1640 medium supplemented with mercaptoethanol (2×10^{-5} M), 10 ng/ml GM-CSF plus 10 ng/ml IL-4 derived from supernatants of murine genetically engineered X63-m-IL-4 cells (Karasuyama and Melchers, 1988). On day 6, the DC were pulsed by 18 h incubation with MK16 or TC-1 tumour lysate at a ratio of one tumour cell equivalent to one DC. Alternatively, the DC was pulsed by 18 h incubation in the appropriate medium containing 100 μg/ml of the peptide (Indrová et al., 2001). For *in vivo* immunization experiments, BMDC pulsed with peptides were matured for 24 h prior to injection into the animals with unmethylated CpG containing phosphorothioate-modified oligodeoxynucleotide CpG 1826 (5'-TCCATGACGTTCCTGACGTT-3', Chu et al., 1997), at a final concentration of 2 μg/ml.

Peptides

The HPV 16 E6/E7 peptides $E6_{41-50}$ (EVYDFAFRDL), $E6_{81-90}$ (YSSVYGTRLPG) (Staus et al., 1992), $E6_{98-107}$ (GYNKPLCDLL) (Azzoury-Ziadeh et al., 1999), $E6_{130-137}$ (GRWTGRCM) (Gao et al., 1995), $E7_{21-30}$ (DLYCYEQLND) (Staus et al., 1992), $E7_{44-62}$ (QAEPDRAHYNIVTFCCKCD = 8Q) (Tindle et al., 1991; Feltkamp et al., 1993), $E7_{48-54}$ (DRAHYNI) (Tindle et al., 1991; Azzoury-Ziadeh et al., 1999), $E7_{48-57}$

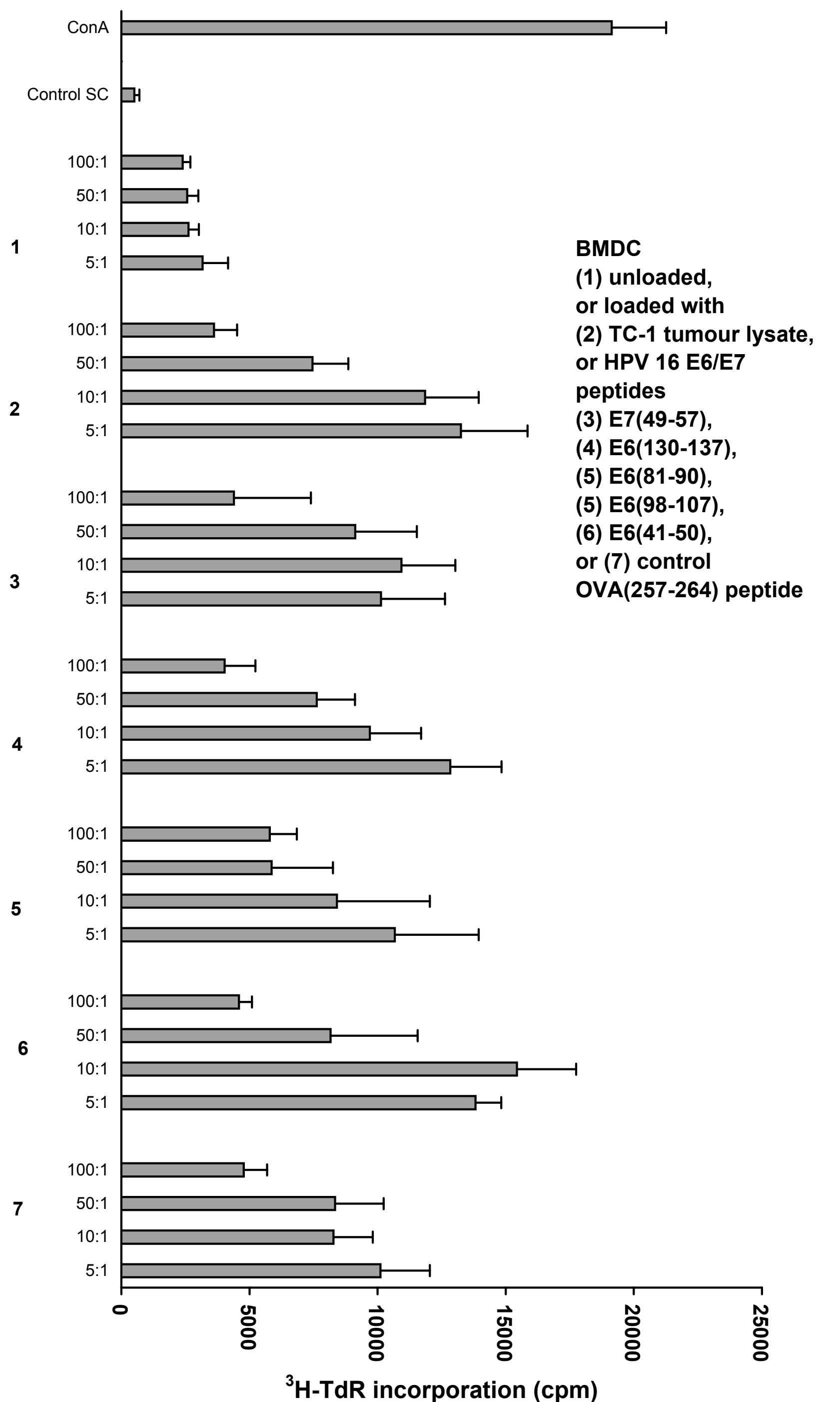

Fig. 1. Comparable level of stimulation of DNA synthesis by BMDC loaded with E6/E7 HPV16 peptides with cytotoxic or helper motifs and with tumour cell lysate

(RAHYNIVTF) (Feltkampf et al., 1993) and control, ovalbumin-derived $OVA_{257-264}$ (SIINFEKL) peptide, were synthesized in the Institute of Organic Chemistry and Biochemistry AS CR, Prague, or custom-synthesized by Sigma Genosys, Cambridge, UK.

Tumour lysates

The tumour cells were irradiated with a dose of 150 Gy and cultivated for three days to obtain apoptotic cells. The collected cells (15–20 × 10^6 cells/ml in serum-free RPMI 1640 medium) were disintegrated by an ultrasonic disintegrator (Sonoprep 150), ten cycles of sonication, with amplitude of 22 microns, in 4°C. After centrifugation (10 min, 250 *g*), the supernatant was collected, checked for disintegration of the cells, frozen (-70°C) and used for loading of dendritic cells.

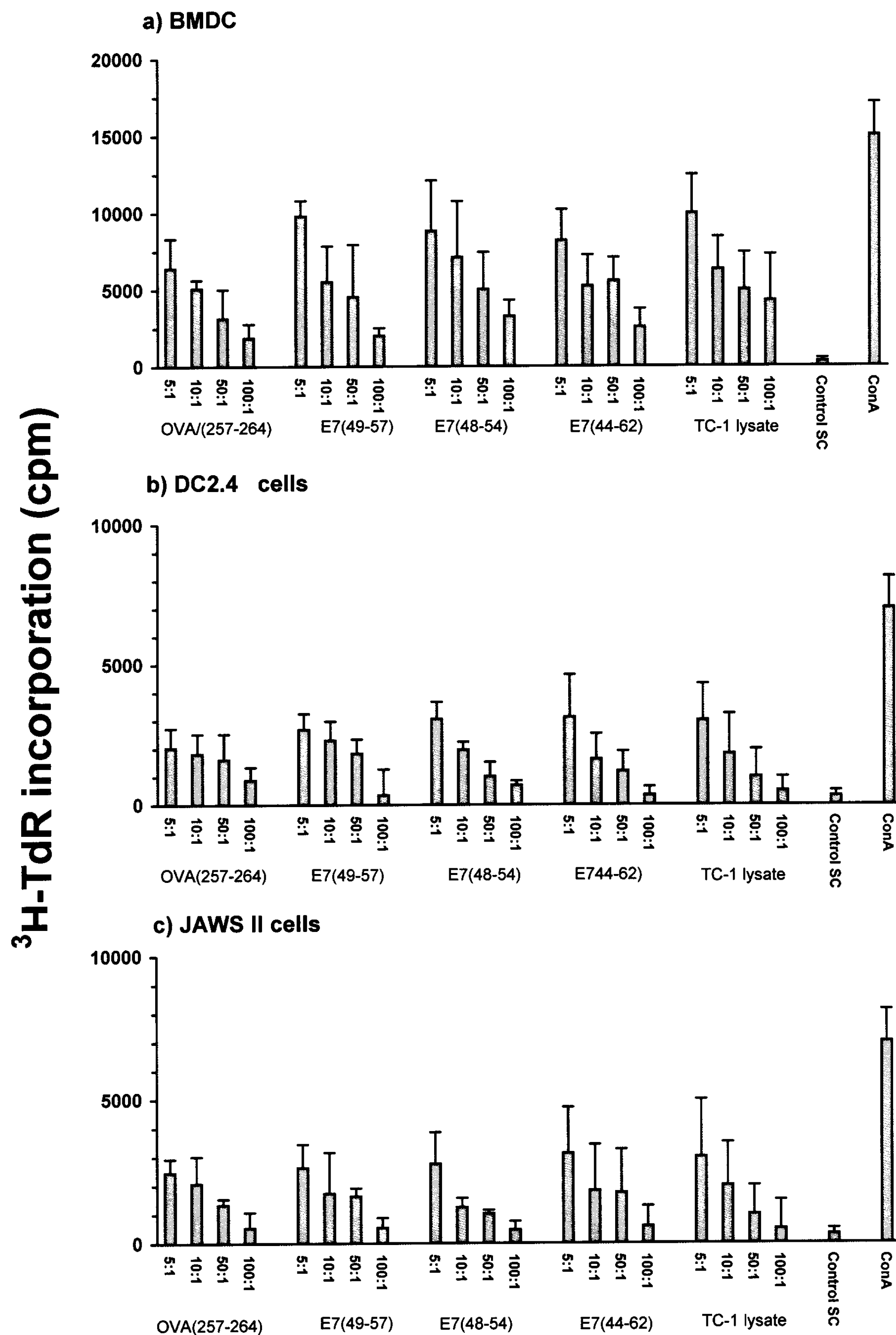

Fig. 2. In vitro priming of spleen cell responses was more efficient with fresh BMDC vaccines (a) than with BMDC-derived cell line vaccines DC2.2.4 (b) or JAWS II (c).

Immunization-challenge experiments

For immunization-challenge experiments, mice were injected s.c., twice in a two-week interval, with 2×10^6 peptide-pulsed BMDC. Seven days after the second immunization mice were challenged s.c. with 1×10^4 TC-1 cells. The tumour incidence and size were recorded twice a week. The tumour-free animals were re-challenged a month after the first challenge with the same dose of the TC-1 tumour cells.

Cultivation and stimulation of spleen lymphocytes

For the *in vitro* tests, mice were sacrificed and the suspension of spleen cells was prepared. After lysis of the erythrocytes with Tris-NH_4Cl buffer, spleen cells were passed through a nylon wool column. The effluent fraction was designated as non-adherent cells. For *in vitro* activation, non-adherent cells were cultivated for three days in complete RPMI 1640 medium supplemented with recombinant IL-2 (20 i.u./ml, Proleukin,

Cetus, Emeryville, CA) and recombinant IL-7 (5 ng/ml, R&D Systems, Minneapolis, MN) together with DC alone or loaded with peptides or lysates (ratio 5 : 1). After one or two cycles of co-cultivation, the cells were collected and used for the ^{51}Cr microcytotoxicity assay.

^{3}H-TdR incorporation assay

For the priming of proliferative spleen cell responses, Mitomycin C (Kyowa, Tokyo, Japan)-treated DC (unloaded, peptides- or lysate-loaded BMDC, JAWS II or DC2.4 cells) were co-cultivated in complete RPMI 1640 medium with syngeneic, nylon wool column non-adherent spleen cells for 4 days in ratios of 1 : 5, 1 : 10, 1 : 50 and 1 : 100 using 96-well round bottom microtitre plates (Nunc, Roskilde, Denmark). Twenty hours before harvesting, the cells were labelled with 0.04 MBq ^{3}H-TdR per well and the uptake of ^{3}H-TdR was measured in the liquid scintillation system (Bubeník et al., 1994; Mendoza et al., 2002).

^{51}Cr microcytotoxicity assay

The mixtures of effector cells with the ^{51}Cr-labelled tumour targets were incubated in three different target-to-effector cell ratios (1 : 25, 1 : 50, 1 : 100) for 18 h in triplicate in 96-well round bottom microtitre plates. Percent specific ^{51}Cr release was expressed according to the formula: [cpm experimental release – cpm control release / cpm maximum release – cpm control release] × 100 (Bubeník et al., 1994; Indrová et al., 2003).

Evaluation of results

For analysis of variance, Newman-Keuls and Tukey-Kramer tests from the NCSS statistical package (Number Cruncher Statistical System, Kaysville, UT) were used. For comparison of tumour takes in experimental and control groups, the χ^2 test was utilized. For statistical analyses of *in vitro* experiments, the Student's t- test was used.

Results

To determine the priming capacity of BMDC loaded with various HPV 16 E6/E7 peptides, five BMDC vaccines loaded with synthetic peptides were compared in their ability to prime the proliferative responses of syngeneic non-adherent murine B6 spleen cells (SC). As positive and negative controls, BMDC loaded with tumour lysate prepared from the TC-1 cells and unrelated synthetic $OVA_{257\text{-}264}$ (SIINFEKL) peptide was used. As shown in Fig. 1, all the tested synthetic HPV16 E6/E7 peptides with cytotoxic $E7_{48\text{-}57}$ (RAHYNIVTF), $E6_{130\text{-}137}$ (GRWTGRCM), $E6_{81\text{-}90}$ (YSVYGTTLEQ), $E6_{41\text{-}50}$ (EVYDFAFRDL) or helper $E6_{98\text{-}107}$ (GYNKPLCDLL) motifs were able to stimulate DNA synthesis at a level comparable with the priming capacity of dendritic cells loaded with TC-1 tumour lysate.

The *in vitro* priming capacity of dendritic cells of BMDC origin was compared to that of two established syngeneic DC lines, JAWS II and DC2.4. These stimulator cells were loaded with synthetic HPV 16 peptides with cytotoxic epitope $E7_{48\text{-}57}$ (RAHYNIVTF), with helper epitope $E7_{48\text{-}54}$ (DRAHYNI) and with a peptide exhibiting both motifs $E7_{44\text{-}62}$ (QAEPDRAHYNIVTFCCKCD). It has been found that both of these established DC lines were able to prime the proliferation of syngeneic non-adherent SC, but the priming activity of the pulsed BMDC was substantially stronger than that of the cell lines JAWS II and DC2.4 pulsed with the same synthetic peptides (Fig. 2).

To generate cytotoxic lymphocytes, non-adherent SC from B6 mice were co-cultivated with BMDC loaded with synthetic HPV 16 E6/E7 peptides exhibiting the cytotoxic epitopes. As positive controls, SC cultivated with DC loaded with TC-1 lysate were used. As negative controls, SC cultivated with unloaded BMDC or BMDC loaded with synthetic unrelated $OVA_{257\text{-}264}$ (SIINFEKL) peptide were used.

SC after a single stimulation with BMDC loaded with the individual peptides carrying the cytotoxic motifs exhibited only a weak cytotoxic effect on syngeneic MHC class I^{+} TC-1 targets (Fig. 3a). However, when the $E7_{44\text{-}62}$ peptide, harbouring both T-helper and CTL epitopes was used, a significantly higher cytolytic effect (Fig. 3a, effector/target cell ratios 100 : 1 and 50 : 1) was observed. The admixture of the $E7_{48\text{-}57}$ peptide to the $E7_{44\text{-}62}$ peptide enhanced the cytotoxic effect of SC stimulated with DC loaded with the $E7_{44\text{-}62}$ peptide at a level statistically not different from the effect of SC stimulated with BMDC loaded with the TC-1 cell lysate (Fig. 3a, effector/target cell ratio 100 : 1).

A significant cytotoxic effect of syngeneic non-adherent SC, sensitized *in vitro* with BMDC loaded with the individual peptides carrying CTL epitopes, comparable with the effect of SC stimulated with DC loaded with the TC-1 cell lysate, was found in the ^{51}Cr microcytotoxicity assay only after repeated sensitization with the loaded BMDC (Fig. 3b).

To characterize the effector cells responsible for the cytotoxic effect of SC, the effectors were examined using syngeneic MHC class I^{-} (MK16) target cells and their MHC class I^{+} variant obtained after cultivation of the MK16 cells in the presence of IFNγ (MK16-IFNγ cells). It has been found that the SC sensitized *in vitro* with BMDC loaded with the examined E6/E7 peptides (or TC-1 lysate) were cytotoxic exclusively for the MHC class I^{+} target cells, the MK16-IFNγ cells (Fig. 2b). The MHC class I^{-} (MK16) target cells were resistant to the cytotoxic effect of these SC effectors (Fig. 4a).

Besides *in vitro* studies, the effectiveness of the peptide-loaded BMDC was assessed in the immunization-challenge experiments (Fig. 5) *in vivo*. The protective potential of BMDC pulsed with the peptides $E7_{49\text{-}57}$,

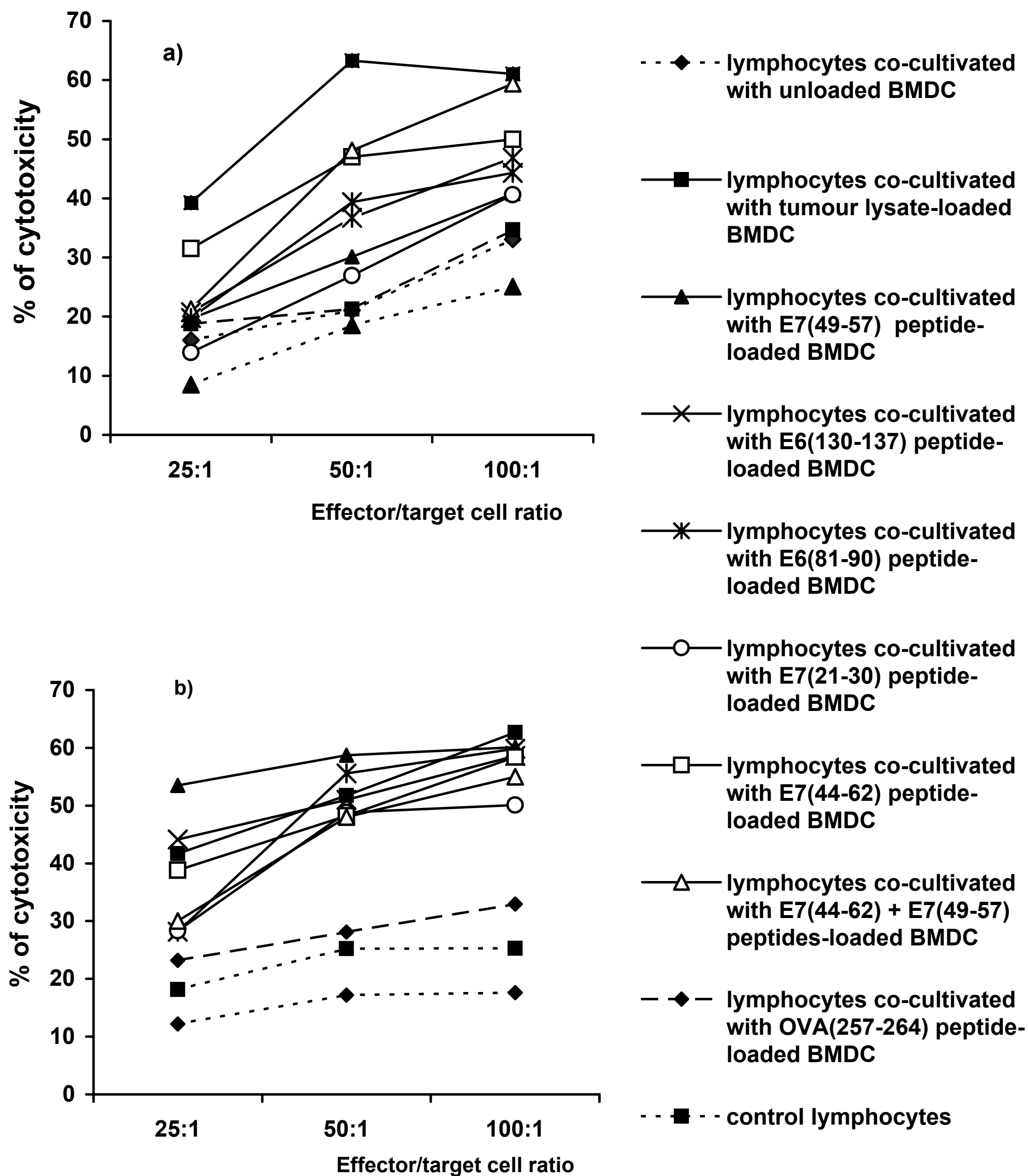

Fig. 3. Repeated *in vitro* stimulation of SC with peptide-loaded BMDC vaccines eliminated differences between the immunization ability of individual peptides. As negative control effectors, lymphocytes cultivated alone, co-cultivated with $OVA_{257-264}$ peptide-loaded BMDC or with unloaded BMDC were used. As positive control effectors, lymphocytes co-cultivated with the BMDC loaded with TC-1 tumour lysate were used. (a) One *in vitro* stimulation ($E7_{44-62}$ peptide group: $P < 0.05$, effector/target cell ratios 50 : 1, 100 : 1 as compared with the effect of negative control effectors, $E7_{44-62}$ plus $E7_{49-57}$ peptide: $P < 0.05$, effector/target cell ratio 100 : 1, as compared with the effect of $E7_{44-62}$ peptide only), (b) two *in vitro* stimulations (HPV 16 E6/E7 peptide groups: $P < 0.01$, effector/target cell ratios 50 : 1, 100 : 1 as compared with the effect of negative control effectors)

$E6_{81-90}$ and $E6_{130-137}$ containing CTL epitopes was determined (Fig. 5a). Mice were twice immunized with the peptide-pulsed BMDC seven days apart and after the second immunization challenged with the TC-1 cells. The most effective inhibition of the tumour growth detectable in growth curves of tumours was achieved with the BMDC pulsed with the E_{49-57} peptide, while the $E6_{130-137}$ peptide-pulsed BMDC did not elicit protective immunity in comparison with the unpulsed BMDC.

Next, we have tested the combinations of the E_{49-57} peptide with the peptides $E7_{44-62}$ and $E6_{98-107}$ harbouring Th epitopes (Fig. 5b). Tumour-free mice were recorded after immunization with the combination of the E_{49-57} and $E7_{44-62}$ peptides. This effect was long-lasting and vaccinated mice were protected against the

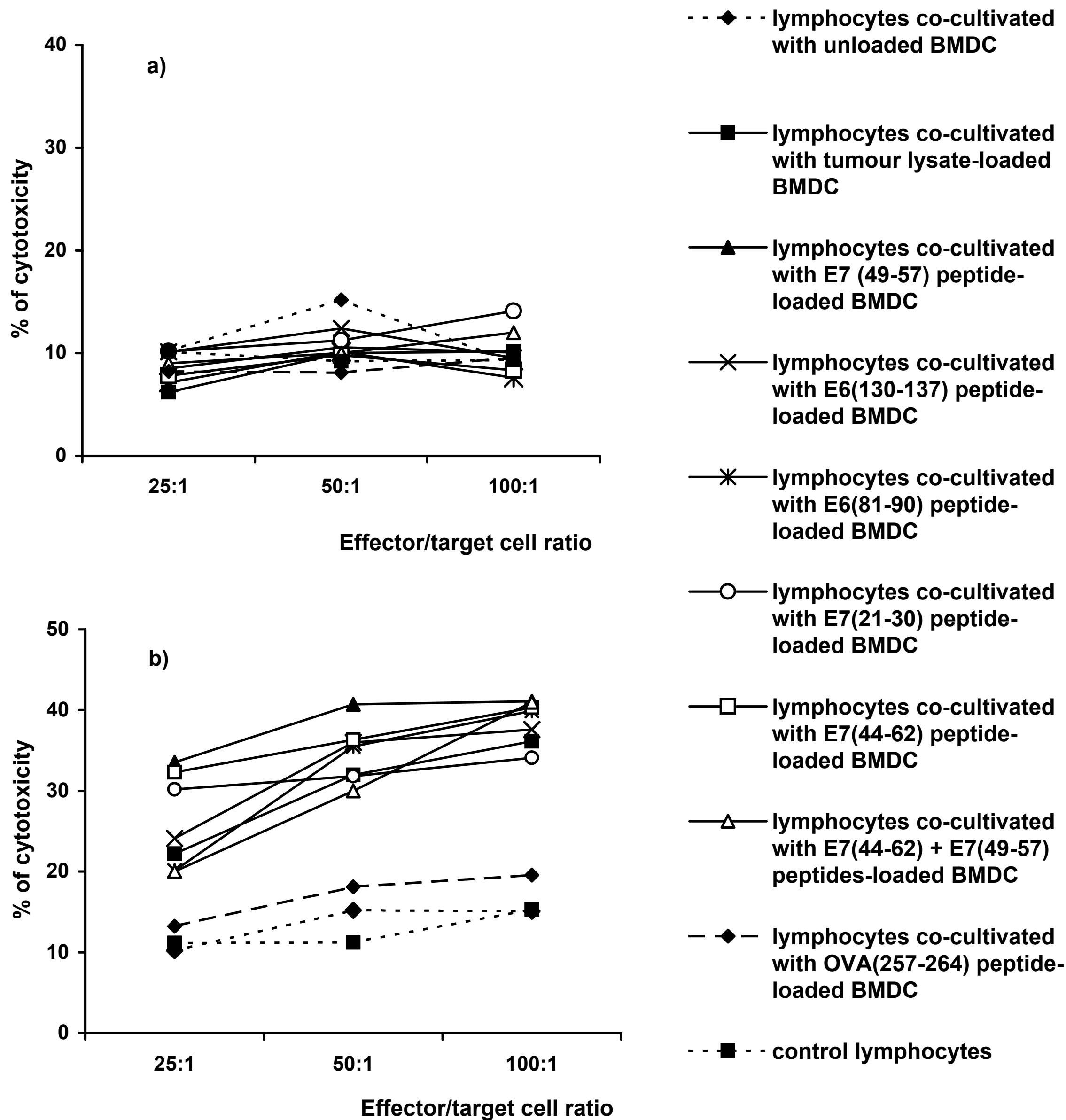

Fig. 4. HPV 16 E6/E7 peptides activate the generation of CTL *in vitro*. As negative control effectors, lymphocytes cultivated alone, co-cultivated with $OVA_{257\text{-}264}$ peptide-loaded BMDC or with unloaded BMDC were used. As positive control effectors, lymphocytes co-cultivated with the BMDC loaded with TC-1 tumour lysate were used. (a) MK 16 (MHC class I^-) target cells, (b) MK16 cells cultivated in IFNγ (MHC class I^+) targets (HPV 16 E6/E7 peptide groups: $P < 0.01$, effector/target cell ratios 50 : 1, 100 : 1 as compared with the effect of negative control effectors)

re-challenge with the TC-1 cells three months after the first challenge (Fig. 6). The second peptide tested, $E6_{98\text{-}107}$, did not increase the effectiveness of the vaccine.

To address the role of the longer $E7_{44\text{-}62}$ peptide in the induction of immune response, we have also compared the effect of this peptide alone and in combination with the $E7_{49\text{-}57}$ peptide. No significant effects were noted, since BMDC loaded solely with the longer peptide were able to elicit as strong protective immunity against the TC-1 challenge as the $E7_{49\text{-}57}$ peptide (Fig. 5c).

Discussion

The results of this study revealed that all BMDC loaded with HPV16 E6/E7-derived peptides, which have been previously characterized as activators of the MHC class I restricted CTL or which exhibited MHC class II restricted Th epitopes, were able to induce spleen cell proliferation *in vitro* in the syngeneic experimental system. The level of the priming capacity of the peptide-loaded BMDC was comparable to that of the tumour cell lysate-loaded BMDC, showing that the presentation of a single epitope or multiple epitopes from

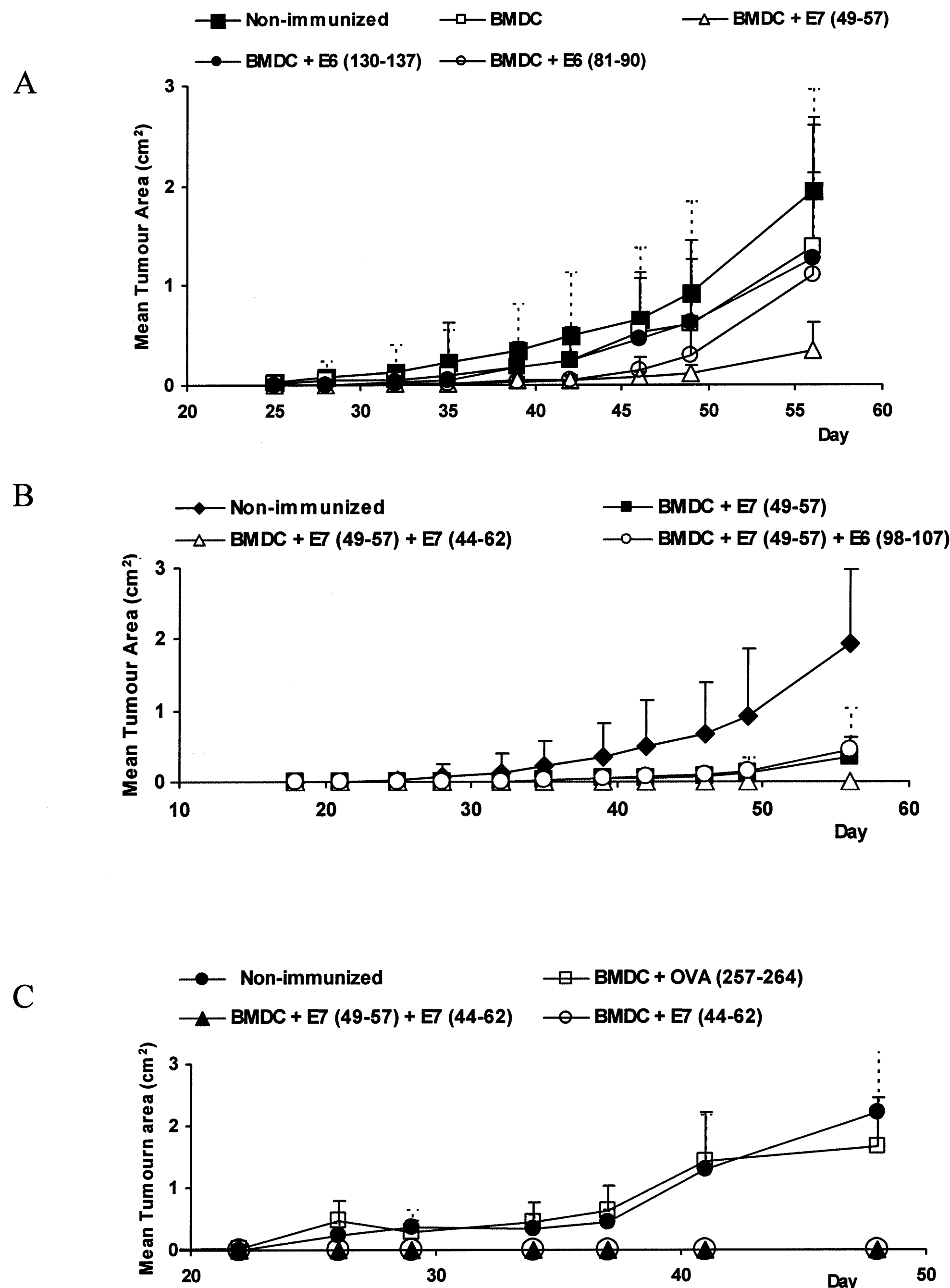

Fig. 5. Tumour-inhibitory effects of immunization with E6/E7 peptide-pulsed BMDC
(A) Mice were immunized with BMDC pulsed with various peptides containing CTL epitopes and s.c. transplanted with the TC-1 tumour cell line on day 0. Significant differences ($P < 0.01$) were observed between the control group (non-immunized) and BMDC+$E7_{49\text{-}57}$ and BMDC+$E6_{81\text{-}90}$ groups, respectively. No. of tumour-bearing/total No. of mice on day 39: 7/8 non-immunized, 6/6 BMDC only, 4/6 BMDC+$E7_{49\text{-}57}$, 4/7 BMDC+$E6_{130\text{-}137}$ and 5/7 BMDC+$E6_{81\text{-}90}$. (B) Mice were immunized with BMDC vaccines pulsed with the $E7_{49\text{-}57}$ peptide alone, mixed with $E7_{44\text{-}62}$ containing both Th and CTL epitopes and with $E6_{98\text{-}107}$ harbouring the Th epitope. Significant differences ($P < 0.01$) were observed between the control group and all three experimental groups. No. of tumour-bearing/total No. of mice on day 39: 7/8 non-immunized, 4/6 BMDC+$E7_{49\text{-}57}$, 0/6 BMDC+ $E7_{49\text{-}57}$+$E7_{44\text{-}62}$. The differences in the numbers of tumour-bearers between all experimental groups were significant ($P < 0.025$). (C) Mice were immunized with BMDC pulsed with the $E7_{44\text{-}62}$ peptide alone or mixed with $E7_{49\text{-}57}$. No. of tumour-bearing/total No. of mice on day 41: 7/7 non-immunized, 0/7 BMDC+ $E7_{49\text{-}57}$+$E7_{44\text{-}62}$ and 0/7 BMDC+ $E7_{44\text{-}62}$. The differences in the numbers of tumour-bearers between control and each experimental groups were significant ($P < 0.001$) as well as tumour growth curves ($P < 0.01$). Controls were non-immunized mice, mice immunized with BMDC and with BMDC pulsed with the ovalbumin-derived peptide containing the CTL epitope.

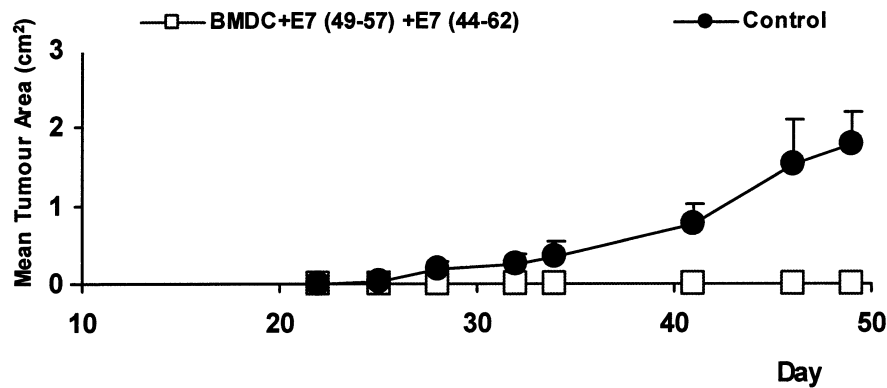

Fig. 6. Long-lasting tumour inhibitory effects of immunization with $E7_{49-57}$ and $E7_{44-62}$ peptide-pulsed BMDC. Tumour-free mice previously immunized with BMDC vaccines pulsed with the $E7_{49-57}$ peptide and with $E7_{44-62}$ and challenged with TC-1 cells were re-challenged three months after the first challenge. No. of tumour-bearing /total No. of mice on day 34 after re-challenge were: 1/6 $E7_{49-57}$ and $E7_{44-62}$ group and 7/7 control group. The differences in the numbers of tumour-bearers and in the tumour growth curves between these two groups were significant ($P < 0.01$).

the tumour cell lysate activates immune cells at a similar level.

Established dendritic cell lines pulsed with the peptides were also capable to induce spleen cell proliferation, although the response was weaker in comparison with BMDC-based vaccines. These findings suggest that the dendritic cell lines, which can be easily cultured *in vitro* and which represent a standard biological material of reproducible quality, can be used as tools for peptide antigen presentation. However, the antigen presentation by these cells is probably less effective than presentation by BMDC.

The differences in the effectiveness of the peptides to induce the development of functional CTL were recorded after single immunization; a significant CTL response arose only when the BMDC-based vaccines were loaded with the $E7_{44-62}$ peptide containing both CTL and Th epitopes. Moreover, the *in vivo* studies, when the maximal protective immunity against the challenge with the E6/E7 expressing TC-1 tumour cells was achieved after two immunizations with vaccines harbouring the $E7_{44-62}$ peptide either alone or after admixture of other peptides, correlated with these results of experiments *in vitro* after single immunization. We have concluded that the most effective and long-lasting immunization with peptide-pulsed dendritic cells is achieved when the longer peptide also exhibiting the Th epitope is used.

After repeated *in vitro* immunizations, all tested BMDC-based vaccines loaded with the peptides harbouring the CTL epitopes were able to induce the development of functional CTL at a comparable level. This indicates that repeated immunization is crucial particularly when a massive specific CTL response should be raised against weaker antigens. Notably, the cytotoxic response induced by all peptides was strictly MHC class I restricted.

It has been shown previously that immunization with peptides and/or with a combination of peptides encompassing both CTL and Th epitopes, whose length suggests their intracellular processing before their cell surface presentation in the context of the MHC class I molecule, is essential for induction of a strong and long-lasting immunity as well as for the development of memory T cells (Shirai et al., 1994; Ossendorp et al., 1998; Zwaveling et al., 2002). The mechanisms underlying the positive effect of the Th lymphocytes probably also involve the activation of dendritic cells as antigen-presenting cells (Ridge et al, 1998). However, although more studies are required in this field, our data strongly suggest that the use of the BMDC for antigenic peptide presentation does not overcome the need of peptides containing both the CTL and the Th epitopes.

Taken together, we have found a good correlation between the vaccine efficacy *in vitro*, determined by chromium release cytotoxic tests, and the level of protection against tumour growth in immunization-challenge experiments. The results from both *in vitro* and *in vivo* experiments revealed that BMDC-based cellular vaccines pulsed with peptides, which exceed the length optimal for binding of the MHC class I and which harbour the Th epitope, are capable to elicit strong and protective CTL-based immunity against HPV-associated MHC class I positive tumours.

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