

Induction of protective immunity against MHC class I-deficient, HPV16-associated tumours with peptide and dendritic cell-based vaccines

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Abstract. Downregulation of MHC class I expression on the cell surface is a common mechanism by which tumour cells, including cervical carcinoma, can escape the T cell-mediated anti-tumour immunity. This downregulation represents an obstacle for the efficacy of anti-tumour vaccines. In this study, we investigated the efficacy of prophylactic peptide and peptide-pulsed dendritic cell-based vaccines in a murine model of experimental MHC class I-deficient tumours (TC-1/A9), expressing E6/E7 oncogenes derived from HPV16, and compared the efficacy of particular vaccination settings to anti-tumour protection against parental MHC class I-positive TC-1 tumours. Peptide vaccine based on the 'short' peptide E7₄₉₋₅₇ harbouring solely the CTL epitope and co-administered to the C57BL/6 mice with CpG oligodeoxynucleotide (CpG ODN) 1826 was effective against MHC class I-positive but not -deficient tumours, while the 'longer' peptide E7₄₄₋₆₂ (peptide 8Q, harbouring CTL and Th epitopes)-based vaccines were also effective against MHC class I-deficient tumours. We have compared the adjuvant efficacies of two CpG ODN, CpG ODN 1826 and CpG ODN 1585. The 8Q peptide immunisation combined with CpG ODN 1585 inhibited growth of the TC-1/A9 tumours more effectively as compared to CpG ODN 1826. Further, we investigated the efficacy of cellular vaccines based on *ex vivo* cultured dendritic cells pulsed with either E7₄₉₋₅₇ or E7₄₄₋₆₂ peptides and matured with CpG ODN 1826. Unlike in the peptide immunisation setting, treatment

with dendritic cells pulsed with a 'short' peptide resulted in the tumour growth inhibition, albeit weaker as compared to the immunisation with the longer peptide. Our data demonstrate that peptide and dendritic cell-based vaccines can be designed to elicit protective immunity against MHC class I-deficient tumours.

Introduction

Downregulation of MHC class I expression on tumour cells represents a frequent event, as well as a serious obstacle in development of effective anti-tumour vaccines based on T cell-mediated immune responses against a variety of tumours, including cervical carcinoma (1-5). Therefore, immunotherapeutic and vaccination protocols should be designed to cope with tumours deficient in the cell surface expression of the MHC class I molecules. Several experimental settings have been shown effective against MHC class I-deficient tumours (6). However, the exact and prevailing mechanisms leading to protective immunity are difficult to determine and may vary in different models. The levels of residual expression of the MHC class I molecules on tumour cells or inducibility of MHC class I expression is of particular importance. Indeed, CD8⁺ cell-mediated immunity can take place thanks to the residual expression of MHC class I molecules on tumour cells or MHC class I-unrestricted mechanism can be involved (7-10). We have shown, using a murine model for HPV16-associated tumours, that immunisation with MHC class I-deficient but not -positive irradiated cells induced immunity against the MHC class I-deficient subline of the cell line expressing the same HPV16 E6/E7 oncoproteins (11,12). This lack of immunologic crossreactivity indicates that the immune responses effective against MHC class I-positive tumours may not be effective against tumours with defects in the expression of MHC class I molecules.

Synthetic peptides derived from HPV16 E7 oncoproteins, used either in combination with adjuvants (13) or as peptide-pulsed dendritic cell-based vaccines (14) are under intensive study as vaccines against cervical carcinoma. Promising results have been obtained both in mice and humans with a mixture of 'long' peptides harbouring both CTL and Th epitopes in combination with unmethylated CpG oligodeoxynucleotides or Montanide ISA-51 as adjuvants (15,16).

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Abbreviations: HPV, human papilloma virus; CTL, cytotoxic T lymphocyte; Th, T helper lymphocyte; NK, natural killer; DC, dendritic cells; CpG ODN, unmethylated CpG oligodeoxynucleotide; TLR, toll-like receptor; ELISPOT, enzyme-linked immunosorbent spot assay

Key words: MHC class I-deficient tumours, CpG oligodeoxynucleotides, peptide vaccines, human papilloma virus

Unmethylated 'CpG' oligodeoxynucleotides are potent immune activators that can be used as non-specific immune activators or as adjuvants for specific immunisation. Importantly, they can induce dendritic cell maturation upon binding toll-like receptor 9 (TLR-9), resulting in effective antigen presentation, expression of co-stimulatory molecules and cytokine production (17). Thus, they have also been used as maturation agents in dendritic cell-based cellular vaccines preparation protocols.

The goal of this study was to optimize peptide vaccines against these tumours, namely to compare the efficacy of a peptide comprising only the CTL epitope with a longer peptide harbouring CTL, Th and B epitopes. Further, we compared the efficacy of peptides admixed to unmethylated CpG ODN as adjuvants to the efficacy of dendritic cells pulsed with the peptides and matured with CpG ODN.

Materials and methods

Synthetic peptides used for vaccination. Synthetic peptides E7₄₉₋₅₇ (RAHYNIVTF) representing H2-D^b-restricted CTL epitope (18), E7₄₄₋₆₂ (8Q, QAEPDRAHYNIVTFCKCD) containing CTL, Th and B cell epitopes (18,19) and control OVA (H2-D^b-restricted) epitope SIINFEKL were synthesized at the Institute of Organic Chemistry and Biochemistry, AS CR, Prague, Czech Republic or custom-synthesised by Vidia (Vestec, Czech Republic).

Synthetic CpG oligodeoxynucleotides. CpG ODN 1826 (5'-TCCATGACGTTCCCTGACGTT-3', optimized for CTL and NK activation) and CpG ODN 1585 (5'-GGGGTCAACGTTGAGGGGGG-3', optimized for NK activation) (20) were purchased from Genosys, Hradec Kralove, Czech Republic. Both CpG ODN were fully sulphur-modified in their backbone (phosphorothioate) to prevent degradation by nucleases. This represents a modification for CpG ODN 1585 that has been previously used as a chimeric ODN with unmodified phosphodiester linkages in the central part of the molecule.

Dendritic cell preparation. Peptide-pulsed dendritic cells used as cellular vaccines were prepared as described previously (21). Briefly, dendritic cells were prepared from bone marrow precursors by culturing in the presence of GM-CSF and IL-4. On day 6, the DC were pulsed by 24-h incubation in the appropriate medium containing 50 µg/ml of peptide and matured for 24 h prior to injection into the animals with unmethylated CpG containing phosphorothioate-modified oligodeoxynucleotide CpG 1826 (or CpG ODN 1585) at a final concentration of 5 µg/ml.

Cell lines. MHC class I-positive cell line TC-1 was obtained by *in vitro* co-transfection of murine lung C57BL/6 cells with HPV16 E6/E7 and activated human Ha-ras (G12V) oncogenes (22). TC-1/A9 [MHC class I-deficient (10)] were obtained from TC-1 tumours developed in immunized mice. Cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, L-glutamine and antibiotics.

Animals. C57BL/6 (B6) mice (2-4 months old) were obtained from Anlab, Prague, Czech Republic. The mice were housed

in the animal facility of the Institute of Molecular Genetics. All experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics, Prague.

Tumour protection assays. Mice were twice immunized with peptide or dendritic cell-based vaccines (days -25 and -10, subcutaneous injection) and challenged on day 0. All tumour cells were injected subcutaneously. The experimental and control mice were observed bi-weekly, and the numbers of tumour-bearing mice and the size of the tumours were recorded. Two perpendicular diameters of the tumours were measured with a calliper and the tumour size was expressed as the tumour area (cm²).

Flow cytometry and the tetramer assay. BMDC were analysed for surface expression of selected molecules by flow cytometry. Labelled antibodies and isotype controls were purchased from BD Pharmingen (San Diego, CA). Cell suspensions were preincubated with anti-CD16/CD32 antibody to minimise non-specific binding. Analysis was performed using BD LSRII flow cytometer (BD, Franklin Lakes, USA). The following antibodies were used: anti-MHC I (PE anti-H-2D^b clone KH95 and PE anti-H-2K^b clone AF6-88.5), PE anti-CD86 (GL1), FITC anti-I-A^b (A_b^b) (AF6-120.1), FITC anti-CD11b (M1/70), PE anti-CD11c (HL3), FITC anti-CD38 (90), PE anti-CD54 (3E2), PE anti-CD80 (16-10A1), PE anti-CD273 (TY25), PE anti-CD274 (MIH5) and biotin anti-CD275 (HK5.3) plus PE-streptavidine. For analysis, 10,000 cells were counted and dead cells were distinguished by Hoechst 33258 staining. For the tetramer assay, 100,000 spleen cells were counted. Cells were stained with PE tetramers containing mouse E7 (49-57) CTL epitope (Sanquin, Amsterdam, The Netherlands), followed by staining APC with anti-CD3 (145-2C11) and FITC anti-CD8 (53-6.7).

ELISPOT analysis. To determine the amount of IFN-γ-secreting cells, ELISPOT kit for detection of murine IFN-γ (BD Pharmingen) was used according to the manufacturer's instructions. Spleen cells from control and experimental mice were placed to the wells of ELISPOT plate at the concentration of 5x10⁵ cells/well in triplicate, restimulated with 0.5 µg/ml of RAHYNIVTF peptide and cultured for 24 h at 37°C and 5% CO₂. Coloured spots were counted with CTL Analyser LLC (CTL, Cleveland, OH, USA) and analysed using ImmunoSpot Image Analyser software.

Statistical analysis. Statistical analysis of the numbers of tumour takes in experimental and control groups was performed using the χ^2 test. Analysis of variance by Newman-Keuls and Tukey-Kramer tests was used for statistical evaluation of the tumour growth curves from NCSS (Number Cruncher Statistical System, Kaysville, UT). The differences between the curves were considered significant at P-value <0.05.

Results

The efficacy of the peptides administered with CpG ODN 1826 as adjuvants. The first goal was to compare the prophylactic

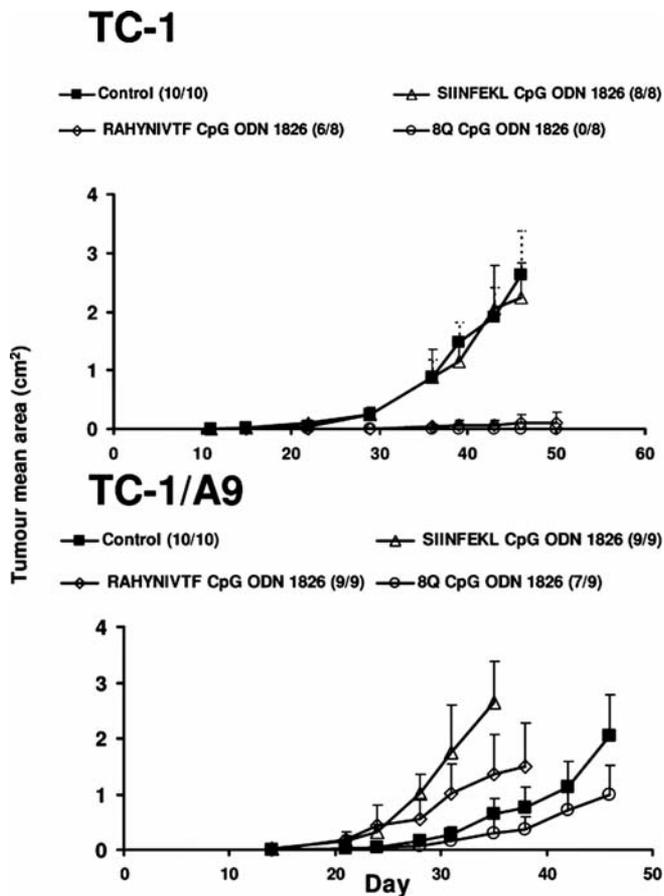


Figure 1. Immunisation against MHC class I-positive and -deficient tumours: the efficacy of the peptides administered with CpG ODN 1826 as adjuvants. Mice were immunized with E7₄₄₋₆₂ peptides (8Q) or E7₄₉₋₅₇ (RAHYNIVTF) peptides with CpG ODN 1826 as adjuvant. Controls were untreated mice and mice immunized with OVA-derived peptide SIINFEKL. (A) Significant inhibition of the TC-1 tumour growth compared to the controls was observed in mice immunized both with 8Q and RAHYNIVTF ($P < 0.05$ determined by Newman-Keuls and Tukey-Kramer tests). (B) The growth of the TC-1/A9 tumours was significantly inhibited by 8Q peptide while immunisation with SIINFEKL or RAHYNIVTF peptides significantly increased the rate of tumour growth. In all experiments, error bars show standard deviations. All experiments were repeated twice with similar results.

efficacy of the E7₄₉₋₅₇ peptide co-administered to the C57BL/6 mice with CpG ODN 1826 harbouring solely the CTL epitope with the 'longer' peptide E7₄₄₋₆₂ (8Q), harbouring CTL and Th epitopes against the parental TC-1, and MHC class I-deficient (TC-1/A9) tumours (Fig. 1). CpG ODN 1826 was used as the adjuvant in this experiment. Both E7₄₉₋₅₇ and E7₄₄₋₆₂ peptides induced immunity against TC-1 tumours while the growth of the TC-1/A9 tumours was partially inhibited only by the E7₄₄₋₆₂ peptide. Interestingly, immunisation with a control peptide derived from ovalbumine, as well as the E7₄₉₋₅₇ peptide rather accelerated than inhibited the TC-1/A9 tumour growth in this experiment.

Comparison of the adjuvant capacities of CpG ODN 1826 and CpG ODN 1585. We have compared the adjuvant efficacies of two CpG ODN, CpG ODN 1826, a potent inducer of both

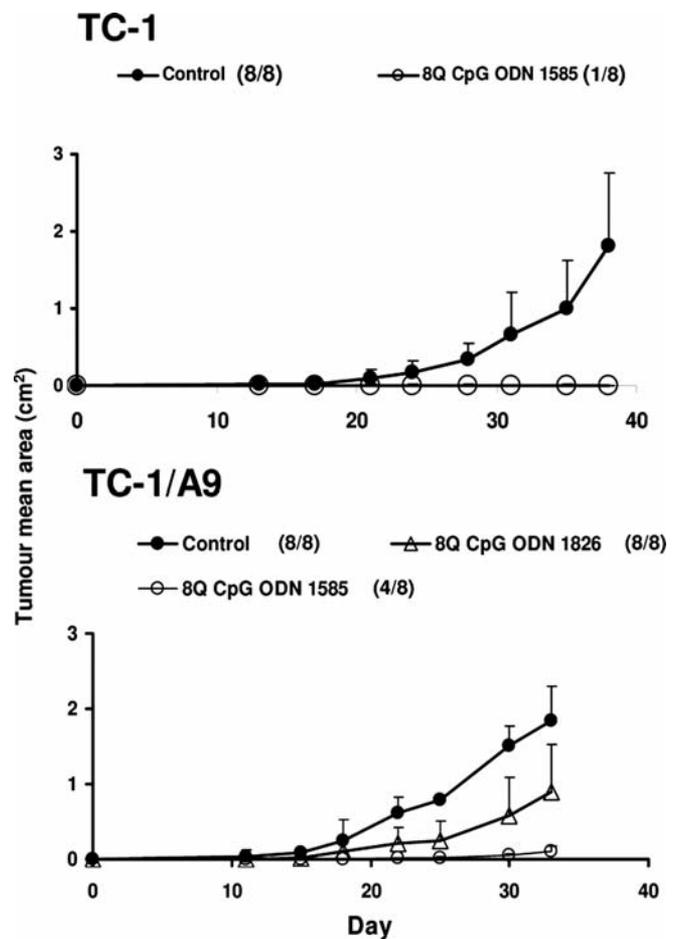


Figure 2. Adjuvant capacity of CpG ODN 1585 in vaccination against TC-1 and TC-1/A9 tumours. The efficacy of the E7₄₄₋₆₂ peptide immunisation with CpG ODN 1585 against TC-1 and TC-1/A9 tumours was compared. Significant inhibition of the tumour growth (according to the number of tumour takes, as well as to the tumour growth rates) was observed in both settings. The prophylactic effects of CpG ODN 1585 and 1826 were compared; CpG ODN 1585 turned out to be more powerful adjuvant as compared to CpG ODN 1826 (according to the number of tumour takes, as well as to the tumour growth rates). The experiments were repeated twice with similar results.

innate and adaptive immunity that is frequently used as an adjuvant in murine model, and CpG ODN 1585, a potent inducer of innate immunity (Fig. 2). Surprisingly, 8Q peptide immunisation combined with CpG ODN 1585 inhibited the growth of the TC-1/A9 tumours more effectively, as compared to CpG ODN 1826. The CpG ODN 1585 therapeutic potential was also demonstrated against the TC-1 cell line. These results indicate that the CpG ODN 1585 adjuvant potential is not restricted to the MHC class I-deficient tumours.

Induction of specific anti-E7₄₉₋₅₇ immune reaction. The capacity of the peptide vaccine preparations to induce specific anti-E7 immunity (against immunodominant epitope E7₄₉₋₅₇) in mice was determined by tetramer assay to quantify the number of anti-E7₄₉₋₅₇ specific CTL (Fig. 3A), as well as by ELISPOT analysis to quantify IFN- γ -producing spleen cells after *in vitro* restimulation with E7₄₉₋₅₇ peptide (Fig. 3B). These experiments revealed that the specific immune response upon

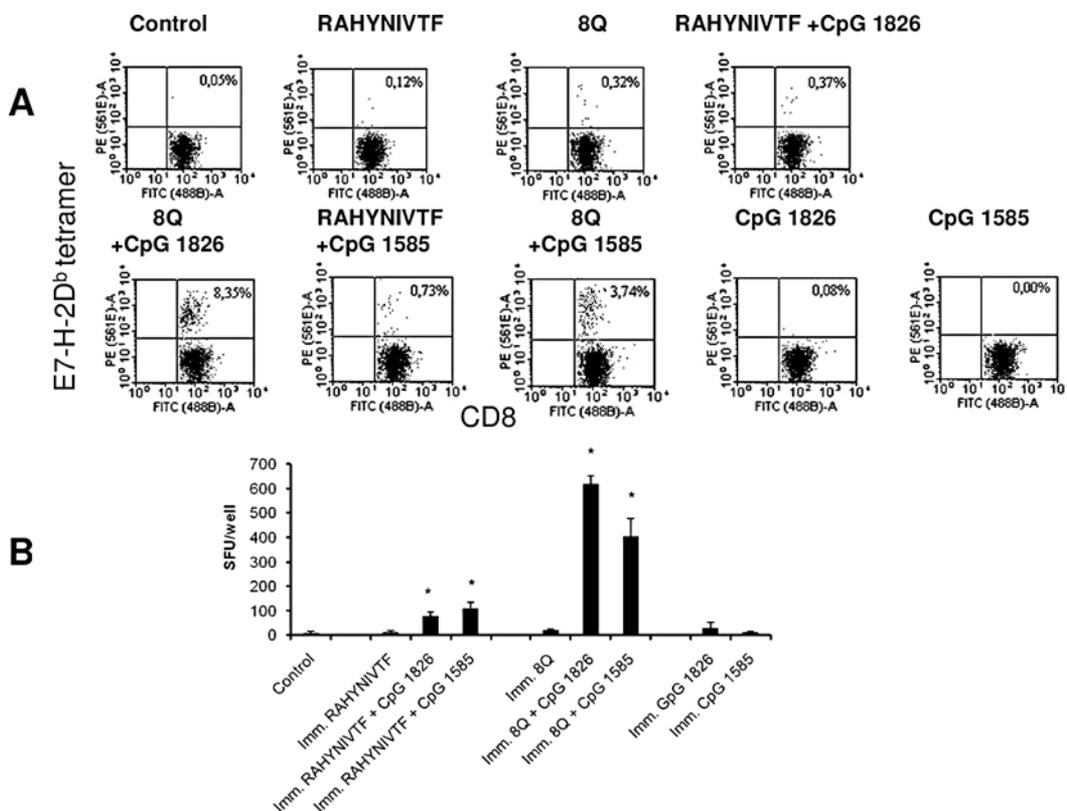


Figure 3. Induction of specific anti-tumour immunity by peptide vaccines. (A) Determination of the anti-E7₄₉₋₅₇ CD3⁺ cells after peptide immunisation. Mice were immunized with E7₄₄₋₆₂ and E7₄₉₋₅₇ and the percentage of specific CD3⁺ cells was determined by FACS analysis using anti CD3 Ab and H2-D^b tetramers loaded with E7₄₉₋₅₇. (B) Determination of IFN-γ-producing spleen cells by ELISPOT after repeated *in vivo* peptide immunisations and *in vitro* restimulation. *P<0.05, as compared to control and respective peptide without CpG ODN.

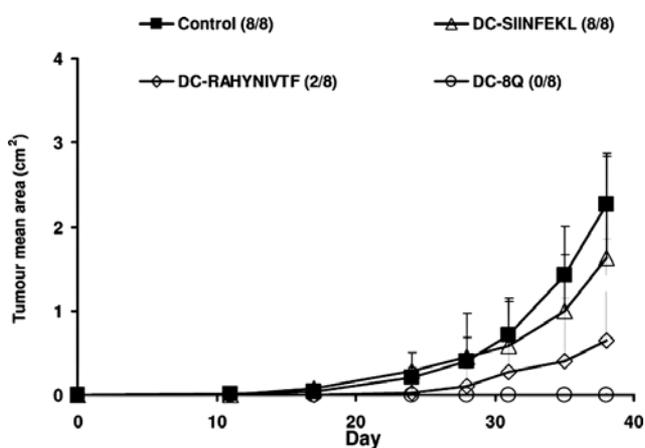


Figure 4. Immunisation against MHC class I-deficient TC-1/A9 tumours: the efficacy of the peptides administered as peptide-pulsed dendritic cells. Mice were immunised with E7₄₄₋₆₂ (8Q) and E7₄₉₋₅₇ (RAHYNIVTF) peptide-pulsed DC matured with CpG ODN 1826. Both vaccines inhibited the TC-1/A9 tumour growth, as compared to untreated controls and to the mice immunized with DC pulsed with a control SIINFEKL peptide. Significant differences were observed in the numbers of tumour takes on day 36 (no. of tumour-bearing mice/no. of mice in a group are presented in parentheses), as well as in the rates of tumour growth.

two immunisations with E7₄₄₋₆₂ peptide with CpG ODN as adjuvant was stronger as compared to the E7₄₉₋₅₇ peptide immunisations. The adjuvant capacity of both CpG ODN was documented (CpG ODN 1826 was stronger compared to CpG ODN 1585). Control immunisations with peptides alone induced very weak immune responses while no induction of specific immunity was observed upon administration of CpG ODN alone.

The prophylactic efficacy of the peptide-pulsed dendritic cells. Further, we investigated the prophylactic efficacy of cellular vaccines based on *ex vivo* cultured dendritic cells pulsed with either E7₄₉₋₅₇ or E7₄₄₋₆₂ peptides and matured with CpG ODN 1826 (Fig. 4). Both vaccines were effective against MHC class I-deficient tumours. Unlike in the peptide immunisation setting, treatment with dendritic cells pulsed with the E7₄₉₋₅₇ peptide resulted in tumour growth inhibition, albeit weaker as compared to the immunisation with the longer peptide.

Immunisation with E7₄₄₋₆₂-pulsed DC induced more intensive anti-E7₄₉₋₅₇ response compared to the E7₄₉₋₅₇-pulsed DC. In order to quantify induction of specific CTL recognizing immunodominant epitope E7₄₉₋₅₇, the percentage of specific CTL after immunisation with E7₄₉₋₅₇ or E7₄₄₋₆₂ peptides was determined by tetrameric assay (Fig. 5). Both immunisations

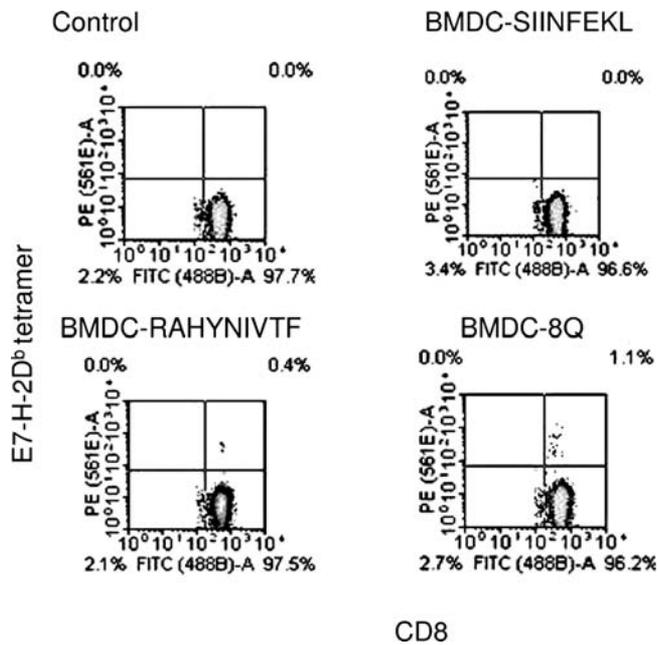


Figure 5. Determination of the anti-E7_{49.57} CD3⁺ cells after peptide-pulsed DC immunisation. Mice were immunized with E7₄₄₋₆₂ and E7_{49.57}-pulsed DC and the percentage of specific CD3⁺ cells was determined by FACS analysis using anti CD3 mAb and H2-D^b tetramers loaded with E7_{49.57} (cells were gated for CD8-positivity).

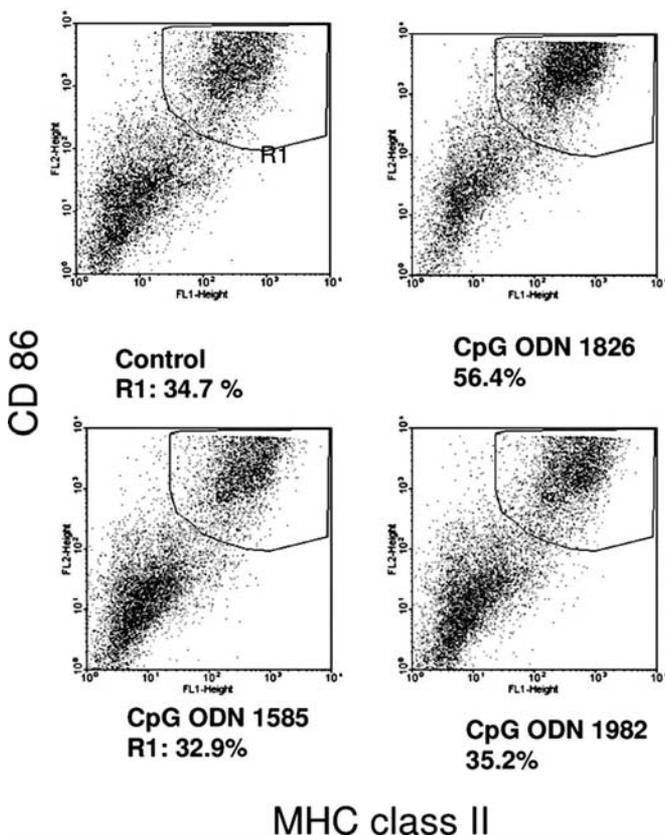


Figure 6. CpG ODN 1826 but not CpG ODN 1982 or CpG ODN 1585 induced BMDC maturation. Distinct CpG ODN (CpG ODN 1826, CpG ODN 1585 and control non-CpG ODN 1982) were analysed for their capacity to induce DC maturation monitored by the MHC class II and CD86 expression by flow cytometry. Only CpG ODN 1826 increased the proportion of MHC class II/CD86.

induced specific immune responses. However, the E7₄₄₋₆₂ peptide-pulsed DC immunisation resulted in the development of 1.1% as compared to 0.4% specific CTL. The tetramer assay was performed after single immunisations with the DC-based vaccines, since in our previous studies of cytotoxic responses after immunisations with DC loaded with different peptides showed significant differences after single but not multiple immunisations (21).

CpG ODN 1826 but not CpG ODN 1585 or control CpG ODN 1982 induce DC maturation. Since CpG ODN 1585 turned out to be a potent adjuvant to peptide immunisation, it was of interest to evaluate its effects on DC maturation. As can be seen in Fig. 6, only CpG ODN 1826 but not CpG 1585 or control CpG ODN 1982 induced DC maturation. Therefore, only CpG ODN 1826 was used for the experiments with dendritic cell-based vaccines in this study.

Discussion

The presented data demonstrated the efficacy of peptide immunisation against MHC class I-deficient tumours in a prophylactic setting. However, they also showed the particular importance of selection of proper peptides and immunisation settings. A number of recent studies and trials indicate that longer peptides that are processed by antigen-presenting cells prior to their presentation in the context of MHC class I molecules and/or also harbouring Th epitopes are necessary for eliciting effective anti-tumour immunity. Our study revealed that the use of short peptides can even accelerate the tumour growth, when administered with CpG ODN as adjuvants. Several previous studies have demonstrated that under some circumstances immunisation against tumours can rather accelerate than inhibit the tumour growth (23,24). Our data emphasize that immunisation solely with CTL epitopes can be very controversial especially in the case of MHC class I-deficient tumours.

An important finding is that the adjuvant effect of the CpG ODN 1585 was more effective as compared to CpG ODN 1826. CpG ODN 1826 is a widely used CpG ODN in murine models, including those for HPV16-associated tumours, either as an adjuvant (15) or as an immunotherapeutic agent (25,26). The sequence of CpG ODN 1585 has been optimized to indirectly activate NK cells (27). Our data also demonstrate that the immune response upon immunisation with CpG ODN 1585 as an adjuvant was not stronger compared to CpG ODN 1826. Therefore its superior adjuvant capacity in combination with peptides was not expected and the mechanism of its action is not clear. Besides the possible role of activated NK cells in the development of anti-tumour immunity we also have to take into account other mechanisms, including some physico-chemical qualities of the CpG ODN 1585, e.g., its tendency to precipitate from the solution.

Different approaches have been tested for peptide immunisation against MHC class I-deficient tumours. We evaluated the effectiveness of vaccination either in the form of a free peptide combined with CpG ODN as an adjuvant or as peptide-pulsed DC. Another approach was to compare CpG ODN 1826, a potent maturation agent for murine dendritic cells, used

either as an adjuvant or as a maturation agent for preparation of DC-based vaccines *in vivo* (CpG ODN 1585 was not compared since its capacity to induce maturation of DC has not been proved). Usage of the peptide-pulsed DC improved the efficacy of the short peptide that became partially protective against MHC class I-deficient tumours and did not hamper anti-tumour immunity. However, peptide E7₄₄₋₆₂ was also superior in this setting. It has been suggested that utilisation of dendritic cells as carriers for peptide-based vaccines can replace the necessity of the Th cell activation by corresponding epitopes (28), although other studies (29) have shown that CTL induction by cross-priming requires the active involvement of CD4⁺ helper T cells and that this CD4⁺ population is effective only when both the helper and CTL determinants are recognized on the same APC. Our analysis of the CTL immune responses by tetramers loaded with E7₄₉₋₅₇ showed more robust expansion of the E7₄₉₋₅₇-specific CTL upon immunisation with the DC loaded with the E7₄₄₋₆₂ peptides, as compared to the E7₄₉₋₅₇-pulsed DC. It is difficult to distinguish based on these experiments whether this increase can be attributed to the processing of the longer peptide by the DC antigen presenting machinery or by the presence of the Th epitope. An important conclusion is that longer peptides presented by *ex vivo* prepared DC represent a promising strategy against MHC class I-deficient tumours.

Taken together, our data indicate that both peptide and dendritic cell-based vaccines (reviewed in ref. 30) represent promising strategies against MHC class I-deficient tumours, but the peptide selection and optimisation of vaccine design are even more important than in the case of MHC class I-positive tumours.

Acknowledgements

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