

CpG-ODN-stimulated dendritic cells act as a potent adjuvant for E7 protein delivery to induce antigen-specific antitumour immunity in a HPV 16 E7-associated animal tumour model

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SUMMARY

We previously reported that both E7 and CpG-oligodeoxynucleotide (ODN) are required for protecting animals from human papillomavirus (HPV) 16 E7-associated tumour challenge. Here we investigate dendritic cells (DC)-based approach in this protection. In the study, we isolated bone marrow-derived DC and stimulated DC with E7 and ODN. *In vitro* stimulation of DC with E7 plus ODN resulted in more production of interleukin-12, as compared to that with E7 or ODN alone. Further injection with E7 + ODN-stimulated DC resulted in more significant tumour protection, as compared to stimulation with E7 or ODN alone. We further evaluated the levels of immune responses induced by DC stimulated with E7 + ODN. We observed little enhancement of E7-specific antibody and T helper cell proliferative responses by E7 + ODN stimulation, as compared to E7 stimulation. However, there was some enhancement of interferon- γ (IFN- γ) production from CD4⁺ T cells and a more significant production of IFN- γ from CD8⁺ T cells by E7 + ODN stimulation, as compared to E7 stimulation alone. This was consistent with intracellular IFN- γ staining levels of CD8⁺ T cells. Tumour protection further appeared to be mediated by CD8⁺ T cells, as determined by *in vivo* T-cell depletion. Thus, these data suggest that upon ODN stimulation DC might function as a potent adjuvant for E7 protein delivery for induction of protective cellular immunity against HPV E7-associated tumour challenge.

Keywords CpG-oligodeoxynucleotide; dendritic cells; human papillomavirus; protein delivery; tumour immunity

INTRODUCTION

Dendritic cells (DC) are the most effective antigen-presenting cells (APC) known that express a high amount

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Abbreviations: APC, antigen-presenting cells; CpG-ODN, CpG-oligodeoxynucleotide; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; HPV, human papillomavirus; HRP, horseradish peroxidase; i.p., intraperitoneally; PBS, phosphate-buffered saline; SI, stimulation index; TLR, Toll-like receptor; IL, interleukin.

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of Class I and II molecules as well as costimulatory molecules.¹ Presently, DC-based tumour immune therapy has been demonstrated to be effective for activating antigen-specific T-cell responses against tumour cells.^{2–6} In human papillomavirus (HPV)-associated tumour studies, application of antigen,^{7,8} peptide,⁹ virus-like particle¹⁰ and viral gene delivery^{11,12} has been tested for its ability to prime DC and then induce antitumour effects. Recent clinical trials using DC suggest a possible efficacy for treatment of HPV-associated cervical cancer.¹³ These findings support potential applications of DC for efficient promotion of antitumour immunity as an anticancer therapeutic modality.

CpG DNA or oligodeoxynucleotides containing unmethylated CpG motifs (ODN) have been reported to stimulate DC for activation and maturation to professional APC.^{14,15} In one study, *in vitro* survival of DC was

increased by treatment with ODN,¹⁶ suggesting that ODN plays a role in continuous activation of DC. ODN can activate B cells, monocytes and natural killer (NK) cells, and induce a Th1 like pattern of cytokine production.^{14,17–21} The CpG sequences drive macrophages to secrete interleukin-12 (IL-12), a potent inducer of interferon- γ (IFN- γ) production *in vivo* from natural killer cells. IFN- γ production drives T helper 1 (Th1) type immune responses through inducing differentiation of type 1 T helper cells which see antigen in the presence IFN- γ from the uncommitted T-cell pool.^{22,23} Moreover, ODN enhances humoral responses, driving them toward immunoglobulin G2a (IgG2a) isotypes (Th1 type indicator)^{22,24} and induces development of enhanced cytotoxic T lymphocyte (CTL) activity.^{19,25} We previously reported that both E7 and ODN are required for protective immunity against E7-associated tumour challenge,²⁶ suggesting an important role of APC, in particular DC in this protection.

Here we investigate whether upon pulsing DC *in vitro* with antigens ODN stimulation might influence antigen-specific immune responses as well as protection against a tumour challenge. In this study, we isolated bone-marrow-derived DC and stimulated them with the recombinant E7 protein of HPV 16 type and/or ODN, and then tested their ability to induce protection against E7-expressing tumours as well as E7-specific immune responses in the animal model system. We observe that injection with DC stimulated with E7 plus ODN results in a more significant protection against tumour challenge, as compared to DC pulsed with E7 alone. Furthermore, ODN stimulation resulted in enhanced E7-specific Th1 type CD4⁺ T-cell responses and more significantly CTL responses, as compared to without ODN stimulation, suggesting an important role of ODN for fostering cellular responses, in particular CTL activities. Furthermore, the enhanced tumour protection appeared to be mediated by CD8⁺ T cells, as determined by *in vivo* T-cell depletion. Taken together, these data illustrate that upon ODN stimulation DC might function as a potent adjuvant for E7 protein delivery for induction of protective cellular immunity against HPV 16 E7-associated tumour challenge.

MATERIALS AND METHODS

Recombinant E7 protein and oligodeoxynucleotides

Recombinant E7 protein was previously generated and used in this study.^{26,27} The immunostimulatory CpG ODN designated as 1826 (5'-TCCATGACGTTTCCTGACGTT-3') was used as a DC stimulant in this study. The ODN was purchased from Biobasic Inc. (Ontario, Canada). ODN was synthesized with a nuclease-resistant phosphorothioate backbone. ODN was dissolved in water and was confirmed to have undetectable endotoxin level.

DC preparation

Bone marrow DC were obtained from C57BL/6 mice. First, red blood cells were removed by adding ACK solution (Biofluids, Camarillo, CA) and the remaining cells were incubated at 37° for 4 hr. Non-adherent cells were secured

and treated further with granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 U/ml) and IL-4 (5 U/ml; Endogen, Woburn, MA) for 7 days with intermittent changes with new complete RPMI media containing GM-CSF and IL-4. For detection of surface markers of DC, CD11c-PE (clone HL3), CD86-fluorescein isothiocyanate (FITC; clone GL1), CD80-FITC (clone 16-10A1), CD40-FITC (clone HM40-3), CD54-FITC (clone 3E2), Class I-FITC (AF6-88.5), Class II-FITC (clone AF6-120.1), hamster IgG (CD11c and CD54 isotypes), rat IgG2a (CD86 isotype), hamster IgG2a (CD80 isotype), hamster IgM (CD40 isotype) and mouse IgG2a (Class I and II isotypes) were purchased from BD (San Diego, CA). DC (1×10^5 cells) were stained with each specific antibodies at 4° for 1 hr, followed by washing with fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline (PBS) + 1% bovine serum albumin + 0.1% sodium azide). Each sample was analysed using flow cytometry (BD). The tested cells expressed surface markers, such as CD11c, Class I, Class II, CD40, CD80, CD86 and CD54 (Fig. 1). These data confirm that the cells are bone marrow-derived DC in their phenotype.

In vitro production of IL-12 from DC by stimulation with E7 and ODN

DC (2.5×10^6 cells per ml) were treated *in vitro* with E7 (10 μ g/ml) and/or ODN (10 μ g/ml). After 2 days incubation at 37° in 5% CO₂, cell supernatants were secured and then used for detecting levels of IL-12 using commercial IL-12 kits (BD) by adding the extracellular fluids to the IL-12-specific enzyme-linked immunosorbent assay (ELISA) plates.

Immunization of mice

Female 4–6 week old C57BL/6 female mice were purchased from Daehan Biolink, Chungbuk, Korea. Mice were injected intraperitoneally (i.p.) with 100 μ l of PBS containing 5×10^5 DC previously pulsed with E7 and/or ODN using a 28-gauge needle (Becton Dickinson, Franklin Lakes, NJ). For DC pulsing, DC ($2.5\text{--}5 \times 10^6$ cells per ml) were treated simultaneously with E7 (10 μ g/ml) and/or ODN (10 μ g/ml) for 5 hr. Primed DC were washed three times with PBS prior to injection.

ELISA

ELISA was performed as previously described.^{28,29} In particular, recombinant E7 protein (1 μ g/ml in PBS) was used as a coating antigen. For the determination of E7-specific IgG, anti-mouse IgG conjugated with horseradish peroxidase (HRP; Zymed, San Francisco, CA) was reacted for ELISA.

T helper cell proliferation assay

T helper cell proliferation assay was performed as previously described.^{30,31} In brief, spleen cells were stimulated with E7 proteins at 1 and 5 μ g/ml concentrations for 3 days. Then, [³H]-labelled thymidine (1 μ Ci per well) was added over night. Next day, the cells were harvested and counts per minute (c.p.m.) were counted using a β -counter (PerkinElmer, Boston, MA). Stimulation index (SI) was

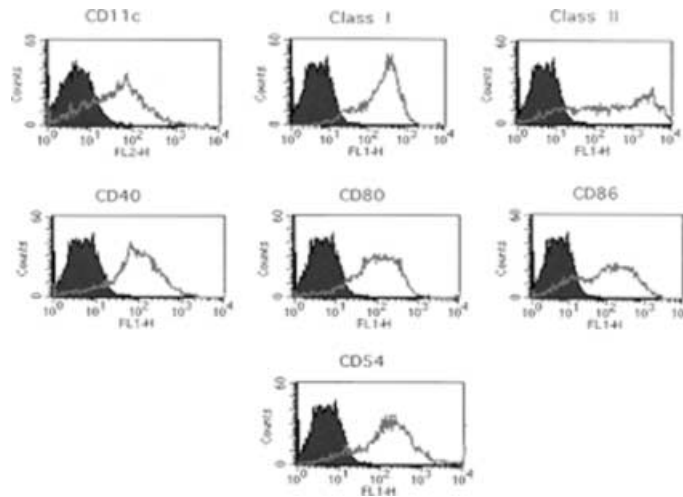


Figure 1. Production of murine dendritic cells from bone marrow stem cells. Murine bone marrow cells were stimulated with GM-CSF and IL-4. Non-adherent cells were harvested after 7 days and expression of surface markers were analysed using flow cytometry. Filled lines show isotype controls.

determined as [(experimental c.p.m. – media control c.p.m.) / [media control c.p.m.)].

IFN- γ release assay

For IFN- γ , a 1 ml aliquot containing 6×10^6 splenocytes was added to wells of 24-well plates. Then, 1 μ g of recombinant E7 protein/ml or 4×10^5 TC-1 cells treated previously with mitomycin C (30 μ g/ml for 3 hr) was added to each well. After 3 days incubation at 37° in 5% CO₂, cell supernatants were secured and then used for detecting levels of IFN- γ using commercial cytokine kits (Biosource, Intl., Camarillo, CA) by adding the extracellular fluids to the IFN- γ -specific ELISA plates.

Intracellular IFN- γ staining and FACS analysis

Mice were killed to obtain spleen cells at 3 weeks following the second injection with DC. Spleen cells (2×10^6) were stimulated with 1 μ g of peptide (amino acids 49–57) containing major histocompatibility complex (MHC) class I epitope³² for 6 h in the presence of 1 μ l GolgiPlug (Pharmingen) per ml and then washed twice with FACS buffer. Cells were incubated with anti-mouse Fc receptor (BD) for 1 hr on ice. The cells were then reacted with PE conjugated anti-mouse CD8 (Pharmingen) for 30 min on ice. In each step, cells were washed twice with FACS buffer. Finally, cells were resuspended in 250 μ l of Cytotfix/cytopermTM for 20 min at 4° and then washed twice with Perm/WashTM soln (Pharmingen). The subsequently fixed/permeabilized cells in Perm/WashTM solution were stained with anti-IFN- γ -FITC (Pharmingen) for 30 min at 4°. After washing three times with FACS buffer, cells were analysed for the percentage of IFN- γ - or CD8 positive cells on a flow cytometer (BD).

Tumour protection assay

Animals were immunized twice i.p. with DC preparations. TC-1 cells were then injected subcutaneously (s.c) into the right flank of C57BL/6 mice. TC-1 tumour cells (a kind gift

from T.-C. Wu, Johns Hopkins Medical Institutions) were grown in complete RPMI supplemented with 400 μ g per ml of G418. The tumour cells were washed twice with PBS and injected into mice. Mice were monitored twice per week for tumour growth. Mice were killed when tumour size reached more than 20 mm in mean diameter.

In vivo depletion of CD8⁺ T cells

Depletion studies were performed as previously described.^{26,27,30,31} First, anti-CD8 (clone 2.43) ascites fluids were generated by injecting hybridoma cells (American Type Culture Collection, Manassas, VA) into pristane-primed nude mice i.p. One hundred μ l of ascites fluids were administered i.p. on days –3, 0 and 3 of tumour challenge. Antibody treatment resulted in more than 98% depletion of specific CD8⁺ T-cell subsets of representative animals over 3-week periods. Depleted mice were subsequently challenged with tumour on day 0.

Statistical analysis

Statistical analysis was done using the paired Student's *t*-test or ANOVA. Values of E7 pulsed group were compared with those of E7+ODN pulsed group or control groups. *P*-values < 0.05 were considered significant.

RESULTS

Production of IL-12 from DC by E7+ODN treatments

It has been reported that ODN can activate DC. We tested whether stimulation of DC with E7+ODN might lead to more activation of DC *in vitro*. As shown in Fig. 2, IL-12 production from DC was more significant when DC were treated with ODN, as compared to non-treatment control showing a background level of IL-12. When DC were treated *in vitro* with E7, there was some level of IL-12 production from DC. Moreover, when DC were treated

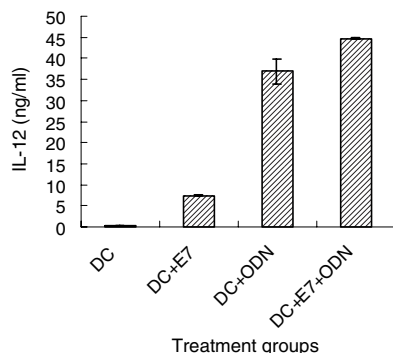


Figure 2. Production of IL-12 upon stimulation of DC with E7 + ODN. Bone marrow-derived DC were prepared as described in Materials and Methods. DC (2.5×10^6 cells/ml) were stimulated with $10 \mu\text{g}$ of E7 and/or ODN per ml for 2 days. Cell supernatants were obtained for detecting IL-12 levels.

in vitro with both E7 and ODN, a more significant production of IL-12 was noted, as compared to E7-treated or ODN-treated DC groups. This supports the idea that DC can be activated by E7+ODN treatment more efficiently than either one of these.

Pulsing DC with E7 plus ODN resulted in a more significant protection from E7-expressing tumour challenge

It is important that ODN-treated DC can be more efficiently pulsed with recombinant E7 proteins for induction of protection against tumour formation. We analyzed protective efficacy of DC pulsed with E7 + ODN in the TC-1 tumour cell challenge model system. Animals were immunized i.p. with DC pulsed with E7 and/or ODN, and then challenged subsequently with 5×10^4 (Fig. 3a) and 2×10^5 (Fig. 3b) of TC-1 cells. As shown in Fig. 3(a), pulsing with E7 plus ODN resulted in 100% protection of animals from tumour formation. In contrast, pulsing with E7 showed 50% of the animals were free from tumour formation. However, all animals showed tumour growth when immunized with DC alone or DC previously stimulated with ODN alone. The similar protection against tumour challenge with TC-1 at a higher dose was also observed (Fig. 3b). This illustrated that stimulation with antigen plus ODN could make DC more efficient for induction of antitumour immunity.

E7-specific IgG production was unaffected by pulsing DC with E7 + ODN

We next investigated the *in vivo* effects of DC pulsed with E7+ODN on the induction of antigen-specific antibody responses. As shown in Fig. 4(a), pulsing DC with E7 resulted in an increase in E7-specific IgG production, as compared to negative control and ODN-treated group. Moreover, pulsing DC with E7 plus ODN resulted in E7-specific IgG production in a manner similar to pulsing with E7 alone. This analysis suggests that DC pulsed with E7+ODN cannot affect an antibody production significantly over DC pulsed with E7 alone.

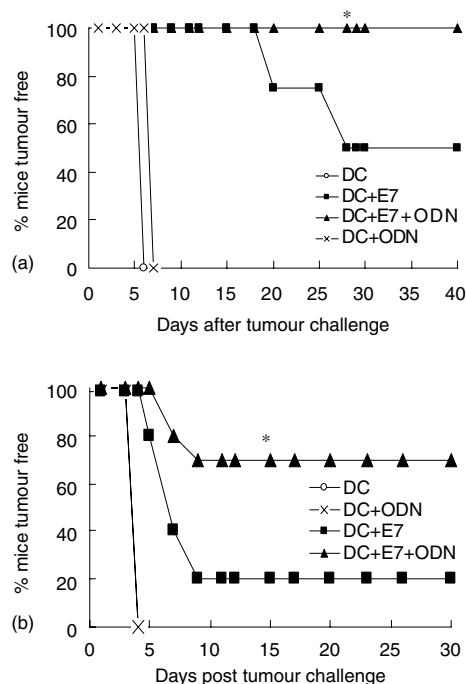


Figure 3. Antitumour effects of ODN application for DC pulsing. Each group of mice ($n = 5-10$) was immunized i.p. with 5×10^5 DC previously pulsed with E7 and/or ODN at 0 and 2 weeks. Three weeks after the second injection, mice were challenged s.c. with 5×10^4 (a) and 2×10^5 (b) TC-1 cells. The number of animals showing tumour growth was counted over time. *Statistically significant at $P < 0.05$ using anova compared to DC + E7 treated groups.

E7-specific T helper cell proliferative responses were unaffected by pulsing DC with E7 + ODN

T helper cell proliferation is a standard parameter used to evaluate the potency of cell-mediated immunity. We measured T helper cell proliferative responses following immunization with DC pulsed with E7 and/or ODN by stimulating the splenocytes of immunized animals *in vitro* with E7 proteins. As shown in Fig. 4(b), pulsing with E7 alone resulted in increased E7-specific T helper cell proliferative responses. However, there was no enhancement in T helper cell proliferative responses by pulsing DC with E7 + ODN, as compared to pulsing with E7 alone. However, little induction of T helper cell responses was observed by injecting DC treated *in vitro* with ODN or none.

IFN- γ production from CD4⁺ and in particular CD8⁺ T cells was enhanced by pulsing DC with E7 + ODN

IFN- γ has been known to play an important role in driving Th1 type immune responses as well as inducing cytotoxic T-cell responses. The protective role of IFN- γ against virus infection or tumour challenge has been reported.³³⁻³⁶ We previously observed that upon stimulation with the recombinant E7 protein CD4⁺ T cells are responsible for IFN- γ production while upon stimulation with TC-1 cells (Class

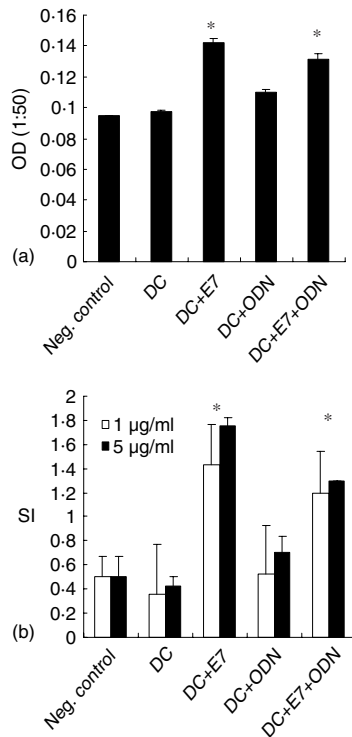


Figure 4. E7-specific IgG (a) and T helper cell proliferative responses (b) were unaffected by injection with DC pulsed in the presence of ODN. Each group of mice ($n = 5$) was immunized i.p. with 5×10^5 DC previously pulsed with E7 and/or ODN at 0 and 2 weeks. Three weeks after the second injection, Mice were bled and sera were tested for ELISA (a). Mice were killed and spleens were pooled for subsequent T helper cell proliferation studies (b). Samples were assayed in triplicate. This was repeated twice with similar results. *Statistically significant at $P < 0.05$ using the paired Student's t -test compared to control groups.

I^+ , Class II $^-$) $CD8^+$ T cells are responsible for IFN- γ production.^{26,27} To evaluate $CD4^+$ T-cell dependent production levels of IFN- γ , we stimulated with the recombinant E7 protein splenocytes of animals injected with DC pulsed with E7 and/or ODN. As shown in Fig. 5(a), IFN- γ production was significantly induced by injection with DC pulsed with E7. Furthermore, IFN- γ production was enhanced by injection with DC pulsed with E7+ODN a bit higher than DC pulsed with E7 alone. However, little induction of IFN- γ production was observed by injecting DC treated with ODN or none. This suggests that ODN has some positive effects on antigen-specific Th1 type $CD4^+$ T-cell responses when added upon pulsing DC with antigens. We next evaluated $CD8^+$ T-cell dependent production of IFN- γ by stimulating splenocytes *in vitro* with E7-expressing syngeneic TC-1 cells. As shown in Fig. 5(b), IFN- γ production was dramatically induced in the animal groups given injections of DC pulsed with E7. Moreover, IFN- γ production was enhanced to a significant level by injection with DC pulsed with E7 plus ODN, as compared to DC pulsed with E7 alone. This was also consistent with intracellular IFN- γ staining levels of $CD8^+$ T cells (Fig. 6). In particular,

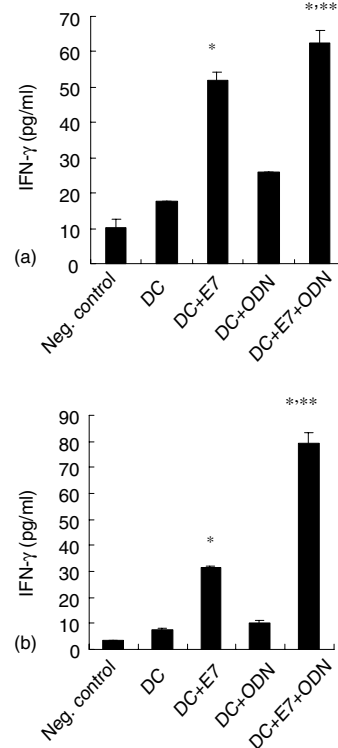


Figure 5. IFN- γ production was enhanced from $CD4^+$ T cells and more significantly from $CD8^+$ T cells by pulsing DC in the presence of ODN. Animals were immunized as shown in Fig. 4. Three weeks after the last injection, mice were killed and spleen cells were pooled. Splenocytes were stimulated with 1 µg of E7 proteins/ml (a) and with mitomycin C-treated TC-1 cells (b) for 3 days. Samples were assayed in triplicate. Values and bars represent mean of released IFN- γ concentrations and the standard deviation. The experiments were repeated twice more with similar results. *Statistically significant at $P < 0.05$ using the paired Student's t -test compared to control groups. **Statistically significant at $P < 0.05$ using the paired Student's t -test compared to DC + E7 pulsed groups.

E7 pulsed group showed 0.11% of both IFN- γ and $CD8$ positive T cells (CTL marker) whereas the E7 + ODN pulsed group showed 0.26% of both IFN- γ and $CD8^+$ T cells. However, little induction of IFN- γ releasing $CD8^+$ T cells was observed in the groups given injections of DC treated with ODN or none. This finding suggests that ODN can be used for enhancing antigen-specific CTL responses upon pulsing DC with antigens.

Involvement of $CD8^+$ T cells in DC-mediated protection

It has been reported that $CD8^+$ T cells are mainly responsible for protecting animals from tumour challenge in a TC-1 tumour model.^{26,27,37–40} We next evaluated possible roles of $CD8^+$ T cells in the DC-mediated protection. As shown in Fig. 7, following immunization with DC pulsed with E7+ODN we depleted $CD8^+$ T cells *in vivo* and then tested effects of $CD8^+$ T cell populations

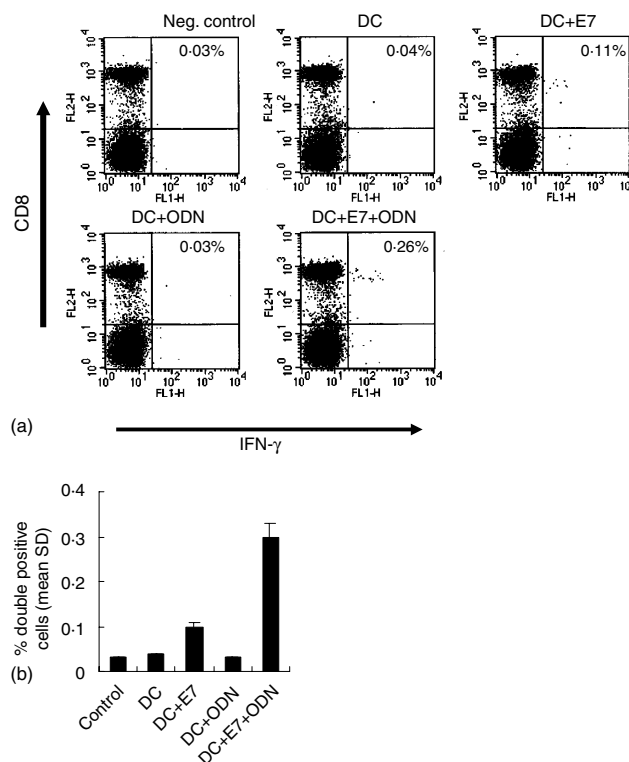


Figure 6. Intracellular IFN- γ staining of splenocytes after stimulation *in vitro* with E7 CTL epitopes. Animals were immunized as shown in Fig. 4. Three weeks after the last injection, mice were killed and spleen cells were pooled. Splenocytes were stimulated *in vitro* with 1 μ g/ml E7 CTL epitopes for 5–6 h and then tested for FACS analysis. (a) Representative figure of the FACS data in three experiments performed. (b) Bar graphs depicting the percent of double positive (CD8⁺/IFN- γ ⁺) cells in total cells tested (mean $\pm$ SD).

on tumour protection. When immunized animals were challenged with tumour cells, complete protection from tumour formation was observed in the absence of immune cell depletion as we observed in Fig. 3(a). However, animals depleted of CD8⁺ T cells showed a bit delayed but complete formation of tumour, as compared to a negative control group, suggesting a critical role of CD8⁺ T cells in protection against tumour formation. Thus, these data

support that DC pulsed with antigen plus ODN can induce protection from tumour growth through effects on CD8⁺ T cells *in vivo*.

DISCUSSION

Application of cytokine or chemokine gene-modified DC for peptide delivery has been shown to augment antitumour immunity.^{41–43} In this report, we demonstrated that ODN treatment in pulsing DC with E7 antigens results in a significant enhancement of antitumour immunity in a HPV 16 E6/E7-associated cervical cancer animal model. This is compatible with our previous observation that both E7 and ODN are required for induction of prophylactic and therapeutic vaccine efficacies.²⁶ In this case, however, vaccination with DC pulsed with E7 alone also showed antitumour immunity, suggesting that direct pulsing of DC with E7 antigens can be responsible for induction of antitumour immunity *in vivo*. Moreover, this is consistent with the previous finding that immunization with DC pulsed with E7 proteins induces CTL-mediated protection against HPV 16-associated tumour cells.⁷ More recently, Hartmann's group reported using a colon cancer animal model that therapeutic efficacy of DC against cancer cell growth is enhanced by stimulating antigen-loaded DC with ODN.⁴⁴ In view of significant enhancement in protection

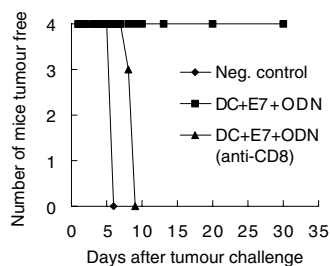


Figure 7. Effects of CD8⁺ T cells on tumour growth. Each group of mice ($n=4$) was immunized i.p. with 5×10^5 DC previously pulsed with E7 plus ODN at 0 and 2 weeks. Three weeks after the second injection, mice were depleted *in vivo* of CD8⁺ T cells, as described in Materials and Methods. Animals were challenged s.c. with 5×10^4 TC-1 cells and then the number of animals showing tumour growth was counted over time.

against tumour cell growth by injection of DC pulsed with antigen plus ODN, it is likely that application of ODN upon pulsing DC with antigens is of significance for augmentation of antitumour immunity in DC-based tumour immune therapy. When manipulating DC, ODN treatment appears to be far easier and less costly than any other methods. Furthermore, ODN is thought to be effective when DC are stimulated along with different types of antigens, such as peptides, tumour cells and tumour cell lysates. However, these should be further confirmed. Our observation also highlights an important *in vivo* role of DC for displaying antitumour immunity driven by injection of both E7 and ODN into animals.

In our observation, there was an enhancement of IFN- γ releasing CD4⁺ T-cell responses and more significantly IFN- γ releasing CD8⁺ T-cell responses (CTL marker) when vaccinated with DC pulsed with E7 plus ODN, as compared to DC pulsed with E7 or ODN alone. This is compatible with our previous observation.²⁶ In agreement with our observation, CTL is generated by using DC transfected with recombinant adenovirus expressing E7 or by priming DC with E6/E7 fusion proteins.^{8,12} It appears that ODN stimulation makes DC switch their antigen processing pathway more toward the MHC class I type than the class II type, resulting in a dramatic induction of CTL responses. It has also been reported that cytokines regulate DC to induce Th1 versus Th2 type cellular responses.⁴⁵ It can be speculated that a Th1 prototype cytokine, IL-12 generated from DC might be involved in priming DC for induction of antigen-specific Th1 type cellular responses. Toll-like receptor (TLR) 9 has been reported to be a specific receptor for unmethylated ODN.⁴⁶ Interaction of ODN with TLR 9 activates APC including DC through the Toll/interleukin (IL) receptor signalling pathway, leading to secretion of cytokines, such as IL-1, IL-6, tumour necrosis factor- α and IL-12 as well as up-regulation of costimulatory molecules.^{14,19,46} We also observed that *in vitro* stimulation of DC with ODN resulted in the secretion of more significant amounts of IL-12, as compared to background levels of DC alone. This pattern of DC activation upon ODN stimulation has been previously reported.^{15,47} Moreover, a small amount of IL-12 production was also detected upon stimulating DC with E7 proteins, suggesting a possible role of E7 proteins for stimulating DC. However, this is unlikely as E7 proteins are known to play a role in inhibition of cytokine production in immune cells.⁴⁸ The E7 effects might be ascribed to undetectable amounts of lipopolysaccharides (less than 100 EU/mg) in E7 proteins we previously prepared.²⁶ In our previous studies, however, we failed to see any endotoxin effects of the E7 preparation in animals.²⁷ This issue, however, should be further investigated.

DC transfected with recombinant IL-12 adenoviruses were reported to augment antitumour immunity through induction of tumour-specific Th1 and CTL responses in an animal tumour model.⁴⁹ However, we observed little enhancement of antibody and T helper cell proliferative responses when DC were treated with E7 plus ODN. This is in contrast to our previous *in vivo* results that injection of

Table 1. Immune responses and protection against tumour challenge

	Antibody responses	Th	CD4 ⁺ T (Th1)	CTL (CD8)	Antitumour effects
DC	–	–	–	–	–
DC + E7	+	+	+	+	+
DC + ODN	–	–	–	–	–
DC + E7 + ODN	+	+	++	+++	+++

Th, T helper proliferative responses; CTL, cytotoxic T lymphocyte; More + represents stronger responses; – represents no responses.

both E7 and ODN results in a significant induction of E7-specific antibody and T helper cell proliferative responses.²⁶ This might be caused by a difference between *in vitro* and *in vivo* stimulation conditions of E7 and ODN. For example, only DC but not other APC (B cells and macrophage) were stimulated *in vitro* by E7 and ODN in this case. Previously, our group and others reported using DNA vaccine models that antibody induction is rather inhibited in an IL-12 dose-dependent manner.^{31,50} This might be one possible explanation for lack of enhancement in both E7-specific antibody and Th cell proliferative responses after pulsing DC with E7 in the presence of ODN. In previous Literatures and ours, CD8⁺ T cells play a major critical role in protective immunity in a HPV 16 E7 tumour challenge model.^{26,27,37–40} We also confirmed this in DC-mediated protection against E7-associated tumour challenge. When animals were depleted of CD8⁺ T cells, tumour growth was observed in all animals injected with DC pulsed with E7 + ODN, suggesting that CD8⁺ T cells are a major effector T-cell population.

In conclusion, we observe that injection with DC pulsed with E7 in the presence of ODN could be a powerful approach to induce protective immunity against HPV-associated tumour cells through augmentation of antigen-specific CTL responses. Moreover, antitumour protective correlate appeared to be IFN- γ releasing CD8⁺ T cells (CTL) (Table 1). Taken together, this study highlights an importance of DC in application of E7 and ODN for protective immunity against HPV 16 E7-associated tumour challenge.

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