TLR agonists activate HPV11 E7-pulsed DCs to promote a specific T cell response in a murine model

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ABSTRACT: Some TLR agonists may up-regulate the activation of dendritic cells caused by viral antigenic peptides and antigen-specific cytotoxic T lymphocytes, which are crucial in HPV vaccine development. We investigated the ability of three TLR agonists, imiquimod, PIC and CpG, to stimulate the maturation of murine BM-DCs loaded with HPV11E7 CTL epitopes, and the subsequent effect on HPV-specific T cell responses and tumour protection in a C57BL/6 mouse model. We found that TLR agonists, mostly PIC and imiquimod, stimulated the maturation of BM-DCs pulsed with HPV11E7 CTL epitope peptide. In combination with the epitope peptide, the TLR agonists CPG and PIC augmented epitope-specific Th1 cytokine production in vivo, while imiquimod and CPG, but not PIC, enhanced Th1 cytokine production in vitro. However, we failed to observe in vivo CTL cytotoxicity and anti-tumour protection upon TLR ligation in our mouse model. Our results demonstrate that TLR agonists activate HPV11E7 CTL epitope pulsed BM-DCs to promote specific Th1 immunity in C57BL/6 mouse model, indicating the promise of TLR agonists as adjuvants for HPV epitope/DC-based multifaceted vaccines against HPV infections such as condyloma acuminiatum.

Keywords: HPV – human papillomavirus; TLR – toll-like receptor; DC – dendritic cell

Human papillomavirus (HPV) infection is one of the frequently occurring sexually transmitted diseases. The most easily recognised symptoms are genital warts, also known as condyloma acuminiatum (CA). Persistent infection with certain HPV subtypes and impaired local cellular immunity are responsible for the high recurrence of CA and development of cervical cancer (Frisch et al., 1997). It has been shown that specific T cell responses contribute to the attenuation of infection caused by the high-risk HPV types 16 and 18 in cervical cancer (Yoon et al., 1998; Scholten et al., 2005). However, less has been elucidated regarding the low-risk HPV types 6 and 11, which account for the majority of CA. Effective antigen-specific cell responses, represented by cytotoxic T lymphocyte (CTL) activity, is considered to be critical to the clearance of virus infection (Stanley, 2008). Indeed, it has been proven that antigen-based HPV vaccines are able to generate specific cellular immunity against HPV-infected cells and subsequently eliminate pre-existing lesions and malignant neoplasms (Hung et al., 2008). Obviously, the key point of such vaccine strategies is not only the identification of an effective epitope-based peptide, but also the proper processing and presenting of the epitope which can subsequently enhance T cell activation (Zhao et al., 2006).

In our previous work, we have demonstrated that HPV11E7 7–15 (TLKDIVLDDL) is an HLA-A*0201-restricted CTL epitope of HPV type 11 by deter-
mining its ability to induce maturation of human monocyte-derived dendritic cells (mdDCs) and activate epitope-specific CTLs (Xu et al., 2008). As is already known, the maturation and activation of DCs are required for the induction of an effective CTL response (Figdor et al., 2004). As the most important antigen-presenting cells (APCs), DCs detect evolutionarily conserved molecular structures through their important surface receptors, the toll-like receptors (TLRs) (Figdor et al., 2004). TLR ligation with a microbial component is crucial in DC maturation and activation, and therefore significant for the pathogen-specific T cell immune response (Bowie and Haga, 2005). DCs pulsed with HPV E7 protein induced specific T cell responses in high-risk HPV-related cervical cancer, and finally led to slow tumour progression. In this respect, the delivery of DCs pretreated with epitope-based peptides may be a promising alternative for a therapeutic vaccine (Santin et al., 2006). It is also conceivable that a HPV11E7 epitope-pulsed DC vaccine may serve as an approach to control low-risk HPV-related CA. On this basis, the addition of effective adjuvants will further boost the vaccination. A recent report revealed that the antigen-presenting efficiency of DCs is dependent on the presence of TLR ligands (Blander and Medzhitov, 2006), underlining the precise regulation of the antigen presentation and immune activating ability of DCs through TLRs and their ligands/agonists. Moreover, it has been demonstrated that some TLR agonists may apparently up-regulate the differentiation and maturation of DCs caused by viral antigenic peptides, giving rise to a stronger antigen-specific T-cell response (Bowie and Haga, 2005; Chen et al., 2010). Thus, the employment of TLR agonists as putative adjuvants for the HPV epitope vaccine regimen has been the subject of increasing interest.

We have found previously that HPV-specific T cell responses were up-regulated in vitro by activating human mDCs with co-stimulation of HPV11E7 CTL epitope peptides and TLR agonists (Chen et al., 2010). However, the immunological profile of the antigenic epitopes in vivo has not yet been completely characterised. In this study, we investigated the ability of three TLR agonists to stimulate the maturation of murine bone marrow-derived dendritic cells (BM-DCs) loaded with HPV11E7 CTL epitopes, and the subsequent effects on HPV-specific T cell response and tumour protection in a C57BL/6 mouse model.

**MATERIAL AND METHODS**

**Cell line and mice**

Murine B16 tumour cells expressing HPV11E7 were established by the Biomedical Research Center of Sir Run Run Shaw Hospital (Zhao et al., 2006), and used as target cells. The cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO₂. Female C57BL/6 mice, 6–8 weeks of age, were purchased from The Animal Laboratory of Chinese Academy of Sciences (Shanghai) and housed at the Central Animal Facility of Zhejiang University. All mice studies were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the institutional guidelines.

**Epitope peptides and TLR agonists**

Immunodominant HLA-A*0201-restricted 9mer-peptide (7–15, TLKDIVLDL) screened previously (Xu et al., 2008), and 17mer-peptide (7–23, TLKDIVLDL QPPDPVGL) of HPV11E7 CTL epitope were synthesised (Invitrogen). The TLR agonists used were: imiquimod (TLR7 agonist, Mingxin Pharmaceutical Ltd. 0.5 mg/ml), polyinosinic acid: polycytidylic acid (PIC, TLR3 agonist, InvivoGen, CA) and CpG oligonucleotide 1826 (CpG, 5’-TCCATCACGTTCCTGACGTT-3’, TLR9 agonist, Invitrogen, USA).

**Generating and pulsing BM-DCs**

Murine BM-DCs were generated as described previously (Inaba et al., 1992). Briefly, bone marrow suspensions were prepared from the femora and tibiae of normal C57BL/6 mice, were subsequently depleted of erythrocytes with ammonium chloride (17mM Tris/144mM NH₄Cl/pH 7.2), and cultured in RPMI 1640 medium with 10% FBS, penicillin/streptomycin, 10 ng/ml GM-CSF(PeproTech, USA), and 1 ng/ml IL-4 (PeproTech, USA). On day 3, the non-adherent cells were gently removed, and the remaining adherent cells were subsequently induced to differentiate into DCs. On day 5, the DCs were co-cultured for two hours with HPV11E7 CTL epitope peptides (20 µg/ml) and for an additional 48 hours with imiquimod (2 µg/ml), PIC
and CpG (20 µg/ml), respectively. As a control, unpulsed DCs were also cultured for 48 hours. All the TLR agonists were used at a concentration that induced significant DC phenotypic differentiation without cell death in initial experiments (data not shown).

**Phenotyping of BM-DCs**

To characterise the populations of CD11c-positive DCs, cells were collected on day 5 and incubated with FITC-labeled CD11c mAB (eBioscience, USA) for 30 minutes at 4 °C. After being washed three times, the cells were resuspended and analysed on an EPICS-XL flow cytometer (Beckman Coulter, USA). To evaluate the maturation of DCs, cells (10^5/100 µl) were collected 48 hours after being pulsed with the indicated combination of peptide and TLR agonists, and stained with FITC-labeled CD80, CD40, phycoerythrin-labeled CD86 (eBioscience), or PE-labeled I-A<sup>k</sup> (Invitrogen, USA).

**Vaccination of mice**

C57BL/6 mice were vaccinated intraperitoneally (i.p.) once a week (a total of three times) with 3 × 10^5/200 µl DCs per mouse, which were pulsed with different combinations of peptides and TLR agonists. Mice vaccinated with unpulsed DCs were employed as controls. Each group consisted of six mice.

**Anti-tumour experiments in vivo**

To evaluate anti-tumour effects, one day after the third vaccination, 1 × 10^5/200 µl/mouse of B16 tumour cells expressing HPV11E7 were injected subcutaneously into the left flank of the C57BL/six mice. Tumour growth was monitored by measuring the diameter of the tumour every two to three days, tumour volumes were calculated according to the formula: \( V = \text{length} \times \text{width}^2 \times \pi/6 \) (Menon et al., 2003).

**ELISA for T cell cytokine release**

For *in vivo* cytokine release experiments, mice were sacrificed 19 days after being challenged with B16 tumour cells. Splenocytes from each group were isolated and plated into six-well plates at 7 × 10^6 cells/ml in RPMI 1640 medium containing 10% FBS and 20 IU/ml murine IL-2, and restimulated with the indicated HPV11E7 epitope peptides (20 µg/ml) at 37 °C for 48 hours. For *in vitro* experiments, DCs were pulsed with the peptides combined with various TLR agonists, and mixed subsequently with fresh splenocytes from untreated six-week old mice (2.1 × 10^7 cells/3 ml/well) at an effector-to-target ratio of 1 : 17 for 48 hours. The culture supernatants were collected and the amount of IFN-γ and TNF-α was separately measured using an ELISA kit according to the manufacturer’s instructions (BioSource, USA).

**Specific CTL assay**

Mouse splenocytes (7 × 10^6/ml) from each group were restimulated with HPV11E7 epitopes at 37 °C for five days to obtain effector T cells. Fifty microlitres of effector and target cells (HPV11E7-B16 cells, approximately 4 × 10^3 cells/50 µl/well) were assigned to 96-well-plates at different effector-to-target ratios (200 : 1, 100 : 1, 50 : 1 and 25 : 1) and incubated at 37 °C for six hours. Supernatants were harvested and cytotoxicity was determined by CytoTox96 Nonradioactive Cytotoxicity Assay (Promega). The percentage of specific lysis was calculated as \([\text{[(experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous)]} \times 100\).}

**Statistical analysis**

All data were expressed as mean ± standard deviation (SD). One-way ANOVA was used to evaluate the significance of group differences. Values of \( P < 0.05 \) were considered to be statistically significant.

**RESULTS**

**TLR agonists stimulate phenotypic maturation of BM-DCs**

To evaluate the maturation of DCs after stimulation with HPV11E7 CTL epitope peptide and TLR agonists, the morphological features and biological behaviour of mouse BM-DCs were observed under an inverted microscope. The DC marker CD11c was measured to reveal the purity of DCs, and the maturation markers CD80, CD40, CD86 and MHC II (I-A<sup>k</sup>) were detected as well by flow cytometry. After
isolation and culture for five days, the BM-DCs had become confluent in typical colonies. After co-culture for an additional two days with different combinations of HPV11 E7 epitope and TLR agonists, the DCs showed maturation signs of suspended growth, increased synapses and typical dendritic processes. The positive rate of CD11c expression on DCs before and after stimulation was 63.5 ± 1.76% and 80.33 ± 1.34%, respectively. When co-stimulated with the TLR agonist imiquimod, PIC or CpG, the CD40, CD80, CD86 and MHC II expressions on BM-DCs were all increased as compared with peptide or medium alone (Figure 1). Imiquimod or PIC together with peptide induced a significant up-regulation of CD40 and CD86 expression by various TLR agonists to a lesser extent than CD40 and CD86 ($P > 0.05$, Figure 1).

**TLR agonists enhance in vivo Th1 cytokine release by T cells stimulated with BM-DCs pulsing HPV11E7 CTL epitopes**

To study the effect of TLR agonists on HPV11E7-specific T cell cytokine production induced by activated DCs in vivo, mice were vaccinated three times with BM-DCs loading various HPV11E7 peptide-TRL agonist cocktails followed with tumour challenge. Five weeks after the first vaccination, mouse splenocytes were isolated and re-stimulated with the associated peptide. Cytokine production was measured by ELISA. As shown in Figure 2, T cells secreted higher levels of TNF-α and IFN-γ than BM-DCs loaded with the peptide alone or unloaded BM-DCs. Of the different cytokines produced at

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**Figure 1.** BM-DCs differentiate in response to TLR ligation. The expression of maturation markers on BM-DCs after 48 h exposure to the indicated combinations of HPV11 E7 epitope peptide and TLR agonists was measured by flow cytometry. All data presented are mean ± S.D. values of three separate experiments.

△Indicates statistical significance ($P < 0.05$) when compared with the untreated control group.

▲Indicates statistical significance ($P < 0.05$) when compared with the peptide alone group.

1 – E7+Imiquimod; 2 – E7+PIC; 3 – E7+CpG; 4 – E7; 5 – Ctrl.
the coexistence of the 9mer-peptide in vivo, similar patterns were noted with various intensities of differences among each group. The CpG and PIC but not imiquimod group showed a striking elevation in TNF-α secretion of the epitope-specific T cell compared to the untreated control group (P < 0.05). TNF-α secretion was also increased when the DCs were pulsed with the 9mer-peptide combined with PIC or CpG (especially the latter) compared to the group which received peptide alone, although this difference was not statistically significant (P > 0.05, Figure 2a). With respect to IFN-γ secretion, the CpG, PIC and 9mer-peptide, but not the imiquimod group had significant effects compared to the untreated control group (P < 0.05); moreover, the IFN-γ enhancement induced by the CpG-peptide combination was also significant when compared to peptide alone (P < 0.05), but not compared to peptide alone (Figure 2c). No significant differences were observed among any of the other groups.

**TLR agonists promote in vitro Th1 cytokine release by T-cells stimulated with BM-DCs pulsing HPV11E7 CTL epitopes**

To further investigate the effects of TLR agonists on HPV11E7-specific T-cell cytokine release induced by activated DCs in vitro, mouse BM-DCs pulsed with different combinations of HPV11E7 CTL epitopes and TLR agonists were mixed with fresh splenocytes from untreated mice, and the amounts of TNF-α and IFN-γ in the culture supernatants were measured separately by ELISA. As shown in Figure 3, the cytokine release in vitro was also elevated in a pattern different from that of the in vivo experiments. Moreover, differences between the modes of cytokine release elicited by a settled stimulation format were likewise noted. In combination with the 9mer-peptide, Imiquimod elicited remark-

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**Figure 2.** T cell cytokine production induced by BM-DCs pulsed with TLR agonists and two HPV11 E7 peptides in vivo. Mice were vaccinated with BM-DCs pulsed with the indicated HPV11 E7 epitope peptides combined with various TLR agonists, and challenged subsequently with B16 tumour cells. The splenic T cells were isolated and re-stimulated with the associated peptide. Cytokine levels were measured by ELISA. (a) TNF-α/9mer-peptide/n = 8; (b) IFN-γ/9mer-peptide/n = 8; (c) TNF-α/17mer-peptide/n = 4. Data were expressed as mean ± SD. △ Indicates statistical significance (P < 0.05) when compared with the untreated control group. ▲ Indicates statistical significance (P < 0.05) when compared with the peptide alone group. 1 – E7+Imiquimod; 2 – E7+PIC; 3 – E7+CpG; 4; – E7; 5 – Ctrl.
able TNF-α secretion \( (P < 0.05, \text{Figure 3a}) \) whereas Imiquimod and CpG induced an obvious elevation in IFN-γ \( (P < 0.05, \text{Figure 3b}) \) as compared to either the untreated or the peptide alone group. No significant differences were observed among any of the other groups. In combination with the 17mer-peptide, Imiquimod and CpG, especially the former, elicited significant TNF-α release \( (P < 0.05) \) compared to either the untreated or the peptide alone group (Figure 3c), while again Imiquimod and CpG, but especially the latter, stimulated much higher IFN-γ secretion \( (P < 0.05) \) than either the untreated or the peptide alone group (Figure 3d). No significant differences were observed among any of the other groups.

Despite the slight differences described above, the 9mer-peptide and 17mer-peptide induced parallel tendencies of cytokine release \textit{in vitro} and \textit{in vivo} (Figure 2 and 3).

**CTL response upon co-stimulation of HPV11E7 CTL epitope peptides and TLR agonists**

To determine the cytotoxicity of the epitope-specific T cells towards the E7-expressing cells in the C57BL/6 mouse model, mouse BM-DCs were isolated and pulsed with either of the two peptides followed by different TLR agonists. After contact with the pre-treated DCs \textit{in vivo} or \textit{in vitro}, the mice splenic T cells were mixed with the HPV11E7-expressing B16 cells at multiple ratios, and a CTL assay was performed to determine the CTL response. The positive expression of HPV11E7 mRNA on B16 cells was confirmed by RT-PCR (data not shown) before initiation of the experiment. In contrast with our previous observations \textit{in vitro} using human mdDCs, the HPV11E7-specific CTL response induced by TLR agonist-pretreated BM-DCs in these groups of mice failed to show significant differences among any of the groups. Nor was an obvious phenomenon of E7-expressing target cell lysis noted.

**Delineating the tumour growth curve in the mouse model**

To evaluate the anti-tumour activity induced by TLR agonist-pulsed DCs, C57BL/6 mice were vaccinated 3 times with the BM-DCs pretreated with the indicated combinations of the 9mer-peptide and TLR agonists, and challenged subcutaneously with
$1 \times 10^5$ HPV11E7-B16 cells. Tumour growth was monitored and volumes were recorded on days 14, 16, 19 after inoculation, when the tumours became visible. As shown in Figure 4, the tumour volumes increased with time in an exponential manner in all the groups. In line with the clinical antiviral activity of imiquimod, the curve also showed a reduction in tumour growth rate in an order from the control group to the E7, E7 + CpG, E7 + PIC, and E7 + imiquimod group, in turn. However, the differences were not considered to be statistical significance.

**DISCUSSION**

TLRs are a family of at least 10 members, which are diversely expressed in different tissues and cell types including DCs (Blander and Medzhitov, 2006). Given that TLR 3, 7, 8, and 9 are mostly involved in anti-viral immunity the immunological properties of their agonists, PIC, CpG and imiquimod were studied in the current set of experiments. A majority of TLR ligands are able to stimulate the maturation of DCs and subsequent activation of T cells (Blander and Medzhitov, 2006; Fajardo-Moser et al., 2008). Our results show that the maturation of BM-DCs from C57BL/6 mice can be augmented by co-stimulation with HPV11E7 CTL epitope and TLR agonists. In agreement with our previous work on human mdDCs (Chen et al., 2010), and work on murine BM-DCs from others (Sharma et al., 2008), PIC and imiquimod were the most effective stimuli for BM-DC maturation in our mouse model.

We subsequently demonstrate that a combination of HPV11E7 CTL epitope peptides and certain TLR agonists, mostly CpG and PIC, can augment Th1 cytokine production to different extents in C57BL/6 mice *in vivo*. Specifically, the release of TNF-α by activated T cells was dependent on the coexistence of specific antigen and certain TLR agonists, while the release of IFN-γ was not. In other words, T cells were more susceptible to IFN-γ than TNF-α secretion by BM-DCs loaded with antigen alone or combined with the TLR agonist CpG. Therefore, IFN-γ is considered to be the predominant Th1 cytokine up-regulated by the TLR9 agonist CpG-activated BM-DCs. We could also infer that PIC orients T cell functions mostly towards TNF-α production whereas CpG favours both TNF-α and IFN-γ. Imiquimod, despite its excellent anti-HPV efficacy in the clinic, failed to exert clear effects on TNF-α and IFN-γ secretion in these groups of mice. Our *in vivo* results agree in most aspects with prior publications reporting the cytokine-stimulating effects of CpG and PIC, but not imiquimod (Sharma et al., 2008), and a preferential Th1 cytokine profile associated with CpG in mice models (Dearman et al., 2009). These findings uncover a corner of the distinct profile and mechanism of different immune pathways in a whole network, and highlight the ability of TLR agonists such as CpG and PIC to elicit production of Th1 cytokines for antigen-specific T cell immunity and their potential as vaccine adjuvants.

Our data also reveal that the TLR agonists imiquimod and CpG, but not PIC, promote HPV11E7 CTL epitope-specific cytokine production in C57BL/6 mice *in vitro*. This cytokine production pattern is inconsistent with our *in vivo* results suggesting that CpG and PIC are the predominant stimuli for cytokine release, possibly due to the disparities between *in vitro* and *in vivo* environments in factors such as antigen presentation and uptake, receptor competition, cytokine milieu, and others.
For instance, a similar experiment in the C57BL/6 mouse model reported a PIC-induced reduction in uptake of soluble antigen, which is independent of TLR-mediated DC activation and T cell stimulating properties. Therefore, PIC can still serve as an effective adjuvant in vivo (Tirapu et al., 2009). Under most circumstances cytokine release in vitro requires the coexistence of CTL epitope and certain TLR agonists, with the only exception being that the 17mer-peptide independently stimulates TNF-α release. It seems as well that T cells were more easily induced towards IFN-γ production than TNF-α by CpG stimulation, but were similarly susceptible with regard to the release of both cytokines by imiquimod stimulation. The different cytokine release patterns in the settled stimulation format illuminate the special characteristics of dissimilar T cell responses. Furthermore, imiquimod and/or CPG together with the HPV11E7 epitopes have additional effects on the production of both cytokine than the peptide alone does in most cases, indicating again their function as immune adjuvants.

In general, longer peptides with more epitopes show stronger immunogenicity and capacity to induce cellular immunity. However our data on the 9mer- and 17mer-peptides in the C57BL/6 mouse model revealed similar levels of TNF-α in vivo and IFN-γ/TNF-α in vitro. Therefore, we now plan to switch to the full length peptide of HPV11 E7 including five epitopes conjugated to TLR ligands.

We summarise herein the results of T cell cytokine release induced by BM-DCs pulsed with different combinations of HPV11E7 epitopes and TLR agonists in vivo and in vitro (Table 1). Along with our previous research on human mdDCs in vitro (Chen et al., 2010), these data suggest that TLR ligation on BM-DCs can further promote E7-specific Th1 immunity with respect to TNF-α and IFN-γ production in murine models. In contrast with the in vivo results showing that cytokine release is mainly induced by CpG and PIC, in vitro cytokine release is mostly induced by imiquimod and CpG. Thus, CpG is regarded to be the most effective stimulus for the CTL epitope-specific Th1 response.

CTLs are of critical importance owing to their anti-viral and anti-tumour properties. However, the molecular mechanisms underlining CTL activation are not fully understood. We and others have provided evidence that TLR agonists can augment the HPV E7-specific T cell response including CTL cytotoxicity through activated DCs in vitro and in vivo (Welters et al., 2007; Chen et al., 2010). Further, vaccination with HPV E7-pulsed DCs can therapeutically inhibit cervical cancer expansion by activating HPV-specific T cells (Santin et al., 2006). It has also been reported that CPG and PIC, but not imiquimod and LPS, exhibit tumour-suppressing effects in a mouse model (Sharma et al., 2008). Unexpectedly, we failed to detect an additional influence on the HPV11E7 epitope-specific cytotoxic activity from TLR ligation using their agonist’s imiquimod, PIC or CpG in our C57BL/6 mouse model. Moreover, the tumour growth curve showed a descending rate, which ran: E7 alone, E7 + CpG, E7 + PIC, and E7 + imiquimod groups compared to the untreated group, in turn, which is in line with the clinical antiviral activity of imiquimod. The differences in tumour growth rates among each group, although very slight, might imply putative tumour-inhibiting effects of TLR agonists such as imiquimod. Notwithstanding this encouraging presumption, the differences were considered ultimately to not be of any statistical significance. Similar results were observed elsewhere in HPV18 E7-expressing tumour models (Sin, 2006). This observation, together with the lack of in vivo CTL cytotoxicity upon TLR ligation, suggests that the activation of DCs and subsequent Th1 cytokine release is mainly induced by CpG and PIC, in vitro cytokine release is mostly induced by imiquimod and CpG. Thus, CpG is regarded to be the most effective stimulus for the CTL epitope-specific Th1 response.

Table 1. Summary of T cell cytokine production induced by BM-DCs co-stimulated with different HPV11 E7 CTL epitopes and TLR agonists in vivo and in vitro

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<th>In vivo</th>
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<td>TNF-α</td>
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<td>9mer-peptide</td>
<td>PIC⁵, CpG⁵</td>
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<td>17mer-peptide</td>
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⁵Indicates statistical significance (P < 0.05) when compared with the untreated group
▲Indicates statistical significance (P < 0.05) when compared with the peptide alone group
release by co-stimulation of the HPV11E7 epitope and TLR agonists is not sufficient for effective anti-tumour protection in the B16 melanoma mice model expressing HPV11E7. This might be interpreted as: (1) insufficient amounts of DCs and duration of vaccination or impaired transport of DCs to the right location to interact with the T cells in these groups of mice; (2) a precise and complicated immunity, especially with respect to the local immune suppressive environment of B16 melanoma; (3) although HPV11E7 mRNA expression on B16 cells was confirmed, it is possible that the E7 antigen was not being expressed by B16 cells in a manner that murine T cells could recognise; (4) interference from other down-regulatory mechanisms. Recently it has been published that the same TLR ligand induced different patterns of expression of cell surface molecules on BM-DCs derived from different murine models, and vice versa, murine BM-DCs show selective responses to TLR ligands with respect to general activation, with differentiated cytokine patterns suggestive of potential priming for divergent immune responses (Dearman et al., 2009). In addition, it is known that cross-talk/regulation happens between various TLRs and their signal transduction pathways (van Aubel et al., 2007; Fahey et al., 2009). Furthermore, the expression and function of certain TLRs can be down-regulated by HPV type 16 (Hasan et al., 2007). Taking these factors into account, a disadvantage of the current work lies in the absence of TLR determination on BM-DCs from C57BL/6 mice, which might influence their reactivity to the ligands. The majority of DCs undergo apoptosis and necrosis early after being injected into animals. The poor migratory capacity of in vitro-generated DC in vivo is considered to be a major drawback of the DC-based vaccines. Possible approaches to maximise the migration of DCs to the T cell areas include, for instance, preconditioning the skin to enhance the migration of DCs, RNA and DNA technology to optimise DC function, and the promising approach of in vivo targeting of the DCs (Aarntzen et al., 2008). A recent publication reported that the combination of TLR-9 adjuvantation by CpG motifs and electroporation (EP)-mediated delivery leads to enhanced cellular immune responses and strong regression of C3 tumors in C57BL/6 mice after vaccination with HPV-16 E7-encoding DNA, suggesting that an optimised application of CpG-enriched DNA vaccines may be an attractive strategy for the treatment of cancer (Ohlschlager et al., 2011).

In conclusion, our data demonstrate that TLR ligation on BM-DCs promotes HPV11E7-specific Th1 immunity in a murine model. The TLR9 agonist CpG is the most effective stimulus for the CTL epitope-specific response in this model, indicating its promising potential as a vaccine adjuvant for T cell immunity against HPV infection. Therefore, the function of TLR9 and its ligands in HPV immunity deserves to be further investigated. The current study will facilitate the development of peptide/DC-based multifaceted vaccines and TLR-based adjuvants for therapeutic strategies against HPV infections such as CA.

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