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The combination of TLR-9 adjuvantation and electroporation-mediated delivery enhances *in vivo* antitumor responses after vaccination with HPV-16 E7 encoding DNA

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Therapeutic DNA vaccination is an attractive adjuvant option to conventional methods in the fight against cancer, like surgery radiotherapy and chemotherapy. Despite strong antitumor effects that were observed in small animals with different antigens, DNA-based vaccines remain weakly immunogenic in large animals and primates compared to protein-based vaccines. Here, we sought to enhance the immunogenicity of a therapeutic nontransforming cervical cancer DNA vaccine (HPV-16 E7SH) by introduction of a highly optimized CpG cassette into the plasmid backbone as well as by an optimized DNA delivery using an advanced electroporation (EP) technology. By integrating the means for agent administration and EP into a single device, this technology enables a simple, one-step procedure that facilitates reproducibility. We found that highly optimized CpG motifs alone triggers an enhanced IFN-γ and granzyme B response in Elispot assays as well as stronger tumor regression. Furthermore, these effects could be dramatically enhanced when the CpG cassette containing plasmid was administered *via* the newly developed EP technology. These data suggest that an optimized application of CpG-enriched DNA vaccines may be an attractive strategy for the treatment of cancer. Collectively, these results provide a basis for the transfer of preclinical therapeutic DNA-based immunization studies into successful clinical cancer trials.

Cervical cancer (cc) is the second largest cause of cancer deaths amongst women around the world. In a global perspective, 370,000 cc cases and 200,000 cc related deaths are diagnosed each year.[1] Despite the successful prevention of cc by monitoring Pap smears in industrial countries, the impact of cc is still growing in economically disadvantaged population areas, due to inadequate access to screening. Therefore, 80% of all cc caused deaths occur in developing countries. But even under optimal treatment (mainly surgery but also chemotherapy and radiotherapy), 40% of cc patients die of the disease.[2]

Key words: gynecology, cervical cancer, virology, immunology, DNA vaccine, electroporation, immunotherapy, tumor regression
Abbreviations: cc: cervical cancer; EP: electroporation; HPV(s): Human Papillomavirus(es); HPV-16 E7SH: rearranged ("shuffled" = SH) gene of the HPV-16 E7 wildtype gene; i.m.: intramuscularly; pAPCs: professional antigen presenting cells; TLR-9: Toll-like receptor 9; WT: wildtype
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Because a persistent infection with high-risk Human Papillomaviruses (HPVs) is necessary for the development of cc, therapeutic vaccination against HPV antigens could be an attractive adjuvant option. One HPV type (HPV-16) alone is responsible for about 50% of all cc.[3] The etiological principle for the transforming activity of the high-risk HPV types has been mainly assigned to the oncoprotein E7,[4] which interferes with the cell cycle mainly through interaction with the Retinoblastom protein (pRb).[5] The exclusive and consistent expression of the E7 in cc tumor cells and in all precancerous lesions renders this antigen to an ideal target for tumor-specific immunotherapy.

Particularly, the usage of DNA vaccination has several potential advantages over vaccines based on recombinant proteins as they are relatively easy to design according to different needs, production cost are relatively low and predictable and DNA is stable. Moreover, there are no unwanted immune reactions against other components of the vaccine observed as it can be the case in vector-based vaccines. Consequently, DNA vaccines can be used for repeated boosting. Therefore, we have previously developed an artificial HPV-16 E7 gene (HPV-16 E7SH) that contains all naturally occurring epitopes, but lacks transforming properties. This gene is immunogenic in mice as well as *in vitro* in human lymphocyte cultures as measured by IFN-γ Elispot assay and Cr^{51} release assays. Moreover, the HPV-16 E7SH gene induces tumor regression of C3 tumors in C57BL/6 animals.[6]

Nevertheless, translation of promising preclinical DNA vaccine candidates into clinical trials has been hampered by

the suboptimal immunogenicity of DNA-based vaccines when administered by conventional injection in larger animals,[7] nonhuman primates[8] and humans.[9] Therefore, strategies to enhance the immunogenicity of DNA vaccines are clearly needed and different approaches have been investigated.[10–12]

One attractive approach is the usage of unmethylated CpG motifs as "danger molecules," which are recognized by the endosomal located Toll-like receptor 9 (TLR-9) resulting in the upregulation of costimulatory molecules on professional antigen presenting cells (pAPCs) and the induction of proinflammatory cytokines.[13–15] Since a number of early studies suggested that these motifs contribute to plasmid immunogenicity[16,17] and because they can be easily introduced into the plasmid backbone, they represent promising candidates for improving the immunogenicity of DNA vaccines.

Another attractive approach to enhance immunogenicity of therapeutic DNA vaccines is the electroporation (EP)-based DNA transfer. EP implies the use of electrodes to apply at least one electric pulse subsequent to the injection of the antigen encoding plasmid. EP has been shown to improve the immune response by facilitating enhanced cellular uptake of the plasmid,[18,19] followed by enhanced magnitude and duration of protein expression in muscle cells.[20] Not all EP systems tested so far are simple to apply and some cause significant tissue damage.[21] The TriGrid™ delivery system of Ichors Medical Systems (San Diego, CA) functions through electrical stimulation of the target tissue in the context of intramuscular plasmid injection. By integrating the means for agent administration and EP into a single device the TriGrid device allows the procedure to be completed in a single application step while ensuring that the EP effect is induced consistently at the site of DNA administration.

In the current study we generated a kanamycin-selectable plasmid encoding for the HPV-16 E7SH antigen and containing a highly optimized CpG motif cassette in the backbone. We analyzed the potency of this plasmid to induce cytotoxic T lymphozytes (CTLs) and tumor regression in mice.

Material and Methods

Generation of the immunization vector pPOE-CpG-E7SH

The HPV-16 E7SH gene was cloned *via* 5′HindIII and 3′XbaI into the pTHamp immunization vector.[22] The ampicillin resistance gene was exchanged by a kanamycin resistance gene: 500 ng of plasmid pTHamp-HPV-16 E7SH and 25 μM oligonucleotides of each primers 5′-AATTGAATTCCTGTCAGACCAAGTTTACTCATATATAC-3′ (pTHamp_EcoRI) and 5′-AATTGGATCCACTCTTCCTTTTTCAATATTATTG-3′ (pTHamp_BamHI) were used to amplify the vector and to introduce flanking EcoRI and BamHI sites, respectively. For the PCR amplification of the kanamycin resistance gene and the introduction of flanking EcoRI and BamHI sites, 500 ng of plasmid pET24a and 25 μM oligonucleotides of each primer (kana_BamHI_for) AATTGGATCCATGAGCCATATTCAACGG and (kana_EcoRI_rev) AATTGAATTCTTAGAAAAACTCATCGAGC were used, respectively. DNA was amplified by 5 min 94°C (1 cycle), [1 min 94°C, 30 sec 56°C, 5 min 72°C (25 cycles)], 7 min 72°C (1 cycle). The resulting plasmid pTHkan-HPV-16 E7SH was analyzed by restriction analysis using EcoRI and BamHI restriction enzymes and was finally sequenced.

The synthetic and optimized CpG cassette was assembled from synthetic oligonucleotides by GENEART (Regensburg, Germany) (5′GGGGGGGGTC**GTCGTT**CTTCTTC**GTCGTT**CTTCTTC**GTCGTT**CTTCTTC**GTCGTT**CTTCBamHITTCGACGTTCTTCTTCGACGTTCTTCTTCGACGTTCTTCTTCGACGTTCTTCTGGGGGGGG 3′, optimized CpG motifs for the murine system are in bold and underlined whereas the optimized CpG motifs for the human system are underlined only). The fragment was cloned into the plasmid pTHkan-HPV-16 E7SH *via* BclI restriction site resulting in the immunization vector pPOE-CpG-E7SH. The final construct was verified by sequencing.

DNA vaccination/EP

Female C57BL/6 mice (own breed) were kept under SPF isolation conditions and standard diet at the animal facilities of the University of Constance, Germany. TLR9$^{-/-}$ mice (BL/6 background) were purchased from animal breeds Füllinsdorf, Swiss. Agarose-gel verified plasmids (>95% supercoiled) (QIAGEN EndoFree Plasmid Kit; preparations contained less than 0.1 endotoxin units / μg plasmid DNA as tested earlier by Limulus endotoxin assay) were applied to 6- to 8-week-old female mice into each *musculus tibialis anterior* (50 μl of plasmid DNA, 1 μg/μl in PBS). Ten to twelve days after vaccination animals were sacrificed and spleens were isolated. In case of EP, the electrode array of the EP unit (rodent model, Ichor Medical Systems, www.ichorms.com) that provides a 0.3 syringe with attached [1/2]″ 30G needle (BD UltraFine, 328431) was directed into the muscle and 4 sec post-DNA injection (50 μl of plasmid DNA, 1 μg/μl in PBS), the pulse generator was activated (0.4-sec duration, comprising pulses applied at 250 V/cm peak amplitude and 10% duty cycle).

Cell lines and culture conditions

All cell lines used were of C57BL/6 origin (H2^b haplotype). RMA cells[23] and RMA-E7 (HPV-16 E7 wildtype gene) transfectants 2F11[24] were cultured in RPMI 1640 supplemented with heat-inactivated 5% (v/v) fetal calf serum (FCS, Gibco, Eggenstein, Germany), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Additionally, G418 (0.8 mg/ml) was added to RMA-E7 cultures to maintain E7 expression. C3 tumor cells derived from embryonic mouse cells transfected with the complete HPV-16 genome[25] were cultured in the same medium, supplemented with kanamycin (0.1 mg/ml).

Splenocytes were cultured in αMEM (Sigma, Deisenhofen, Germany) supplemented with 10% FCS, 0.1 mM β-mercaptoethanol, 4 mM glutamine and antibiotics as above for the first 4–5 days after splenectomy. Subsequently, the spleen

cells were cultured in αMEM+ supplemented with 2.5% supernatant of a concanavalin-A-induced rat spleen cell culture as a source of murine IL-2 and 25 mM methyl-α-mannopyranosid (Sigma).

In vitro restimulation of murine CTL lines

Spleen cells (2×10^7, pretreated with ACT lysis buffer [17 mM Tris/HCl, 160 mM NH_4Cl, pH 7.2] to deplete erythrocytes) were cocultured with 2×10^6 irradiated (100 Gy) RMA (controls) or RMA-E7 cells in 25-cm^2 culture flasks. First *in vitro* restimulation was performed at the day of the spleen isolation and was repeated weekly up to 4 times. Five days after the first *in vitro* restimulation, the spleen cell cultures were distributed into 24-well plates (every single culture was titrated over 6 wells) using 2 ml of αMEM+ medium per well. Beginning with the second *in vitro* restimulation additionally to the RMA/RMA-E7 cells (1×10^5 per well), irradiated (100 Gy) DC2.4 cells (kindly provided by Rock, K., University of Massachusetts Medical School Worcester, MA, USA) were added. Cultures were grown at 37°C and 7.5% CO_2 in a humidified incubator.

IFN-γ/granzyme B Elispot assays

Murine IFN-γ Elispot assays were performed *ex vivo* and 5 or 6 days after each *in vitro* restimulation as described earlier.[6] The granzyme B Elispot assay was performed similarly to the IFN-γ Elispot Assay. For this assay, the anti-mouse granzyme capture antibody (100 ng/well, AF1865; R&D Systems, Minneapolis, USA) and the biotinylated anti-mouse granzyme detection antibody (50 ng/well, BAF1865; R&D Systems) were used. Splenocytes were seeded in triplicates in 2-fold serial dilutions from 200,000 to 25,000 cells per well. One of the triplicates was left untreated (negative control), the second received 200 ng of pokeweed mitogen/well (Sigma) in 2 μl of PBS (positive control), whereas the third received 0.2 μmol of $H2D^b$-restricted $E7_{49\text{-}57}$ peptide in 2 μl of PBS/well (test sample). Spots of the negative control (untreated) were subtracted from the spot number in the corresponding test sample.

^{51}Cr-release assays

The ^{51}Cr-release assays were performed 5–6 days after an *in vitro* restimulation of murine spleen cells in parallel to the Elispot assays. $Na_2{}^{51}CrO_4$ (1×10^4) labeled (0.05 mCi) target cells/well (RMA or RMA-E7) were incubated together with decreasing numbers of effector cells in 200 μl per well of a 96-well round bottom plate (Costar, Corning, USA) for 4 hr. Subsequently, 50 μl of supernatant was harvested from each well and the released radioactivity was measured in a Microbeta counter (Wallac, Turku, Finland). Specific lysis was calculated according to the formula: percent specific lysis = [(cpm of the sample-spontaneous release) / (total release-spontaneous release)] × 100, where total release and spontaneous release are measured in counts per minute (cpm). Spontaneous chromium release was determined by using ^{51}Cr-labeled target cells without effector cells, and total chromium release was determined by adding 2% Triton X-100 to lyse the labeled target cells. An animal was scored positive when the specific lysis of a specific target (RMA-E7 cells) was at least 20% above the lysis of the control (RMA cells).

Tumor regression studies

C57BL/6 mice received 0.5×10^6 HPV-16 E7 expressing C3 (Feltkamp, 1993) cells in 100 μl of PBS subcutaneously in the right shaved flank (needles: 20G 1[1/2]″ BD Microlance 3). When small tumors were palpable in all animals (Days 6–16), the first DNA-injection (recombinant or control plasmid) was applied intramuscularly (i.m.) in both *musculus tibialis anterior*. The boost-vaccination was performed 10–14 days later. In case of EP, the procedure was as indicated above. Tumor sizes were measured with a caliper and were determined every 2–4 days until mice had to be sacrificed (tumor size of 400 mm^2 or when tumors were bleeding). Tumor sizes of the mice within a group were calculated as arithmetic means with standard error of the means. In the tumor regression experiments an individual was counted as "regressor," when the tumor area at the endpoint of each experiment was within the "0–25 mm^2" field. All operations on live animals were performed under Isoflurane anesthesia (CuraMed Pharma, Karlsruhe, Germany). The institutional review board approved the study.

Statistical analysis

Differences of means between experimental and control group were considered statistically significant when p was <0.05 by unpaired Student's t-test.

Results

The aim of this study was to enhance the cellular immune response of a therapeutic DNA vaccine directed against cc. In this study, an artificial HPV-16 E7 gene (HPV-16 E7SH) was used for which an induction of E7-wildtype specific CTLs and tumor regression were already shown in mice.[6] Since the extrapolation of DNA vaccine candidates from rodent models to larger animals and humans has typically been associated with a reduction in immunogenicity, we aimed to boost the antitumor effect of this DNA vaccine. For this purpose, we combined the introduction of a highly optimized CpG cassette into the plasmid backbone and an EP-based DNA delivery using an advanced EP system suitable to support eventual clinical evaluation of the HPV-16 E7SH gene.

Generation of a kanamycin-selectable plasmid vector containing an optimized CpG cassette

Because of the concerns associated with the use of ampicillin selected plasmid-DNA in humans, we exchanged this gene for kanamycin resistance gene that is already used in clinical trials (http://clinicaltrials.gov/). In Elispot assays (IFN-γ,

granzyme B) and tumor regression experiments, we have shown that the exchange of the antibiotic resistance gene does not affect the cytotoxic T lymphocyte response or tumor regression (data not shown).

Previous studies have demonstrated that translation of promising preclinical DNA vaccine candidates into clinical trials led to rather disappointing results due to the very modest immunogenicity of the plasmids in larger animals and humans.[7–9] The addition of adjuvants represents an important approach for improving the immunogenicity of cancer vaccines.[11] In numerous tumor vaccination approaches, CpG elements increased T-cell responses to a variety of tumor-associated antigens.[26,27] The number and composition of unmethylated CpG motifs within the plasmid backbone are shown to be critical to induce killer cells, to secrete IFN-γ[28] and to stimulate pAPCs to induce Th1-cytokines.[13–15] We have composed a CpG cassette consisting of four 5′ GTCGTT 3′ motifs and four 5′GACGTT 3′motifs for which an optimal activation in mice and in humans were shown.[29–31] We have introduced a 5′ TpC dinucleotide and a pyrimidine-rich region on the 3′end, respectively, as an immune stimulatory effect of both arrangements has been shown.[30] Moreover, we have flanked the CpG cassette by a poly G sequence because immunostimulatory effects were described that are distinct from CpG-mediated effects.[32] The CpG cassette was inserted into the backbone leading to the HPV-16 E7SH gene encoding immunization vector pPOE-CpG-E7SH.

Improvement of the cellular immune response and inhibition of the tumor growth by the introduction of a highly optimized CpG-containing cassette

We wanted to know if the CpG-enriched plasmid (pTHkan-E7SH plus optimized CpG cassette in the backbone, designated as pPOE-CpG-E7SH) induces enhanced cellular immune responses compared to its counterpart pTHkan-E7SH. For this purpose, we immunized mice ($n = 4$ per group) i.m. either with pPOE-CpG-E7SH or pTHkan-E7SH (100 μg per animal), respectively. Control animals received the respective vector devoid of the encoded antigen (pTHkan or pPOE-CpG). Again, 10–12 days after the immunization the animals were sacrificed and Elispot assays were performed for IFN-γ and granzyme B. Consistent with the previous finding, immunization with the pTHkan-E7SH construct induced an antigen-specific cellular response (24 ± 3 IFN-γ and 18 ± 4 granzyme B secreting cells per 1×10^4 splenocytes). Importantly, the magnitude of the CTL response could be significantly enhanced by administration of the CpG-enriched plasmid pPOE-CpG-E7SH (38 ± 4 IFN-γ and 46 ± 8 granzyme B secreting cells per 1×10^4 splenocytes) (p-values: 0.03 for IFN-γ and 0.02 for granzyme B). Empty vectors revealed comparable background levels (see Table 1, Supporting Information Tables 1 and 2).

In consequence, we wanted to know if the increased cytokine production observed with the CpG-enriched vector correlates with an improved therapeutic response against established tumors. Therefore, groups of 10 animals were vaccinated with the respective plasmids (pPOE-CpG-E7SH *vs.* pTHkan-E7SH) as soon as previously transplanted C3 tumors became palpable (Day 0, means of the tumor sizes: 5–8 mm^2) and a boost-immunization was given 12–15 days after the prime. It was necessary to end the regression experiment at Day 45 when the tumor size of the first animals of the control group (empty vectors pTHkan and pPOE-CpG) reached 400 mm^2 (358 ± 17 mm^2 and 329 ± 21 mm^2, respectively). At this time point, 2 complete tumor regressors (absence of palpable tumor) were found in the pPOE-CpG-E7SH (48 ± 9 mm^2) but none in the pTHkan-E7SH group (79 ± 11 mm^2) (Fig. 1, Supporting Information Fig. 1). The therapeutic effect of the CpG-enriched plasmid was significantly enhanced (p-value: 0.04). Collectively, these data suggest that a plasmid enriched by optimized CpG motifs can induce enhanced cellular immune responses as measured by IFN-γ and granzyme B Elispot assays and, more importantly, stronger tumor regression.

Table 1. Elispot responses after DNA immunization

pTHkan-E7SH *vs.* pPOE-mCpG-E7SH (Experiment I)	Secreting cells/1×10^4	
	IFN-γ	Granzyme B
pTHkan	2 ± 0.6	2 ± 0.5
pPOE-CpG	4 ± 2	5 ± 2
pTHkan-E7SH	24 ± 3	18 ± 4
pPOE-CpG-E7SH	38 ± 4	46 ± 8

Four mice per group were immunized i.m. with 100 μg empty vectors (pTHkan or pPOE-CpG) or with E7SH-encoding vectors (pTHkan-E7SH or pPOE-CpG-E7SH). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1×10^4 splenocytes ± SEM after one *in vitro* restimulation. One representative of 3 experiments performed is shown. The results of the second and third experiment are given in Supporting Information Tables 1 and 2.

Electroporation-based transfer of the E7SH gene further improves cellular immune responses and antitumor responses

In the past, EP has been shown to facilitate increased plasmid uptake through the cytoplasma membrane resulting in an enhanced immune response.[18,19] Problematically, most of the already used EP systems are not simple to apply and are not suited to generate reproducible results. Indeed, one of the main challenges for efficient EP in larger animals and humans is to consistently assure a correct match between the electric field and the injected DNA. In this study, we utilize an integrated EP system providing the "colocalization" of DNA injecting needle and electrodes that may be a more clinically relevant alternative. In this experiment, we immunized 4 mice per group in 3 independently performed settings (total: $n = 12$/group, 100 μg/plasmid/animal) with or without EP using Ichor's TriGrid EP delivery system. Because the CpG-enriched vector was superior over the pTHkan

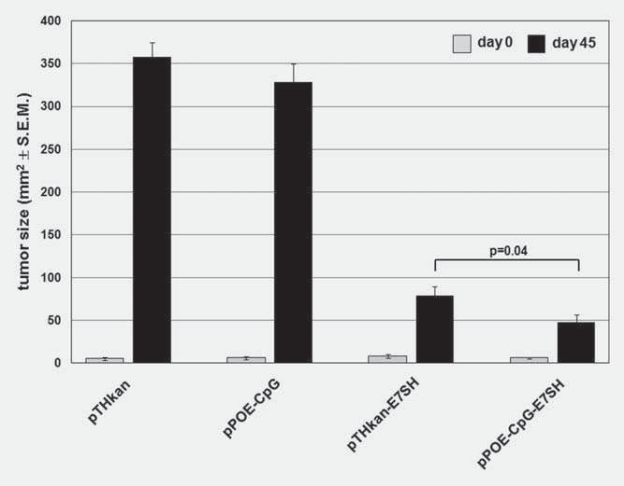

Figure 1. Growth of C3 tumors in mice after immunization with HPV-16 E7SH encoding vectors pTHkan *vs.* pPOE-CpG. Mice (n = 10/group) received tumor cells and were immunized with DNA (empty vector, E7SH encoding vector plasmids pTHkan or pPOE-CpG) when the tumors were clearly palpable and surface tumor sizes were measured over time. Data give the average tumor sizes ± S.E.M. at Day 45 when the experiment was terminated. One representative of two tumor regression experiments is shown. The results of the second experiment are given in Supporting Information Fig. 1.

Table 2. *Ex vivo* Elispot responses after DNA immunization

pPOE-mCpG-E7SH ± electroporation (Experiment I)	Secreting cells/1 × 10^4	
	IFN-γ	Granzyme B
pPOE-CpG	6 ± 2	4 ± 2
pPOE-CpG + EP	12 ± 4	9 ± 4
pPOE-CpG-E7SH	28 ± 7	26 ± 8
pPOE-CpG-E7SH + EP	281 ± 24	254 ± 18

Animals were immunized i.m. with 50 μg empty vector (pPOE-CpG) or with E7SH-encoding vector (pPOE-CpG-E7SH) in each *musculus tibialis anterior* either with or without electroporation (n = 4/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 × 10^4 splenocytes ± SEM. One representative of 3 experiments performed is shown. The results of the second and third experiment are given in Supporting Information Tables 3 and 4.

plasmid, we decided to use only the pPOE-CpG plasmid in this part of the study. Interestingly, we observed a slightly enhanced background when the empty vector pPOE-CpG was administered *via* EP. In IFN-γ Elispot assays, EP-treated animals displayed 12 ± 4 secreting cells (*vs.*-EP: 6 ± 2, p-value: 0.2) and 9 ± 4 (*vs.* 4 ± 2, p-value: 0.3) in granzyme B Elispot assays. Importantly, we found a markedly increased CTL response of pPOE-CpG-E7SH electroporated mice in comparison to nonelectroporated animals for IFN-γ secretion (-EP: 28 ± 7, +EP: 281 ± 24 IFN-γ secreting cells per 1 × 10^4 splenocytes, respectively) and for granzyme B (-EP: 26 ± 8, +EP: 254 ± 18 granzyme B secreting cells per 1 × 10^4 splenocytes, respectively, Table 2, Supporting Information Tables 3 and 4) (p-value for IFN-γ and granzyme B: < 0.0001, respectively).

To clarify if there is a correlation between IFN-γ secretion, granzyme B secretion and specific cell lysis after EP, we also performed ^{51}Cr-release assays. After one round of *in vitro* restimulation, all pPOE-CpG-E7SH-treated animals displayed an E7WT-specific lysis of RMA-E7 target cells (Fig. 2, Supporting Information Fig. 2). The strongest specific lysis of E7-expressing cells was observed in the electroporated group (78% ± 8%), whereas the same plasmid injected without EP induced a specific lysis up to 28% ± 6% (p-value: 0.002). The splenocytes of the control animals (empty vector) did not show any E7-specific lysis, demonstrating that E7-specific priming was induced *in vivo*.

Next, we wanted to know if the observed enhanced cellular immune response after vaccination with the CpG-enriched plasmid is abrogated in TLR9$^{-/-}$ mice. In 2 independently performed immunization experiments, we electroporated TLR9 knock out and wildtype animals with the antigen expressing pPOE vector (n = 4/group). The increased cellular immune response was abrogated in the knock out animals as measured in IFN-γ and granzyme B Elispot assays *ex vivo*. In the wildtype animals, we detected 247 ± 17 IFN-γ secreting cells per 1 × 10^4 splenocytes whereas only 64 ± 8 spots were counted in the TLR9$^{-/-}$ group (p-value: 0.0001). A similar observation was made in the granzyme B Elispot assay (251 ± 21 *vs.* 47 ± 10, p-value: 0.0001) (Table 3, Supporting Information Table 5). This outcome suggests that under the conditions used in this study, the CpG cassette within the pPOE backbone contributes to the immunogenicity of the pPOE-mCpG-E7SH DNA vaccine.

A comparable effect was observed when therapeutic immunizations in the C3 tumor model were performed (prime-boost, Days 0, 12–15). Although the control animals (pPOE-CpG ± EP) after 48 days developed similar tumor sizes of 244 ± 14 mm^2 (-EP) and 233 ± 21 mm^2 (+EP), pPOE-CpG-E7SH-treated mice displayed strong control of the tumor growth. One out of ten animals of the pPOE-CpG-E7SH -EP collective underwent complete regression (44 ± 18 mm^2). Interestingly, the tumor size was moderately reduced when the pTHkan-E7SH plasmid was administered by EP (88 ± 8 *vs.* 57 ± 12 mm^2, p-value: 0.05). But the therapeutic effect was dramatically enhanced when the pPOE-CpG-E7SH vector was used in combination with EP (7 complete regressors, 4 ± 3 mm^2, p-value: 0.04) (Fig. 3, Supporting Information Fig. 3). The experiment was performed twice with very similar results. From this set of experiments we conclude that EP using the Ichor's TriGrid EP delivery system leads to a significantly improved cellular immune response and a commensurate increase in antitumor response.

Together, these experiments imply that the combination of a CpG-enriched vector combined with an optimized application regime significantly enhances the CTL responses as

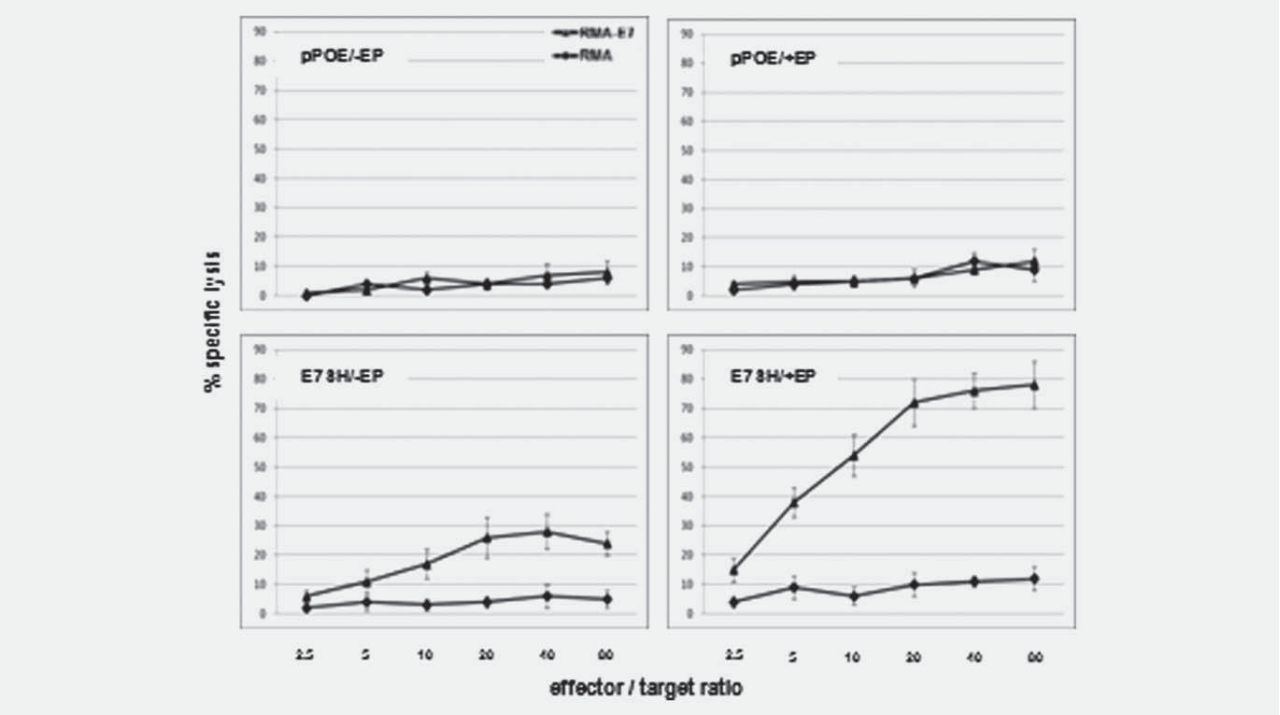

Figure 2. CTL activity against wildtype HPV-16 E7. Animals were treated with empty vector (pPOE) or with E7SH-encoding plasmid (E7SH) ± EP and splenocytes were tested by ^{51}Cr-release assays after one round of *in vitro* restimulation for lysis of syngeneic parental RMA (diamonds) or E7-wildtype expressing RMA-E7 transfectants (triangles). Data give the mean ± S.E.M. of the indicated group ($n = 4$), respectively. One representative of two experiments performed is shown. The results of the second experiment are given in Supporting Information Fig. 2.

measured *in vitro* by IFN-γ and granzyme B Elispot and ^{51}Cr-release assays and demonstrated *in vivo* in tumor regression experiments.

Discussion

In the present study, we have demonstrated to the best of our knowledge for the first time that the introduction of a highly optimized CpG cassette into the backbone of an immunization vector in combination with EP-mediated delivery improves synergistically the cellular immune responses and the tumor response of a DNA vaccine directed against HPV-16 induced cc, tremendously.

To day, a number of clinical trials based on immunization with plasmid DNA have been performed. In general, DNA-based vaccines offer important advantages compared to protein- or peptide-based vaccines, for example, the production process is much less expensive and DNA does not need a cold chain due to its stability. Moreover, there are no unwanted immune reactions against other components of the vaccine as it is observed in case of vector-based vaccines; thus DNA vaccines can be used for repeated boosting. Clinical trials have demonstrated a favorable safety profile of DNA vaccines[9,33] but, at the same time, DNA-based vaccines have demonstrated suboptimal immunogenicity, especially when extrapolated for use in large animals and primates.[7–9] Because of these circumstances, multiple approaches have been investigated with the aim of enhancing the immunogenicity of DNA-based vaccines.[10–12] The inability to reliably recapitulate the results obtained in rodent models in the clinical setting clearly demonstrates the need for improvements of the vaccine as well as the delivery technology.

One promising approach for enhancing the immunogenicity of DNA-based vaccines is the combination with adjuvants, like CpG containing DNA. Bacterial DNA contains unmethylated phosphodiester-linked cytosine and guanine (CpG) motifs capable of activating the innate and adaptive immune system that is mediated by binding to the TLR9 of pAPCs.[13–15] Because of the fact that unmethylated CpG elements are much less common in vertebrates than in bacteria, they act as a danger signal for the immune system during bacterial infections.[34] Binding of CpG motifs to TLR9 induces an activation of transcription factors resulting finally in the upregulation of the expression of cyokines and chemokines.[26,35] Because the TLR9 molecule differs remarkably between different organisms, diverse CpG/TLR9 interactions are known. For example, for the murine system optimal CpG motifs are flanked by two 5′ purines and two 3′ pyrimidines (5′GACGTT3′) whereas 5′GTCGTT3′ motifs are reported to be superior in the human system.[29–31] In the past, CpG-

Table 3. *Ex vivo* Elispot responses after electroporation in *TLR9*$^{-/-}$ mice

	Secreting cells/1 × 10^4	
WT *vs.* TLR9$^{-/-}$ (Experiment I)	**IFN-γ**	**Granzyme B**
WT/pPOE-CpG	9 ± 3	11 ± 2
WT/pPOE-mCpG-E7SH	247 ± 17	201 ± 21
TLR9$^{-/-}$/pPOE-CpG	3 ± 1	4 ± 2
TLR9$^{-/-}$/pPOE-mCpG-E7SH	64 ± 8	47 ± 10

Animals were immunized i.m. with 50 μg empty vector (pPOE-CpG) or with E7SH-encoding vector (pPOE-CpG-E7SH) in each *musculus tibialis anterior* either in wildtype (WT) or TLR9$^{-/-}$ mice ($n = 4$/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 × 10^4 splenocytes ± SEM. One representative of 2 experiments performed is shown. The results of the second experiment are given in Supporting Information Table 5.

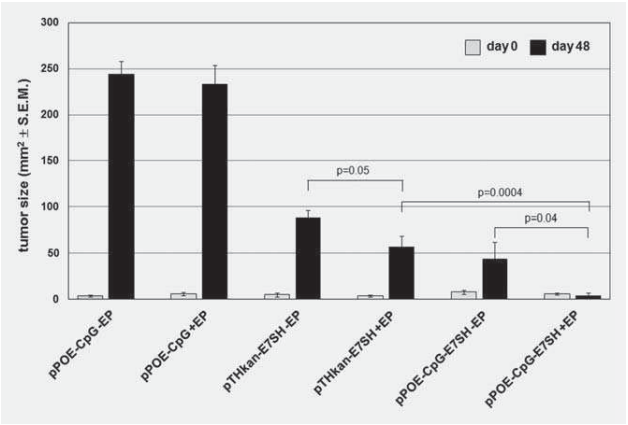

Figure 3. Growth of C3 tumors in mice after immunization with HPV-16 E7SH encoding vectors pTHkan *vs* pPOE-CpG with or without electroporation. Mice ($n = 10$/group) received tumor cells and were immunized with DNA (empty vector, E7SH encoding vector plasmids pTHkan *vs.* pPOE-CpG ± electroporation) when the tumors were clearly palpable and surface tumor sizes were measured over time. Data give the average tumor sizes ± S.E.M. at Day 48 when the experiment was terminated. One representative of two tumor regression experiments is shown. The results of the second experiment are given in Supporting Information Fig. 3.

containing DNA was widely used with success as adjuvant displaying enhanced cellular as well as humoral immune responses[26] (Klinman, 2004) and, moreover, CpG motifs were already used in clinical trials against numerous tumors (http://clinicaltrials. gov/ct2/show/NCT00254904? Term= NCT00254904&rank=1). For this study we have designed a CpG cassette consisting of 4 optimized murine and human CpG motifs, respectively. We have flanked each motif by 1 TpC dinucleotide on the 5′ end and a pyrimidine-rich region on the 3′ end due to the finding of Hartmann *et al.* of an immunostimulatory effect. Effects on the immune system were also described for poly G sequences,[32] which we have added to both flanks of the CpG cassette. Here, we were able to demonstrate improved CTL and tumor responses of the CpG-enriched plasmid in direct comparison to its naïve counterpart. Because the plasmid contains murine as well as human optimized CpG motifs, the vector could be transferred without changes into a clinical trial of phase I against cc. This is possible due to the exchange of the ampicillin against the kanamycin resistance gene in the pPOE vector. Indeed, it is not feasible to inject ampicillin-selected plasmid-DNA in humans, due to the relatively common β-lactam-antibiotic allergy (small residues of the antibiotic will remain in the preparation).[36] Moreover, β-lactam-antibiotics are commonly used in humans—after vaccination the ampicillin resistance gene could be transferred to other bacteria, resulting in the insensitivity of these bacteria against this important antibiotic group.[36]

Probably, the major hurdle for DNA vaccines is the uptake through the plasma membrane after its injection into the muscle tissue. *In vivo* EP was shown in the past to be a very potent method for increasing the immunogenicity of DNA-based vaccines by enhancing the intracellular uptake in targeted tissue regions. This effect is achieved by electrical fields, resulting in a transient increase in membrane permeability in cells of the target tissue. The improvement of the cellular and the humoral immune system has been shown mostly in smaller animals and for tumor systems,[37,38] but also in the nonhuman primate model.[39] Up to now, different EP technologies have been used—mostly with success—for *in vivo* DNA delivery during the past decade, but transfer into the clinic has been hampered by the lack of procedures suitable for widespread clinical application. To facilitate eventual clinical translation of this work, in this study we have utilized the rodent version of an EP technology, which is now in clinical testing with multiple DNA vaccine candidates (see www.clinicaltrials.gov#NCT00545987and#NCT00471133). In IFN-γ and granzyme B Elispot assays, we were able to detect about 10-fold increase in the magnitude of response following EP-based DNA delivery and in *in vitro* cytotoxicity assays we found a 2.6-fold enhancement in CTL response in electroporated mice. More importantly, in tumor regression experiments we detect a 10-fold decrease in tumor burden in EP-treated animals that was associated with a higher number of complete tumor regressors (7 animals *vs.* 1 animal). Probably, the use of EP was a substantial factor in the success of our preclinical study. In our experience, the integration of the electrodes and syringe into a single device characteristic of the TriGrid EP device provided a simple and effective method for procedure application. This device format facilitated consistent application by assuring the correct match between the electrical field and the injected DNA in a user independent fashion. In contrast to user controlled devices that may be susceptible changes in administration conditions, this approach reduces concerns that negative results are due to the inconsistency of the delivery method. In addition, the reduced time and complexity associated with an integrated,

single step EP application is likely to be more favorable for both subject and operator in the clinical setting.

Interestingly, it is believed that EP leads to a transient increase in membrane permeability resulting in an enhanced DNA uptake into the cytoplasm. On the other hand, an accumulation of CpG DNA and TLR9 in the endosomes is necessary for triggering the TLR9 pathway. In the present study, we have not investigated if EP allows an endosomal entry of plasmid DNA probably by a transient membrane permeability of this organelle. Another possibility is that only one part of the plasmid molecules is transferred by EP from the extracellular to the intracellular space by a transient increase of the cytoplasm membrane permeability. In this setting, remaining plasmid molecules could enter the cells by the "conventional" endosomal pathway and interact with TLR9. These scenarios are not unlikely, but at the same time in the last few years other intracellular DNA binding receptors ("DNA sensors") were described (for overview, see Ref. [40]). Currently, it could not be excluded that at least one of these receptors is additionally involved in a CpG associated activation pathway. This hypothesis would be supported by the finding of Spies *et al.* that TLR9 knock out mice surprisingly do respond unaltered to plasmid DNA vaccination, indicating that T cell priming is TLR9 independent.[41] But it should be mentioned that in this study a non-CpG enriched backbone was used, thus providing an only very limited and probably less effective number of CpG motifs. Here, we found contrary to this study that the CpG-enriched pPOE immunization vector induces an enhanced cellular immune response compared to its nonenriched counterpart in wildtype animals that is abrogated in TLR knock out mice. This finding suggests that under the conditions used in the present study the TLR9 is responsible for the improved immune responses. That CpG motifs act *via* TLR9 is also supported by the observation of Tudor *et al.* that TLR9-deficient mice were able to induce a cellular immune response after DNA vaccination, which is lower than in wildtype animals.[42] They concluded that TLR9 signaling enhances CpG effects on antigen-specific immune responses.

In conclusion, our findings provide a rational basis for the development of an effective DNA-based delivery approach. The combination of a highly optimized immunization vector and clinical stage EP technology used in this study may be a route to overcome DNA vaccine limitations and may have important implications for designing DNA vaccine strategies to treat cancer as well as infectious diseases. By enhancing the potency of the vaccine candidate, this combination of technologies may also improve the magnitude and consistency of response as it is extrapolated for application in larger species. Further preclinical studies could also support a reduction in the dose and frequency of administration necessary to achieve target levels of immune response.

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