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Title: Preclinical development of DNA vaccine candidates for the treatment of HPV16 induced malignancies
Issue Date: 2012-06-27
CHAPTER 3
PRECLINICAL DEVELOPMENT OF HIGHLY EFFECTIVE AND SAFE DNA VACCINES DIRECTED AGAINST HPV16 E6 AND E7
**ABSTRACT**

To allow vaccination irrespective of HLA type, DNA vaccines encoding full-length antigens are required. However, here we demonstrate that the immunogenicity of DNA vaccines encoding the full-length human papilloma virus (HPV) type 16 E7 and E6 proteins is highly reduced compared to a vaccine encoding only the immunodominant epitope. Furthermore, the low remaining immunogenicity is essentially lost for both E7 and E6 when a non-oncogenic ‘gene-shuffled’ variant is utilized. To address these issues we tested whether alterations in transgene design can restore the immunogenicity of full-length and gene-shuffled DNA vaccines. Remarkably, genetic fusion of E7 with tetanus toxin fragment C resulted in a dramatic increase in immunogenicity both for the full-length and the gene-shuffled version of E7. Moreover the TTFC fusion vaccines were more immunogenic than a vaccine encoding a fusion of E7 and mycobacterial heat shock protein-70, that has recently been tested in a clinical trial. Interestingly, vaccination with these TTFC fusion vaccines also resulted in extremely persistent T cell responses. The E7-specific CD8+ T cells induced by TTFC fusion vaccines were functional in terms of IFN-g production, formation of immunological memory, in vivo cytolytic activity and tumor eradication. Finally, we show that genetic fusion with TTFC also improves the immunogenicity of a gene-shuffled E6 DNA vaccine. These data demonstrate that genetic fusion with tetanus toxin fragment C can dramatically improve the immunogenicity of full-length and gene-shuffled DNA vaccines. The DNA fusion vaccines developed here will be evaluated for the treatment of HPV positive carcinomas in future studies.

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INTRODUCTION

Persistent infection with “high risk” HPV genotypes, is strongly associated with the development of anogenital cancers (1,2). Of the “high risk” genotypes, HPV16 alone is known to be responsible for about half of the cervical cancer cases worldwide (3). Because persistent expression of the oncogenic HPV proteins E6 and E7 is required for carcinogenesis, these viral antigens are ideal targets for immunotherapeutic interventions. Since E6 and E7 are solely expressed intracellularly, such therapeutic interventions should induce cellular immune responses in order to control existing HPV induced lesions (3,4).

DNA vaccination forms an attractive approach for the induction of cellular immune responses as the DNA encoded antigens are by definition produced intracellularly. Furthermore DNA vaccines are safe, easy to produce, stable and do not suffer from the drawback of preexisting immunity or induction of anti-vector immunity (5,6). In murine models, numerous DNA vaccines directed against either HPV16 E6 or E7 have been tested with promising results (7-13). However, to date the clinical translation of these approaches has met little success (14,15). Recently we developed a novel DNA vaccination strategy named DNA tattoo vaccination that can potentially overcome this translational block. This strategy was shown to lead to the rapid induction of cellular immunity as compared to conventional methods of DNA vaccination in mice (16). Furthermore, DNA tattooing outperformed classical intramuscular DNA vaccination by 10-100-fold when tested in non-human primates (17). Currently, DNA tattoo vaccination is being evaluated in a phase I clinical trial for the treatment of melanoma, using a DNA vaccine that was produced in house in a GMP compliant plasmid production facility (18).

In this study we describe the preclinical development of DNA vaccines directed against HPV16 E6 and E7, aiming for optimal safety and immunogenicity. In earlier work we have established that DNA vaccines that encode single defined antigen-derived T cell epitopes are highly immunogenic (19). However, the extensive polymorphism of HLA alleles precludes the broader application of such epitope-directed DNA vaccines, and we therefore set out to develop effective DNA vaccines that encompass the full epitope-encoding potential of the HPV16 E6 and E7 proteins. Furthermore, as HPV16 E6 and E7 are oncogenes through their ability to induce degradation of the tumor suppressors p53 and pRb respectively (20,21), the transforming potential of these genes needs to be eliminated before application in humans. Two strategies have previously been put forward to disrupt the oncogenic potential of E6 and E7 in DNA vaccines. Firstly, point mutations in the pRb binding site for E7 and in the p53 binding site for E6 can prevent degradation of these targets and thus prevent cellular transformation (11,22). Secondly, a more drastic approach – termed gene-shuffling – has been developed recently to prevent the risk of cellular transformation by HPV16 E7 (9,23). In this approach, the gene sequence of E7 was taken apart at exactly those positions that are critical for the known transforming properties of the protein, and the resulting fragments were reassembled in a ‘shuffled’ order. To avoid the loss of putative CD8+ T cell epitopes at the junctions, sequences encoding the 9 amino acids at either side of the different junctions in the natural protein were added as an ‘appendix’. Since the 3-dimensional structure of the resulting protein product will be markedly different from that of the parental protein, it is plausible that for thus shuffled proteins not only the binding to known cellular targets, but also interaction with other potential targets (20,21,24,25) is prevented. Consequently, gene shuffling can be considered a preferred approach from a safety perspective.

Here we demonstrate that the immunogenicity of DNA vaccines that encode the full-length HPV16 E6 or E7 proteins is highly reduced as compared to vaccines in which only the
immunodominant epitope is present. Furthermore, this low remaining immunogenicity is essentially lost when the preferred shuffled E6 and E7 vaccine formats are utilized. We subsequently demonstrate how DNA vaccines with a superior capacity for CD8+ T cell priming can be generated through the genetic fusion of either full-length or gene-shuffled HPV genes with domain 1 of Tetanus toxin fragment C (TTFC). Collectively, these experiments define the transgene formats for HPV16 E6 and E7 DNA vaccines for use in an upcoming phase I clinical trial.

MATERIALS AND METHODS

Mice
C57BL/6 mice (6-10 weeks) were obtained from the experimental animal department of The Netherlands Cancer Institute (Amsterdam, The Netherlands). All experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of The Netherlands Cancer Institute and in accordance with institutional and national guidelines.

DNA vaccines
DNA vaccines based on HPV16 E6 and E7 genes were generated by the introduction of target genes or gene fragments into pVAX 1 (Invitrogen, Carlsbad, CA, USA). The generation of GFP-E7a49-57 has been described elsewhere (26). GFP-E6a48-57 encodes the immunodominant H-2Kb–restricted epitope EVYDFAFRDL as a genetic fusion with GFP and was constructed in an analogous manner as GFP-E7a49-57 in between the BamHI and Not I sites of pVAX. E7WT, E7GGG (11), E6WT and E6GG (22) were obtained from GeneArt (Hilden, Germany), with codon optimization for expression in human cells, and were all cloned between the HindIII and XbaI sites of pVAX. The generation of E7SH has been described elsewhere (23), and E6SH was constructed in a similar fashion. In brief, E6 was cut at positions corresponding to aa 31/32, aa 64/65, aa 104/105 and aa 137/138, the resulting five segments were reassembled in the order ADCBE, and the original junctions destroyed by the dissection were added as an “appendix”. The design of E6SH is shown in Suppl. Fig. 1. For this study, both E6SH and E7SH were cloned between HindIII and XbaI sites of pVAX. The TTFC fusions, TTFC-E7WT, TTFC-E7GGG, TTFC-E7SH and TTFC-E6SH were generated by C terminal fusion of the gene of interest to Tetanus Toxin Fragment C domain 1 (TTFC) through PCR. All PCR products were cloned into the HindIII and XbaI sites of pVAX. pNGVL4a-sig/E7(detox)HSP70 (15) was a kind gift from T.C. Wu. Sequences were confirmed by sequence analysis. Plasmids were expressed and amplified in E. Coli DH5α and were purified using an endotoxin free DNA purification kit (Qiagen, Hilden, Germany). DNA vaccines for intradermal tattoo application were dissolved in water for injections (Aqua B. Braun, Melsungen, Germany).

Tattoo vaccination
Intradermal DNA tattoo vaccination was performed at day 0, 3 and 6, as described previously with minor modifications (16). One day prior to the first DNA tattoo, the hair on the hind leg was removed using depilating cream (Veet®, Reckitt Benckiser, Hull, U.K.). On the day of vaccination, mice were anesthetized and 10 µl of a 2 µg/µl DNA solution in water was applied to the hairless skin of the hind leg. The DNA vaccine was applied with a Permanent Make Up (PMU) tattoo machine (kindly provided by MT Derm GmbH, Berlin, Germany), using a sterile
Disposable 9-needle bar with a needle depth of 1 mm and oscillating at a frequency of 100 Hz for 30 seconds.

Detection of HPV-specific T cells in peripheral blood

Peripheral blood cells were obtained via tail bleeding, and erythrocytes were removed by incubation in erythrocyte lysis buffer (155mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA (pH 7.4)) on ice. The cells were subsequently stained in FACS buffer (1× PBS, 0.5% BSA and 0.02% sodium azide) with allophycocyanin (APC)-conjugated anti-CD8a mAb (BD Pharmingen, San Jose, USA) plus phycoerythrin (PE)-conjugated H-2Db E749-57 or H-2Kb E648-57 tetramers for 15 min at 20 °C. Subsequently, cells were washed two times in FACS buffer before analysis. Live cells were selected based on PI exclusion. MHC tetramers were produced by UV-induced peptide exchange, as described previously (27).

IFN-γ assays were performed using the BD Cytofix/Cytoperm kit (Becton Dickinson Sciences, Franklin Lakes, NJ, USA) according to the manufacturer’s protocol. Peripheral blood cells were stimulated for 4 h at a 1µg E749-57 peptide (RAHYNIVTF) concentration and subsequently stained using PE-conjugated anti-INF-γ mAb (BD Pharmingen, San Diego, CA, USA), and APC-conjugated anti-CD8a mAb (BD Pharmingen,). All samples were analysed on a FACScalibur (Becton Dickinson), using Flow-Jo software (Three Star, Ashland, USA) for data analysis.

In vivo cytotoxicity assay

The capacity to kill peptide loaded target cells in vivo was assayed as described previously (28). Briefly, splenocytes from naive mice were labeled with either 0.1 µM CFSE (low) or 1 µM CFSE (high). The cells labeled with 1 µM CFSE were subsequently pulsed with 10 µM RAHYNIVTF peptide for 1 h at 37°C, and the cells labeled with 0.1 µM CFSE were pulsed with a control (influenza A-derived ASNENMDAM) peptide. After peptide loading, cells were washed three times and subsequently injected into mice in a 1:1 ratio for a total of 2 × 10⁶ cells per mouse. After 20 h, spleen cells were isolated and the ratio of CFSEhigh/ CFSELow cells was determined by flow cytometry. The percentage antigen-specific cytotoxicity was determined as follows: 100 - ([% CFSEhigh in vaccinated/ % CFSELow in vaccinated] / [% CFSEhigh in naive / % CFSELow in naive]) * 100).

TC-1 tumor challenge

C57BL/6 mice were injected subcutaneously with 1×10⁵ TC-1 tumor cells that express both HPV16 E6 and E7 (29). DNA tattoo vaccination was subsequently performed on day 3, 6 and 9 after tumor challenge. Tumor growth was monitored 1-3 times per week using caliper measurements in two dimensions. The volume of the tumors was calculated as follows: volume = (width² x length)/2 (30) Mice were sacrificed when the tumor diameter reached 15 mm or when the tumor volume exceeded 1000mm³.

Statistical analysis

Statistical analysis was performed using a student’s t-test. A p-value <0.05 was considered to be significant (two-tailed). All findings were confirmed in at least one additional independent experiment.
RESULTS

Immunogenicity of HPV16 E7 and E6 DNA vaccines is highly dependent on transgene design

We first compared the immunogenicity of the immunodominant HPV16 E7\(^{49-57}\) epitope when either present within the full-length E7 gene, or as a C-terminal fusion of the single epitope with GFP (see fig. 1 A, B, C). Consistent with earlier results (16,19), \textit{ex vivo} MHC tetramer staining of peripheral blood of mice that had received a DNA vaccine encoding GFP-E7\(^{49-57}\) demonstrated that this epitope-directed vaccine was highly immunogenic (peak T cell response of 4.97\% +/- 2.15). In contrast, immune responses against the same epitope remained low in mice that were vaccinated with the E7WT encoding vaccine (0.83\% +/- 1.14, \(p<0.01\) versus GFP-E7\(^{49-57}\)). The immunogenicity of the full-length E7 was not altered by the introduction of point mutations, as immunogenicity of E7GGG was equally low (0.63\% +/- 0.66, \(p<0.01\) versus GFP-E7\(^{49-57}\)). However, the immune responses elicited by a DNA vaccine in which the same epitope was present in the ‘shuffled’ version of the E7 gene (E7SH) were not significantly different from the background responses detected in mock-vaccinated animals (\(p=0.57\)). Similar to what was observed for E7, immune responses induced by a shuffled HPV E6 DNA vaccine (E6SH) were close to background, and substantially lower than those induced by E6WT, E6GG, and in particular GFP-E6\(^{48-57}\) DNA vaccines (5.8 fold lower, \(p<0.05\)) (figure 1D). Taken together, these results demonstrate that the context in which a defined HPV16 E7 or E6 CD8\(^+\) T cell epitope is delivered strongly influences its immunogenicity. Importantly, the poor performance of the full-length or shuffled versions necessitated the optimization of the DNA vaccine design before moving to clinical evaluation.

Fusion of full length E7 with Tetanus toxin fragment C domain 1 results in a dramatic increase in CD8\(^+\) T cell responses

As CD8\(^+\) T cell responses to the gene shuffled DNA vaccines were essentially undetectable, we first focused on improving the immunogenicity of the wild-type and point-mutated versions, choosing HPV16 E7 as a model vaccine. Prior work by Stevenson et al. has demonstrated that fusion of antigenic peptides with the C-terminus of domain 1 of Tetanus toxin fragment C domain 1 (hereafter referred to as TTFC) enhances CD8\(^+\) T cell responses against these peptides (31). To evaluate whether this strategy would also be successful for the full length E7 protein, we generated DNA vaccines consisting of C-terminal fusions of E7WT and E7GGG with TTFC (Fig. 2A). Vaccination of mice with TTFC-E7WT and TTFC-E7GGG resulted in E7\(^{49-57}\) specific CD8\(^+\) T cell responses that were markedly increased relative to those induced by E7WT, with mean peak CD8\(^+\) T cell frequencies of 17.6\% +/- 9.83 and 16.6\% +/- 9.85 respectively (fig. 2B). Importantly, the responses induced by the TTFC-fusion vaccines were also markedly higher than those induced by sig/E7(detox)HSP (mean peak CD8\(^+\) T cell frequencies of 5.79\% +/- 3.15) that has recently been tested in a clinical trial (15). Surprisingly, fusion to TTFC did not only increase the peak height of the CTL response, but also caused a marked change in response kinetics. Specifically, whereas classical vaccine-induced cellular immune responses (as induced by all other vaccine formats tested) are characterized by a rapid contraction after the peak of the T cell response, tattoo vaccination with TTFC-E7 DNA vaccines induced CD8\(^+\) T cell responses that remained near constant for about 3 weeks after the peak of the CD8\(^+\) T cell response was reached (Fig. 2b). Furthermore, also in the months following vaccination, marked DNA vaccine-induced T cell responses remained detectable directly \textit{ex vivo}. 


Since the kinetics of the E7-specific CD8+ T cell responses induced by TTFC-E7 vaccines were so markedly different from classical vaccine-induced CD8+ T cell responses, we investigated the functional properties of these cells. Firstly, the ability of CD8+ T cells to produce IFN-γ upon E749-57 peptide stimulation was tested by intracellular cytokine staining of peripheral blood samples taken from DNA tattoo vaccinated mice (fig. 3). Four weeks after vaccination, the production of IFN-γ could be detected in 4.4% +/- 3.03 and 3.6% +/-1.14 of CD8+ T cells of TTFC-E7WT and TTFC-E7GGG vaccinated mice, respectively. In contrast, essentially no IFN-γ production above background could be observed in samples taken from E7WT and E7GGG vaccinated mice.

Secondly, the ability of the vaccination-induced T cell pool to respond to secondary antigen encounter was evaluated by a single homologous booster vaccination 3 months after priming (Fig. 3C). Peak secondary T cell responses were comparable in size to the primary response for both TTFC-E7WT and TTFC-E7GGG. In contrast, in E7WT and E7GGG vaccinated mice, secondary T cell responses were markedly reduced relative to the primary response. As a result, the differences between E7WT and TTFC-E7WT (7.7 fold; p< 0.05) and E7GGG and TTFC-E7GGG (12.2 fold; p< 0.001) were even more pronounced during the secondary response. As a third and final test for T cell functionality of the long-term persisting HPV E7-specific CD8+ T cell pool,
T cells, the ability to kill peptide loaded target cells was tested in an \textit{in vivo} cytolytic assay 6 weeks after priming with either TTFC-E7GGG or E7GGG (Fig. 4). In TTFC-E7GGG vaccinated mice, 59.1 $\pm$ 13.7\% specific lysis of target cells was observed compared to only 15.9 $\pm$ 4.3\% in E7GGG vaccinated mice ($p=0.0013$). Taken together, these results demonstrate that DNA tattoo vaccination of mice with TTFC-E7 fusion vaccines induces superior T cell reactivity compared to E7 vaccines as revealed by both antigen-specific T cell numbers and their function.

TTFC fusion also enhances the immunogenicity of the E7SH and E6SH DNA vaccines

Because of the perceived greater safety of shuffled HPV16 E6 and E7 genes as compared to point mutated HPV16 E6/E7 genes, we evaluated whether the immunogenicity of the very weakly immunogenic shuffled DNA vaccines could also be enhanced by TTFC fusion. As shown in figure 5A, the use of TTFC-E7SH resulted in a strong E7-specific CD8$^+$ T cell response with an 11.7 fold ($p<0.01$) difference at the peak of the response compared to E7SH. Moreover, both the magnitude and persistence of the primary and secondary CD8$^+$ T cell response induced by TTFC-E7SH was comparable to that induced by vaccination with TTFC-E7WT. Evaluation of

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\caption{TTFC fusion enhances DNA vaccine immunogenicity. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific CD8$^+$ T cells by MHC tetramer staining. \textbf{A} Schematic representation of E7 variants that were expressed as C-terminal fusions with Tetanus Toxin Fragment C domain 1 (TTFC) (31). \textbf{B} Plot depicting the mean percentage +/- S.D. of H-2D$^b$ E7$^{49-57}$-specific CD8$^+$ T cells for the indicated groups over time.}
\end{figure}
the magnitude of CD8+ T cell responses induced by TTFC-E6SH relative to those induced by E6SH (fig 5B) revealed a modest but non-significant increase in primary T cell responses (3.0 fold p=0.27). The delayed contraction of the vaccination-induced immune response, observed for TTFC-E7 fusions, was not seen for TTFC-E6SH. However, following homologous boost vaccination, E6-specific CD8+ T cell responses in TTFC-E6SH vaccinated mice were markedly higher than those in E6SH vaccinated mice (14.6 fold at the peak of the response, p< 0.01).

Figure 3. Long-term persisting E749-57-specific CD8+ T cells are functional in terms of interferon-γ production and secondary expansion. Interferon-γ production of peripheral blood CD8+ cells from indicated groups of the experiment displayed in fig. 2 was assayed 4 weeks after start of vaccination. A) Representative dot plots of IFN-γ staining of the indicated groups. B) Bar diagram showing the mean percentage +/- S.D. of interferon-γ positive CD8+ T cells after 4h stimulation with the E749-57 peptide. C) Secondary T cell responses of TTFC-E7WT or TTFC-E7GGG vaccinated mice. Mice from the experiment displayed in fig. 2 were boosted with a single homologous DNA tattoo vaccination at the indicated time point. Peripheral blood was analyzed for antigen-specific CD8+ T cells by MHC tetramer staining. The mean percentage +/- S.D. of H-2D^b E749-57-specific CD8+ T cells for the indicated groups is displayed over time.
Taken together these results demonstrate that TTFC fusion enhances the immunogenicity of both E7SH and E6SH DNA vaccines.

Comparing the anti-tumor effect of E7SH and TTFC-E7SH DNA vaccines
To evaluate whether the difference in immunogenicity resulting from the TTFC fusion translated into a measurable difference in the ability of vaccine-induced T cells to eradicate HPV16 E6/E7 transformed tumors, mice were inoculated subcutaneously with 1*10^5 TC-1 tumor cells. At day three post tumor cell injection, at which point most of the animals carry palpable tumors, tattoo vaccination with E7SH, TTFC-E7SH, or a control DNA vaccine was initiated. In the animals treated with empty vector, E7-specific T cell responses remained below the level of detection indicating that the TC-1 tumor itself is not immunogenic (Fig. 6A). Also in animals treated with the E7SH DNA vaccine, E7-specific T cell responses remained close to background, and tumor outgrowth and survival was indistinguishable between the two groups. In contrast, in animals treated with the TTFC-E7SH fusion vaccine, a robust E7-specific T cell response emerged (26.7 +/- 20.6%). This T cell response was accompanied by tumor regression and resulted in a prolonged survival for all mice (Fig. 6B, 6C). In spite of the high frequencies of E7 specific CD8^+ T cells in TTFC-E7SH vaccinated mice, tumors did eventually recur in 60% of the mice.

Figure 4. Cytolytic activity of long-term persisting E7_{49-57}-specific CD8^+ T cells. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific CD8^+ T cells by MHC tetramer staining. A) Plot depicting the percentage +/- S.D. of H-2D^b E7_{49-57}-specific CD8^+ T cells over time. The arrow indicates the time point at which mice were injected with peptide loaded target cells. B) Representative histograms displaying the ratio of target cells pulsed with specific peptide (CFSE high) or irrelevant peptide (CFSE low) 20h after injection. C) Plot depicting the percentage of specific kill of E7_{49-57}-pulsed target cells. Displayed are the individual values and the mean of each group.
indicating that the TC-1 tumors can grow out in the face of an ongoing high-level E7-specific T cell response. This immune evasion by TC-1 tumors has been described previously, and has been attributed to both mutation of the immunodominant epitope and increased expression of vascular cell adhesion molecule-1 (VCAM-1) on the tumor cells (32,33). In summary, the increased immunogenicity of the shuffled E7 DNA vaccine obtained by TTFC fusion resulted in a highly reduced tumor outgrowth and a significant increase in median survival ($p<0.002$; Log-rank test).

**DISCUSSION**

The aim of this study was to design safe and highly immunogenic DNA vaccines that encode the full-length HPV16 E6 and E7 oncogenes. E6 and E7 encoding genetic vaccines thus far used in clinical trials contain point mutations that avoid binding of the encoded proteins to p53 and pRB, respectively (7,34). However, it is well recognized that both HPV16 E6 and E7 also interact with other cellular targets than p53 and pRB, and that these interactions may play an additional

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**Figure 5. TTFC fusion enhances the immunogenicity of E7SH and E6SH.** C57Bl/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and boosted with a single homologous tattoo vaccination at the indicated time point. Peripheral blood was analyzed for antigen-specific CD8+ T cells by MHC tetramer staining. **A)** Plot depicting the mean percentage +/- S.D. of H-2D$^b$ E749-57 specific CD8+ T cells for the indicated groups is displayed over time. **B)** Plot depicting the mean percentage +/- S.D. of H-2K$^b$ E648-57 specific CD8+ T cells for the indicated groups over time.
role in cellular transformation (1,20,21,24,25,35). Because of this concern, the use of E6 and E7 vaccine formats in which the potential of E6 and E7 to interact with cellular targets is altered in a more drastic manner appears preferable. Therefore, we aimed to develop DNA vaccines that encode gene-shuffled variants of E6 and E7. Prior studies have already demonstrated the loss of oncogenic potential of the shuffled variant of E7 in in vitro assays (9,23). Likewise, shuffled E6 shows a complete lack of transforming potential (Öhlschlager et al., unpublished observations).

While shuffled E6 and E7 genes therefore appear suitable for use in genetic vaccines from a safety perspective, we here observed that the immunogenicity of E7SH and E6SH upon DNA tattoo is strongly reduced as compared to both the unmodified and the point mutated variants of E6 and E7. How can the low immunogenicity of E6SH and E7SH be explained? A possible explanation for the reduced immunogenicity of these shuffled gene products is that these proteins are misfolded and hence rapidly degraded. Prior work has demonstrated that cross presentation of MHC class I-restricted epitopes is biased towards epitopes that accumulate in the antigen-producing cell (36,37), and induction of CD8+ T cell responses upon DNA tattoo administration is known to depend at least partially on cross presentation (38).

**Figure 6. Tumor regression by vaccination with the TTFC-E7SH fusion vaccine.** C57BL/6 mice (n=5-7 per group) were injected with \(1\times10^5\) TC-1 tumor cells on day 0. Subsequently, mice were immunized by DNA tattoo vaccination on day 3, 6 and 9 after tumor challenge with the indicated vaccines. Tumor sizes were determined by caliper measurements 2-3 times weekly. Peripheral blood was analyzed for antigen-specific CD8+ T cells by MHC tetramer staining. A) Plot depicting the mean percentage +/- S.D. of H-2D\(^b\) E749-57-specific CD8+ T cells for the indicated groups over time. B) Plot depicting the mean tumor size +/- S.D. (mm\(^3\)) for the indicated groups over time. C) Plot depicting the percentage survival for the indicated groups over time.
In further support of this, we have previously demonstrated that in vivo antigen stability and immunogenicity of DNA vaccine encoded antigens that are introduced via intradermal DNA tattooing are correlated, and that the destabilization of a model vaccine via an approach very similar to gene shuffling also resulted in the loss of vaccine immunogenicity (38). As a second potential explanation, the gene shuffling procedure could conceivably disrupt CD4+ T cell epitopes present within the parental protein. However, as a deleterious effect of gene shuffling is observed for both E6 and E7, and as the regions that encompass the sites at which the parental protein is taken apart are rescued in the ‘appendix’, this explanation seems less likely. It has to be noted that the immunogenicity of (non-fused) E7SH and E7WT were comparable in a previous report (23). Potentially, differences in the route of administration (intramuscular injection versus intradermal tattoo), or number of administrations (single injection versus 3 DNA tattoo applications) could influence the immunogenicity of shuffled DNA vaccines. Regardless of this, from the current analyses it is apparent that for the planned clinical application, i.e. intradermal DNA tattoo, gene shuffling is highly detrimental to the immunogenicity of both HPV E6 and E7.

We and others have previously demonstrated that DNA vaccines can be improved by fusing genes encoding an antigen or an epitope of interest to that of a carrier protein (7,8,12,19). Here we demonstrate for both E6SH and E7SH that their immunogenicity can be fully remedied by genetic linkage to TTFC, priorly developed as a fusion partner in DNA vaccines by the group of Stevenson (6,39). In these studies, the optimal configuration for the induction of CD8+ T cell immunity consisted of a C-terminal fusion of a minimal epitope with domain I of FrC (here referred to as TTFC for simplicity) (6,31,40). Here we show for the first time that the beneficial effects of TTFC fusion do also apply to full-length gene products, thereby allowing antigen presentation via multiple HLA class I alleles. What is the mechanism by which fusion with a carrier molecule enhances DNA vaccine immunogenicity? In the case of TTFC a likely explanation is that fusion of genes of interest to TTFC promotes the induction of CD8+ T cell responses through the provision of CD4+ T cell help via one of the TTFC encoded “promiscuous” CD4+ helper epitopes (6,39). Support for this notion comes from prior work demonstrating that a carrier protein needs to be of non-self origin in order to improve DNA vaccine immunogenicity and that CD8+ T cell responses towards a carrier-epitope fusion encoding DNA vaccine are dependent on MHC-II mediated antigen presentation (19). In line with this MHC-II/-/- mice were not able to mount measurable CD8+ T cell responses after vaccination with either TTFC-E6SH or TTFC E7SH (Suppl. Fig. 3). However, it is plausible that the presence of helper T cell epitopes is not the only relevant factor, as -as discussed above- antigen stability correlates with the immunogenicity of DNA vaccines. To assess whether TTFC fusion may increase the accumulation of E7(SH) protein, we transfected HEK293 cells with either E7WT or E7SH, or with TTFC-E7WT or TTFC-E7SH and detected E7 protein expression by western blot analysis 24 hrs after transfection. As shown in Suppl. Fig. 2, E7 accumulation was substantially higher in the TTFC E7(SH) transfected cells, indicating that the stability of E7 is indeed improved by this fusion.

A remarkable observation in our study is the delayed contraction of the CD8+ T cell responses upon DNA tattoo vaccination with all TTFC-E7 fusion vaccines tested. Conventional CD8+ T cell responses rapidly contract after the peak of the response has been reached, and levels of CD8+ T cells that remain after contraction are generally around 5% of peak levels (41,42). In all our previous studies, immune responses induced by DNA tattoo showed similar kinetics, with contraction being close to complete about 1 week after the peak of the response (16,38). In contrast, upon tattooing TTFC-E7 encoding DNA vaccines, we consistently observed that contraction of the induced CD8+ T cell response was delayed for about 3 weeks, and that the
remaining frequencies after 4 weeks were still around 20% of the initial peak height (Fig. 3). At present we do not know the cause of this delayed contraction, but the data available suggest a combination of epitope-intrinsic and epitope-extrinsic factors is required. Specifically, the fact that delayed contraction is observed for TTFC-E7 but not for TTFC-E6 DNA vaccines indicates that epitope identity does play a role. On the other hand, the fact that delayed contraction is observed for TTFC-E7 but not for GFP-E7<sub>49-57</sub> DNA vaccines shows that the identity of the carrier also forms a crucial component. Clearly, understanding the molecular basis underlying both the increase in peak height and the improved persistence of CD8<sup>+</sup> T cell responses induced by these fusion vaccines, would be highly useful for future DNA vaccine development, and will be the focus of our future work.

In conclusion, we have constructed DNA vaccines targeting full length HPV16 E6 and E7 with good immunogenicity and safety profiles, by successfully combining strategies to “detoxify” and improve DNA vaccine encoded antigens. The resulting vaccine format outperformed a vaccine encoding sig/E7(detox)HSP that was recently tested in humans (15), providing a strong rationale for clinical evaluation of our vaccine format. We are currently planning to evaluate a combination of TTFC-E7SH and TTFC-E6SH encoding constructs, applied via DNA tattoo vaccination, for treatment of HPV16 positive carcinomas in a phase 1 clinical trial.

ACKNOWLEDGEMENTS

This work was supported by the Netherlands organization for health research en development (ZonMw) grant 432-00-001.

The vector pNGA4a sig/E7(detox)HSP was kindly provided by Prof. T.C. Wu, Johns Hopkins Medical Institutions, Baltimore, Maryland.

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40. Chaise C, Buchan SL, Rice J, Marquet J, Rouard H, Kuentz M et al. DNA vaccination induces


Supplementary figure 1. Schematic representation of E6SH. HPV16 E6 was taken apart at the amino acids positions indicated in the figure, thereby dissecting the C-X-X-C- motifs crucial for interaction with p53 (Crook et al, Cell, 1991). The resulting domains were reassembled in the indicated order, resulting in the E6SH core sequence. To avoid the loss of putative CD8+ T cell epitopes at the junctions, sequences encoding 12-18 amino acids at either side of the different junctions in the natural protein were added as an ‘appendix’.

Supplementary figure 2. TTFC-E6SH and TTFC-E7SH specific CD8+ T cell responses are dependent on MHC-II-restricted antigen presentation. WT C57BL/6 mice (n=5 per group) or MHC-II-/- mice (n=4 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific CD8+ T cells by MHC-I tetramer staining. A) Plot depicting the percentage +/-S.D. of H-2D\textsuperscript{b} E7\textsubscript{49-57}-specific CD8+ T cells of the TTFC-E7SH vaccinated mice over time. B) Plot depicting the percentage +/-S.D. of H-2K\textsuperscript{b} E6\textsubscript{48-57}-specific CD8+ T cells of the TTFC-E6SH vaccinated mice over time.
Supporting information figure 3. TTFC fusion results in enhanced accumulation of both E7WT and E7SH in vitro. HEK 293 cells were transfected with a mixture of 1µg GFP encoding DNA and 4 µg of DNA encoding either E7WT or E7SH, or their respective TTFC fusions. Cells were harvested 24 hours after transfection and both HPV16 E7 and GFP were detected by western blot analysis, using a mouse monoclonal antibody against E7 and a rabbit monoclonal antibody against GFP, respectively. Note that E7SH has a slightly higher MW than E7WT (11kDa) due to the addition of the appendix, and that the addition of TTFC Dom 1 (~30 kDa) results in a protein product of the expected size.