Cover Page

The handle http://hdl.handle.net/1887/19165 holds various files of this Leiden University dissertation.

Author: Oosterhuis, Koen
Title: Preclinical development of DNA vaccine candidates for the treatment of HPV16 induced malignancies
Issue Date: 2012-06-27

PRECLINICAL DEVELOPMENT OF DNA VACCINE CANDIDATES FOR THE TREATMENT OF HPV16 INDUCED MALIGNANCIES

Koen Oosterhuis

ISBN: 978-94-6182-127-0

Layout & printing: Off Page, www.offpage.nl

Copyright © 2012 by K. Oosterhuis. All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means without permission of the author and the publisher holding the copyright of the articles.

PRECLINICAL DEVELOPMENT OF DNA VACCINE CANDIDATES FOR THE TREATMENT OF HPV16 INDUCED MALIGNANCIES

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 27 juni 2012
klokke 16.15 uur

door

Koen Oosterhuis
geboren te Breukelen
in 1980

PROMOTIECOMMISIE

Promotor: Prof. Dr. J.B.A.G. Haanen

Co-promotores: Prof. Dr. T.N. Schumacher
Dr. B. Nuijen (NKI-AVL/SLZ)

Overige leden: Prof. Dr. S.H. van der Burgh
Prof. Dr. G. Kenter (NKI-AVL)
Prof. Dr. C.A.H.H. T. Daemen (UMCG)
Dr. R.D.M. Steenbergen (VUmc)

The research described in this thesis was performed at the Department of Immunology at the Netherlands Cancer institute (NKI-AVL) and was financially supported by the Netherlands organization for health research en development (ZonMw) grant 432-00-001.

The printing of this thesis was financially supported by the Netherlands Cancer Institute.

"How many roads must a man walk down, before you call him a man?"

Bob Dylan

Voor mijn ouders,

voor Annelies, Eline & Merel

TABLE OF CONTENTS

Chapter 1	General Introduction and outline	9
Chapter 2	DNA Vaccines and Intradermal Vaccination by DNA Tattooing *Curr. Top. Microbiol. Immunol.: 351, 221-50 (2012)*	17
Chapter 3	Preclinical development of highly effective and safe DNA vaccines directed against HPV16 E6 and E7 *Int. J. Cancer: 129, 397–406 (2011)*	43
Chapter 4	Preclinical safety evaluation of DNA vaccines encoding modified HPV16 E6 and E7 *Accepted for publication in Vaccine*	63
Chapter 5	Rational design of DNA vaccines for the induction of HPV16 E6 and E7 specific cytotoxic T cell responses *Submitted for publication*	83
Chapter 6	Shielding the cationic charge of nanoparticle-formulated dermal DNA vaccines is essential for antigen expression and immunogenicity *J. Control. Release: 141(2), 234-40 (2010)*	105
Chapter 7	Summarizing discussion and future outlook	123
Annex	Nederlandse Samenvatting	136
	Curiculum Vitae	140
	List of Publications	141

CHAPTER

GENERAL INTRODUCTION AND OUTLINE

HPV AND CANCER

Human papilloma viruses (HPV) are non-enveloped DNA viruses that infect human skin and mucosa and are the causative agents of mostly benign proliferative lesions such as common (genital) warts (1). However, persistent infection with sexually transmitted, mucosal 'high-risk' HPV subtypes is strongly associated with the development of anogenital malignancies such as cervical, vulvar-, penile- and anal cancer, and also a subset of oropharyngeal cancers (1-4). The association is strongest for cervical cancer: illustrated by the finding that in over 99% of cervical cancers HPV DNA can be detected (5, 6). Notably, cervical cancer is the third most common cancer in women world wide, with an estimated death toll of almost 300.000 women annually, mostly in developing countries (7, 8). The much lower burden in the developed world is due to screening programs (most often Pap testing) that aim to detect early lesions, which can most often be cured by surgical removal of the lesion (2, 8, 9). As the immune system operates by the principle of non-self recognition, the involvement of a virus in the development of these types of malignancies provides a unique opportunity for the immune system to prevent or eradicate these types of malignancies.

PREVENTIVE VACCINATION

Recently two vaccines have become available for the prevention of HPV induced malignancies, namely Cervarix® and Gardasil® (10-12). Both vaccines are directed against the two most prevalent high-risk subtypes, HPV16 and 18, accounting for about 50% and 20% of cervical cancer cases respectively (13). Gardasil is also directed against the mucosal low-risk sub types 6 and 11, together accounting for 90% of genital warts (14). Both vaccines are composed of viral like particles (VLPs) that self-assemble when the major capsid protein L1 is expressed in eukaryotic cells. These VLPs are highly immunogenic structures that resemble the virus particle, but without the genetic content of the virus and thus without the risk of inducing disease. The VLPs provoke a strong B cell mediated immune response against L1, resulting in viral capsid specific antibodies, that are believed to neutralize/shield the virions before they can infect, thereby providing sterile protection against infection with the corresponding HPV virus sub-types (11, 15). However, these vaccines have no value for the treatment of pre-existing lesions (see below) and as a consequence these vaccines need to be administered to individuals before they get infected. For optimal prophylaxis, the complete population has to be vaccinated before the onset of sexual activity (12). So far, long-term (up to 6 years of follow-up) clinical trials in young (15-26 year old) women have shown nearly 100% protection against the development of precancerous lesions, caused by HPV16 and 18, upon vaccination with these preventive vaccines (16, 17). Although this efficacy is impressive, the estimated costs involved in the prevention of a single case of cervical cancer are extremely high: approximately 5 million US dollar based on an incidence of 7 per 100.000 (the age standardized incidence of cervical cancer in Western Europe (8)) and the cost per vaccination of 360 US dollar. This is explained by the fact that only very few HPV infections will eventually result in the formation of malignancies (18, 19). It has to be noted that the prevention of precancerous lesions (that have a much higher incidence) as such already provides a significant clinical benefit as the treatment of such lesions often requires surgical intervention (16, 17).

NEED FOR THERAPEUTIC VACCINE DEVELOPMENT

Beside the poor cost-effectiveness, a major drawback is that the preventive vaccines do not generate therapeutic effects against pre-existing lesions (20, 21), as also mentioned above. This is explained by the fact that upon infection the virus is maintained inside cells where antibodies can not reach it because they cannot pass the cell membrane. Moreover, expression of the viral capsid protein L1, that is recognized by these antibodies, is lost upon malignant transformation (22, 23). Therefore, a different approach is needed in order to generate an immune response that can eradicate existing lesions. The type of immune response required to eradicate pathogen-infected cells is called a cytotoxic T cell response. Cytotoxic T cells can kill pathogen infected cells upon recognition of virus-derived peptides presented at the cell surface on MHC class I molecules. (24). As it is well established that the viral proteins E6 and E7 of the high-risk sub-types play an essential role in the transformation process (25, 26), and are expressed in all HPV transformed cells, they are excellent targets for therapeutic vaccine development (21, 27). Importantly, the spontaneous clearance of HPV induced (pre-)malignancies is associated with T cell mediated immune responses against these proteins (28-30). Over the past two decades, numerous therapeutic vaccine candidates, targeting mostly HPV16 E6 and E7, have been developed in preclinical models (15, 21, 27, 31). Disappointingly clinical success has been rather limited with response rates usually not exceeding the rate of spontaneous regression (15). One recent study in patients suffering from grade 3 vulvar intraepithelial neoplasia (VIN 3) vaccinated with a vaccine consisting of E6 and E7 based long-peptides in incomplete Freunds adjuvant, showed a durable and complete regression in 47% of patients (32, 33). Also another recent study in which protein based vaccine (TA-CIN), that had no clinical effect as such (34), was combined with local immune modulation using Imiquimod (a TLR-7 agonist) in VIN 2/3 patients showed complete regression in 63% of patients (35). These two recent successes demonstrate the true value of therapeutic vaccination.

DNA VACCINATION

The therapeutic vaccines developed so far consist of broadly three categories: protein or peptide based vaccines, viral vectored vaccines or DNA vaccines (15). Among these strategies we consider DNA vaccination particularly attractive as outlined below. Uptake of the DNA by cells at the vaccination site will lead to local intracellular production of the antigen, thereby mimicking natural viral infection. As a consequence the immune system will be primed to produce predominantly cytotoxic T cells (36, 37). In contrast, injection of the proteins as such would in contrast predominantly result in the production of antibodies, which are considered useless, as E6 and E7 are intracellular proteins. An important advantage over vectored vaccines is that DNA vaccines can be administered repeatedly without the risk of inducing vector specific immunity (37). Other advantages of DNA vaccination are the fact that DNA can be relatively easily produced at large scale, the fact that DNA is stable at room temperature, the good safety profile of the DNA vaccination platform compared to for example live vector vaccines, and finally DNA can be easily manipulated in order to affect the properties of the encoded protein (37) (see also chapter 2 of this thesis for a detailed review on DNA vaccination in general). Over the past years many candidate DNA vaccines targeting E6 and E7 have been developed in rodent models (reviewed in (38, 39) and several clinical trials have been performed, or are currently ongoing (15, 38, 40, 41). Although vaccine specific immune responses could be

detected in some cases, the clinical outcome of these trials so far has been rather disappointing (15). Therefore, there is a strong need for optimization of E6 and E7 directed DNA vaccines.

AIM OF THE THESIS AND OUTLINE

The aim of this thesis was to develop highly immunogenic and safe candidate DNA vaccines for the treatment of HPV16 induced malignancies. Furthermore, we wanted to obtain insight in the mechanisms that contribute to the enhanced immunogenicity of so called 'DNA fusion vaccines'. The content of the individual chapters is summarized below.

Chapter 2 is provides a detailed review on DNA vaccination in general and DNA tattoo vaccination in particular. Among the subjects discussed in this review are: the advantages of DNA vaccination compared to conventional vaccine platforms, the mechanisms of T cell priming upon DNA vaccination, the origin of the "danger-signal" in DNA vaccine preparations and the value of DNA tattooing, a technique developed in our lab, compared to other DNA delivery methods.

Chapter 3 describes the development of highly effective and safe HPV16 E7 and E6 directed DNA vaccine candidates. As E6 and E7 are known oncogenes, we selected so called "gene-shuffled" versions of E6 and E7 in order to avoid cellular transformation at the vaccination site in case genomic integration might occur. The gene-shuffling results in the production of a completely rearranged protein that can be expected to have lost its oncogenic potential, while individual T cell epitopes are not altered. We found that these shuffled versions of E6 and E7 are no longer immunogenic upon DNA tattoo vaccination. Therefore, we had to develop a strategy to overcome the loss in immunogenicity. We constructed genetic fusions with Tetanus Toxin fragment C (TTFC), a bacterial protein that had been shown previously to improve the immunogenicity of C-terminally coupled antigenic peptides in DNA vaccination, and evaluated the effect of this fusion on the immunogenicity of the shuffled versions of E6 and E7.

Chapter 4 describes the preclinical safety studies performed to demonstrate that the vaccine candidates, TTFC-E6SH and TTFC-E7SH developed as described in chapter 2, indeed lost the oncogenic potential that is associated with E6 and E7 wild-type genes. For this purpose we selected two different model systems. In the first model system we made use of murine fibroblasts (NIH 3T3 cells) that were transfected with either our vaccine candidates, or wild-type E6 and E7 containing plasmids. Next we introduced a model system based on the viral transduction of primary human foreskin keratinocytes (HFKs). The latter model system can be regarded as more relevant as it comprises the use of the natural target cell of vaccination (the human keratinocyte). In addition, since we used retroviral vectors and grew the cells under selective pressure, we mimicked the worst-case scenario of stable integration of our vaccine candidates in the genome of keratinocytes, thereby increasing the likelihood of detecting residual oncogenic activity.

Chapter 5 describes the rational design of DNA vaccines encoding modified HPV16 E6 and E7. This chapter can be regarded as a follow up study of chapter 3. The exact mechanisms by which fusion with so called "carrier-proteins" (such as TTFC) enhances the immunogenicity of HPV16 E6 and E7 are not entirely clear. Often the biological function of such carrier-proteins is considered to play an important role. We hypothesized that rather more general mechanisms, such as provision of CD4+ T cell help, improvement of antigen stability or alteration of the subcellular localisation of the antigen, can explain the immune-potentiating effect observed

after fusion with such carrier-proteins. To test this hypothesis we developed modular DNA vaccines in which the presence of different components could be systemically altered.

Chapter 6 focuses on the improvement of the delivery of dermal DNA vaccines by formulating the DNA into nano-particles. It is estimated that only 1 out of $5x\,10^6$ to $5x\,10^9$ DNA copies is taken up after DNA tattoo vaccination. Therefore, if it would be possible to only slightly increase the efficiency of DNA uptake this could hypothetically result in an enormous increase in the amount of produced antigen. This can be expected to strongly improve the immunogenicity of DNA vaccination, as the amount of antigen expressed is considered to be a limiting factor. However, we found that complexation of DNA with cationic polymers, a method that strongly improves DNA uptake *in vitro*, completely blocks DNA tattoo mediated gene expression in intact human skin or in mice *in vivo*. We hypothesised that the positive charge of the resulting nanoparticles might lead to immobilization of the DNA in the extracellular matrix by charge interactions. Therefore we shielded the cationic charge of such particles by the addition of charge neutral PEG chains to the particles and evaluated the effect of this modification on the immunogenicity of the DNA-nanoparticles.

Finally **Chapter 7** contains a summarizing discussion and provides suggestions for future research.

REFERENCE LIST

1. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond)* 2006;110:525-41.
2. Scheurer ME, Tortolero-Luna G, Adler-Storthz K. Human papillomavirus infection: biology, epidemiology, and prevention. *Int J Gynecol Cancer* 2005;15:727-46.
3. Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;2:342-50.
4. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* 2002;55:244-65.
5. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999;189:12-9.
6. Snijders PJ, Steenbergen RD, Heideman DA, Meijer CJ. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* 2006;208:152-64.
7. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893-917.
8. Arbyn M, Castellsague X, de Sanjose S, Bruni L, Saraiya M, Bray F et al. Worldwide burden of cervical cancer in 2008. *Ann Oncol* 2011.
9. Wright TC, Jr., Massad LS, Dunton CJ, Spitzer M, Wilkinson EJ, Solomon D. 2006 consensus guidelines for the management of women with abnormal cervical cancer screening tests. *Am J Obstet Gynecol* 2007;197:346-55.
10. Pomfret TC, Gagnon JM, Jr., Gilchrist AT. Quadrivalent human papillomavirus (HPV) vaccine: a review of safety, efficacy, and pharmacoeconomics. *J Clin Pharm Ther* 2011;36:1-9.
11. Kwak K, Yemelyanova A, Roden RB. Prevention of cancer by prophylactic human papillomavirus vaccines. *Curr Opin Immunol* 2011;23:244-51.
12. Lowy DR, Schiller JT. Prophylactic human papillomavirus vaccines. *J Clin Invest* 2006;116:1167-73.
13. Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004;111:278-85.
14. Greer CE, Wheeler CM, Ladner MB, Beutner K, Coyne MY, Liang H et al. Human papillomavirus (HPV) type distribution and serological response to HPV type 6 virus-like particles in patients with genital warts. *J Clin Microbiol* 1995;33:2058-63.
15. Frazer IH, Leggatt GR, Mattarollo SR. Prevention and treatment of papillomavirus-related cancers through immunization. *Annu Rev Immunol* 2011;29:111-38.
16. Dillner J, Kjaer SK, Wheeler CM, Sigurdsson K, Iversen OE, Hernandez-Avila M et al. Four year efficacy of prophylactic human papillomavirus quadrivalent vaccine against low grade

cervical, vulvar, and vaginal intraepithelial neoplasia and anogenital warts: randomised controlled trial. *BMJ* 2010;341:c3493.

17. Paavonen J, Naud P, Salmeron J, Wheeler CM, Chow SN, Apter D et al. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. *Lancet* 2009;374:301-14.
18. Holowaty P, Miller AB, Rohan T, To T. Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999;91:252-8.
19. Nobbenhuis MA, Helmerhorst TJ, van den Brule AJ, Rozendaal L, Voorhorst FJ, Bezemer PD et al. Cytological regression and clearance of high-risk human papillomavirus in women with an abnormal cervical smear. *Lancet* 2001;358:1782-3.
20. Hildesheim A, Herrero R, Wacholder S, Rodriguez AC, Solomon D, Bratti MC et al. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. *JAMA* 2007;298:743-53.
21. Hung CF, Ma B, Monie A, Tsen SW, Wu TC. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. *Expert Opin Biol Ther* 2008;8:421-39.
22. Griesser H, Sander H, Walczak C, Hilfrich RA. HPV vaccine protein L1 predicts disease outcome of high-risk HPV+ early squamous dysplastic lesions. *Am J Clin Pathol* 2009;132:840-5.
23. Roden R, Wu TC. How will HPV vaccines affect cervical cancer? *Nat Rev Cancer* 2006;6:753-63.
24. Yewdell JW. Designing CD8+ T cell vaccines: it’s not rocket science (yet). *Curr Opin Immunol* 2010;22:402-10.
25. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. *Virology* 2009;384:324-34.
26. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. *Virology* 2009;384:335-44.
27. Brinkman JA, Hughes SH, Stone P, Caffrey AS, Muderspach LI, Roman LD et al. Therapeutic vaccination for HPV induced cervical cancers. *Dis Markers* 2007;23:337-52.
28. Nakagawa M, Stites DP, Farhat S, Sisler JR, Moss B, Kong F et al. Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia. *J Infect Dis* 1997;175:927-31.
29. Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* 2007;67:354-61.
30. Stanley M. Immunobiology of HPV and HPV vaccines. *Gynecol Oncol* 2008;109:S15-S21.
31. Albers AE, Kaufmann AM. Therapeutic human papillomavirus vaccination. *Public Health Genomics* 2009;12:331-42.
32. van der Burg SH, Melief CJ. Therapeutic vaccination against human papilloma virus induced malignancies. *Curr Opin Immunol* 2011;23:252-7.
33. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 2009;361:1838-47.
34. Smyth LJ, van Poelgeest MI, Davidson EJ, Kwappenberg KM, Burt D, Sehr P et al. Immunological responses in women with human papillomavirus type 16 (HPV-16)-associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV-16 oncogene vaccination. *Clin Cancer Res* 2004;10:2954-61.
35. Daayana S, Elkord E, Winters U, Pawlita M, Roden R, Stern PL et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. *Br J Cancer* 2010;102:1129-36.
36. Nagata T, Aoshi T, Uchijima M, Suzuki M, Koide Y. Cytotoxic T-lymphocyte-, and helper T-lymphocyte-oriented DNA vaccination. *DNA Cell Biol* 2004;23:93-106.
37. Liu MA. DNA vaccines: an historical perspective and view to the future. *Immunol Rev* 2011;239:62-84.
38. Monie A, Tsen SW, Hung CF, Wu TC. Therapeutic HPV DNA vaccines. *Expert Rev Vaccines* 2009;8:1221-35.
39. Huang CF, Monie A, Weng WH, Wu T. DNA vaccines for cervical cancer. *Am J Transl Res* 2010;2:75-87.
40. Sheets EE, Urban RG, Crum CP, Hedley ML, Politch JA, Gold MA et al. Immunotherapy of human cervical high-grade cervical intraepithelial neoplasia with microparticle-delivered human papillomavirus 16 E7 plasmid DNA. *Am J Obstet Gynecol* 2003;188:916-26.
41. Trimble CL, Peng S, Kos F, Gravitt P, Viscidi R, Sugar E et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clin Cancer Res* 2009;15:361-7.

CHAPTER 2

DNA VACCINES AND INTRADERMAL
VACCINATION BY DNA TATTOOING

2

CONTENTS

1. An Introduction on Two Decades of DNA Vaccination

2. Advantages of DNA Vaccination Compared to Conventional Vaccine Platforms

- 2.1 Ease and Speed of Production
- 2.2 Ability to Induce Cellular Immunity
- 2.3 Lack of Vector-Specific Immune Responses
- 2.4 Favorable Safety Profile

3. Mechanism of T Cell Priming upon DNA Vaccination

- 3.1 Direct- Versus Cross-Priming
- 3.2 Influencing Antigen Properties

4. Origin of the "Danger Signal" in DNA Vaccines

- 4.1 Danger in 'Naked' DNA
- 4.2 Administration-Induced Danger

5. Optimizing DNA Vaccination by Intradermal Tattooing

6. Mechanism of Immune Induction upon DNA Tattooing

- 6.1 Antigen Expression and Priming
- 6.2 Provision of Danger Signals

7. DNA Tattoo Versus other DNA Delivery Techniques

- 7.1 Intramuscular Injection
- 7.2 Particle-Mediated Epidermal Delivery
- 7.3 Electroporation-Mediated Gene Transfer
- 7.4 Jet Injection
- 7.5 Microneedle-Assisted Gene Transfer
- 7.6 Concluding Remarks on the Different DNA Vaccine Delivery Methods

8. Clinical Translation of Intradermal DNA Tattooing

- 8.1 Ex Vivo Human Skin Model
- 8.2 Ongoing and Planned Clinical Trials

9. Opinion on Usefulness of Intradermal DNA Vaccination, Large-Scale Use of DNA Tattoo, and Future Perspectives

10. Conclusion

K. Oosterhuis, J.H. van den Berg, T.N. Schumacher and J.B.A.G Haanen

Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands

Curr. Top. Microbiol. Immunol.: 351, 221-50 (2012)

ABSTRACT

Over the past two decades, DNA vaccination has been developed as a method for the induction of immune responses. However, in spite of high expectations based on their efficacy in preclinical models, immunogenicity of first generation DNA vaccines in clinical trials was shown to be poor, and no DNA vaccines have yet been licensed for human use. In recent years significant progress has been made in the development of second generation DNA vaccines and DNA vaccine delivery methods. Here we review the key characteristics of DNA vaccines as compared to other vaccine platforms and recent insights into the prerequisites for induction of immune responses by DNA vaccines will be discussed. We illustrate the development of second generation DNA vaccines with the description of DNA tattooing as a novel DNA delivery method. This technique has shown great promise both in a small animal model and in non-human primates and is currently under clinical evaluation.

1. AN INTRODUCTION ON TWO DECADES OF DNA VACCINATION

It is now two decades ago since it was first demonstrated that injection of naked plasmid DNA into mouse muscle results in expression of the encoded protein (1). Soon thereafter it was demonstrated that both cellular and humoral immune responses can be elicited against DNA vaccine-encoded proteins, when applied intradermally using a 'gene gun' (2) or upon intramuscular (IM) injection (3;4). Furthermore, these DNA vaccination-induced immune responses were shown to confer protection in various preclinical disease models, including models of viral, bacterial, and parasitic diseases and various tumor models (reviewed in ref (5) and (6)). Based on these encouraging preclinical data and a number of perceived advantages of DNA-based vaccines (see below), a series of clinical trials was initiated during the late 1990s that evaluated the efficacy of DNA vaccines in the induction of immune responses against pathogen- (HIV, malaria, hepatitis B) and cancer-associated antigens (7-9). While these trials provided overwhelming evidence for the overall safety of DNA vaccines (7;8), immunogenicity of this first generation DNA vaccines was at best modest.

Following the observation of low immunogenicity of DNA vaccines in the early human trials, the field has taken two directions. 1). It has been argued that while DNA vaccines may not induce high-level immune responses as a single modality, these vaccines would nevertheless be valuable to provide low-level priming. Such low-level immune responses can then subsequently be amplified by administration of a virus-based vaccine (10;11). Such DNA-prime viral vector-boost regimens can reduce the issue of vector-specific immune responses that are a common problem in viral vector-based vaccines. 2). As a second and more ambitious goal, a large effort has been made to develop (what we here will loosely call) "second generation DNA vaccines" that should be able to induce robust immune responses without a requirement for booster vaccination by virus-based vaccines. In these vaccines, optimization has either focused on *i*) improvement of the expression vectors, *ii*) improvement of the vaccine formulation, *iii*) enhancement of the immunogenicity of the vaccine-encoded antigen, or *iv*) the provision of molecular adjuvants in order to boost immunogenicity. A selected set of examples of such

optimizations will be provided. Furthermore a large effort has been made to develop novel physical delivery methods that aim to increase DNA vaccine efficiency, of which intradermal (ID) DNA tattooing forms an example.

Is it plausible that DNA vaccines will become available for human use in the foreseeable future? The licensing of 3 different DNA vaccines in the field of veterinary medicine (against West Nile virus in horses, against infectious haematopoietic necrosis virus in salmon and for treatment of melanoma in dogs) (12), and a recent report showing DNA vaccination-mediated protection of human subjects against influenza challenge (13), both illustrate the therapeutic potential of DNA vaccines as single modalities. Because of this, there is presently renewed optimism that DNA vaccines may within the next years be approved for applications in humans (11;14).

2. ADVANTAGES OF DNA VACCINATION COMPARED TO CONVENTIONAL VACCINE PLATFORMS

DNA vaccines have a number of attractive properties that contribute to the strong interest in their development. Among these properties are the ease and speed of vaccine production, the ability to induce both cellular and humoral immunity and the favorable safety profile as compared to other gene-based vaccine platforms that are able to induce strong cellular immunity. These aspects are discussed in more detail below.

2.1 Ease and Speed of Production

Plasmid DNA is relatively easy to produce in small to large quantities in a generic way, with little if any need for adaptation of the production process for different individual plasmids. This is in sharp contrast to in particular protein-based vaccines, for which the production process needs to be specifically designed for each new vaccine. Moreover, since DNA vaccine-encoded proteins are synthesized by the host cells upon delivery, difficulties associated with recombinant protein-based vaccine production, such as protein folding and post translational modifications (e.g. glycosylation) are circumvented (32;40). Another important advantage of DNA vaccines is the excellent stability of DNA as compared to other vaccine modalities, thereby likely circumventing the need for a 'cold chain' for vaccine distribution.

2.2 Ability to Induce Cellular Immunity

While direct experimental evidence is limited, there is some reason to assume that DNA vaccines are more suitable for the induction of $CD8^+$ ('cytotoxic') T cell immunity than recombinant peptide or protein vaccines (6;41-43). Due to the fact that by definition, vaccination-induced antigen expression takes place by host cells, there is ample opportunity for the transfected cells to present peptide fragments of the antigen in MHC-class I molecules at the cell surface. In contrast, in many other vaccine formats such as protein, peptide, or inactivated pathogen-based vaccines, antigen is offered within the extracellular space. As extracellular antigens are mainly presented via MHC-class II molecules, induction of $CD4^+$ ('helper') T cell and antibody responses can be expected to predominate (43). This discussion is somewhat complicated by the observation that induction of T cell responses upon DNA vaccination occurs at least in part by cross-priming rather than direct interaction between naive $CD8^+$ T cells and transfected skin or muscle cells (see below). However, as cross-priming is also more efficient for cell-associated than for soluble antigens (44), the advantage of vaccine formats that induce intracellular antigen expression remains.

2.3 Lack of Vector-Specific Immune Responses

While the presumed advantage of DNA vaccines in the induction of CD8^{+} T cell responses is shared with live attenuated viral vaccines or viral vector based vaccines, the latter modalities bear greater risks in terms of production and safety (45;46). Furthermore, viral vector-based vaccines such as recombinant adenovirus or vaccinia virus can suffer from pre-existing immunity towards the vector or can induce vector-directed immunogenicity, thereby preventing repeated administration of these vectors (47). In the case of DNA vaccines the only immunogenic structure produced is the antigen itself, thereby allowing repeated administration.

2.4 Favorable Safety Profile

For the large scale use of new vaccine formats in the general population their safety profile obviously needs to be well-established (48;49). Because of their non-infectious and non-replicating nature, DNA vaccines are considered more safe than live attenuated viruses or recombinant viral vectors. Furthermore, DNA vaccines have proven to be well tolerated and non-toxic in both preclinical- and clinical studies (9;14;50-52). However a few safety issues unique to plasmid DNA vaccines may potentially hamper their widespread use.

The main safety concern associated with DNA vaccines is the risk of genomic integration into the host genome. Genomic integration could potentially lead to activation of oncogenes, inactivation of tumor suppressor genes, or, when integrated into the chromosomal DNA of germ line cells, to vertical transmission. Several studies have examined the frequency of integration upon DNA vaccination. Collectively, these studies indicate that integration can occur but with a frequency that is manifold (around 3 orders of magnitude, depending on the system) lower than the spontaneous gene-inactivating mutation frequency of the genome. (50;53;54). Vertical transmission due to genomic integration in germ line cells has been observed after direct injection of DNA into the gonads (55). However, genomic integration into germ-line cells has not been observed after DNA vaccination at sites distant from the gonads (52;56). In conclusion, because of the low frequency of genomic integrations at the vaccination site and the absence of integrations in germ-line cells, the risks associated with genomic integration upon DNA vaccination are at present considered negligible. An important exception to this is formed by DNA vaccines that encode proteins with known or suspected transforming activity (e.g. the HPV E6 and E7 oncoproteins). Proteins with transforming activity are attractive targets for vaccination as they can serve as unique tumor associated antigens. However, for such DNA vaccines, the survival advantage of cells that express the encoded proteins could conceivably lead to outgrowth of those (extremely) few cells in which genomic integration has occurred (57). Because of this concern, the use of engineering strategies that abolish the transforming properties of the vaccine-encoded antigen should be considered essential.

A second potential safety concern in the use of DNA-based vaccines is the induction of anti-DNA antibodies and the subsequent development of auto-immune disease. This concern is increased by the fact that the bacterial derived DNA contains unmethylated phosphodiester-linked cytosine and guanine (CpG) motifs in the plasmid backbone that have an immunostimulatory activity via triggering of Toll-like receptor 9 (TLR9) (58), see also below. Anti-DNA antibodies are considered a hallmark of certain autoimmune diseases such as systemic lupus erythematosus (SLE), as most (but not all) patients manifest this characteristic of disease (5;59). Although induction of anti-DNA antibodies has been observed in some animal models after injection of plasmid DNA, thus far no evidence has been found that these antibodies are

associated with the development of systemic autoimmune diseases, either in healthy animals or in animals that are at risk for the development of autoimmune disease (reviewed in reference (50;60)). Furthermore, in human DNA vaccination trials no statistically significant increase in the presence of antinuclear antibodies and anti-DNA antibodies amongst vaccinees has been detected (50).

In conclusion, all preclinical and clinical studies that have aimed to evaluate potential safety concerns of DNA vaccines have not provided any compelling evidence for substantial risks associated with the use of DNA vaccines. Because of this, we currently see no major obstacles for the application of DNA vaccines for therapeutic purposes, or for prophylaxis against high-risk disease. It is noted however that the potential toxicities of DNA vaccines would primarily concern long-term effects that may be difficult to address in the studies discussed above. Because of this, it would seem prudent to await the long-term outcome of clinical trials for high-risk indications before widespread application of DNA vaccination for low-risk disease is considered.

3. MECHANISM OF T CELL PRIMING UPON DNA VACCINATION

At first glance, the general mechanism by which plasmid DNA vaccines induce immunity seems straightforward. Upon administration the plasmid DNA is taken up by host cells, leading to production of the antigen by these cells and to the release of 'danger' signals as dictated by the danger model. However, there is still substantial uncertainty about the antigen-presentation pathway that leads to the display of antigen-derived epitopes to naive T cells and also by which molecular mechanisms 'danger' is perceived upon DNA vaccination. Importantly, a better understanding of both of these factors is likely to result in more efficient DNA vaccine formats.

3.1 Direct- Versus Cross-Priming

Through the use of bone marrow chimeras it has been demonstrated that the induction of cellular and humoral immune responses upon DNA vaccination is absolutely dependent on antigen presentation by bone marrow derived professional antigen-presenting cells (APCs) (61). On the other hand, for various routes of administration it has been demonstrated that antigen expression upon DNA vaccination primarily results in antigen expression in non-immune cells in peripheral tissues, such as myocytes in the muscle and keratinocytes in the skin (62;63). An important question therefore is whether immune activation primarily occurs by the action of a small number of APCs that have become directly transfected, or whether antigen produced by the much larger number of non-immune cells serves as a source of antigen that is handed over to APCs that subsequently present the antigen (a process termed cross-presentation in the case of $CD8^+$ T cell activation). This issue is of more than academic interest as it has previously been demonstrated that the efficiency with which antigens are cross-presented can vary markedly depending on the context in which an epitope is provided (see also below) (64;65).

Most DNA vaccination studies performed to address this question have used gene gun or IM needle injection as a delivery platform. From these studies there is clear evidence that both direct presentation of antigen by transfected APCs (63;66-68) and cross-presentation of antigen acquired from non-immune cells (1;69;70) can occur *in vivo* after DNA vaccination. The design of most of these studies however does not allow a conclusion on the relative contribution of these two processes to $CD8^+$ T cell activation *in vivo*. An exception to this is formed by a study in

which a DNA vaccine encoding the influenza A nucleoprotein (NP) under control of either the keratinocyte-specific K14 promoter or the APC-specific CD11b promoter was applied via gene gun (69). This study revealed that keratinocyte-directed transgene expression induced both higher cellular and humoral immune responses than APC-directed transgene expression, thus providing strong evidence for a dominant role for cross-presentation in $CD8^+$ T cell priming upon gene gun immunization. These data are in apparent contrast to a second study that – again using gene gun application – provided evidence for a dominant role for directly transfected APCs in $CD8^+$ T cell activation (63). In this study, co-transfection, but not co-immunization of plasmids encoding co-stimulatory molecules was shown to restore the immunogenicity of an otherwise non-immunogenic nuclear protein (NP) variant. This observation seems most consistent with antigen presentation by directly transfected APCs, as cross-presentation would not be expected to result in cell surface expression of the vaccine-encoded costimulatory molecules on the APC. It is noted however, that the NP variant used in the latter study may form a poor substrate for cross-presentation, as the mutations within this antigen may prevent proper folding and thereby reduce antigen accumulation within the donor cell or by other means disrupt the transfer of antigen from the antigen-producing cells to specialized APCs (see below) (36).

Taken together, to date no definitive answer exists regarding the exact mechanism of T cell priming upon DNA vaccination (71;72), and it is plausible that the mechanism of immune induction will differ between different methods of immunization (68;73), between target tissues (e.g. skin versus muscle) (68), and between different DNA vaccine designs.

3.2 Influencing Antigen Properties

Several strategies have been developed in which an antigen of interest is genetically fused to a 'carrier' protein. Carrier proteins that have been shown to (sometimes strongly) increase the immunogenicity of the fused antigen include tetanus toxin fragment C (TTFC), heat shock protein 70 (HSP 70), MHC class II invariant chain (Ii), calreticulin (CRT), herpes simplex virus viral protein 22 (HSV VP 22) and *E. coli* b-glucuronidase (Table 1). The exact mechanism(s) by which these carrier proteins enhance the immunogenicity of the fused antigen remain largely unclear and may vary between different carrier molecules. However, based on our current understanding of DNA vaccines, two broad categories are likely to play dominant roles.

Provision of $CD4^+$ T cell help: There is abundant evidence that $CD8^+$ T cell responses induced by DNA vaccination are dependent on $CD4^+$ T cell help (74). However, $CD4^+$ T cell responses are likely to be weak or lacking when using DNA vaccines that either encode self proteins or single $CD8^+$ T cell epitopes. In such cases, the provision of $CD4^+$ T cell help via carrier encoded helper epitopes is likely to be an important factor in the immune-enhancing effect of foreign carrier molecules, like TTFC and *E. coli* β-glucoronidase (23;27).

Enhancement of antigen presentation: There is strong evidence that improvement of antigen stability enhances DNA vaccine immunogenicity. First, many of the above mentioned fusions result in increased steady state antigen levels (26;27;29). Second, formal evidence for the notion that the stability of DNA vaccine-encoded antigens in the transfected cell contributes to vaccine immunogenicity has been provided using a set of engineered luciferase variants with a variable *in vivo* half-life (75). For this set of variants, immunogenicity was directly correlated to antigen stability. Also the observation that covalent linkage of an epitope towards a carrier protein, but not the simultaneous expression of the epitope and the carrier using a bicistronic vector, improves vaccine immunogenicity, is consistent with the notion that carrier proteins can influence vaccine

immunogenicity by increasing antigen half life (76). At present, the most straightforward explanation for the observed effect of antigen stability on vaccine immunogenicity is that it would enhance cross-presentation, although a direct analysis of epitope density on APCs would be required to provide formal evidence for this model. Genetic fusion to carrier proteins may also influence antigen presentation through other mechanisms. For VP-22 it has been proposed that it enhances antigen spreading to neighbouring cells (29). For HSP-70 it has been proposed that it increases uptake of the antigen by APCs via a HSP specific receptor (77). Finally, some carrier molecules such as Ii (26) and calreticulin (25) alter the subcellular localization of an antigen and might thereby improve the immunogenicity of the DNA encoded antigen. This is in line with the finding that the sole addition of signals influencing subcellular localization (such as ER targeting signals) to DNA vaccine-encoded antigens can improve their immunogenicity (29;78-80). Also in this case, enhanced immunogenicity may be due to increased cross-presentation, but again, formal evidence is lacking. Clearly, improved insight into the mechanisms by which different carrier influence vaccine immunogenicity will enable more rational DNA vaccine optimization and should be an important area of future research.

4. ORIGIN OF THE “DANGER SIGNAL” IN DNA VACCINES

Although the addition of various adjuvants (Table 1) can enhance their immunogenicity, DNA vaccines are also able to induce strong immune responses in animal models without the addition of adjuvants that provide inflammatory signals. As the induction of adaptive immune responses requires not only the presence of antigen, but also the presence of signals that induce APC activation (something often referred to as the danger model) (73;81;82), this implies that either DNA vaccines themselves or the DNA vaccination procedure provides elements that result in a sense of danger.

4.1 Danger in ‘Naked’ DNA

For many years it has been assumed that unmethylated CpG motifs were the primary source of danger in DNA vaccine preparations. Unmethylated CpG motifs form one of the so called ‘pathogen-associated molecular patterns’ (PAMP) that are recognized by pattern recognition receptors (PRR), in the case of CpG the TLR9. TLR9 is expressed in the endocytic pathway, providing endocytosing cells with the ability to detect CpG motifs within ingested material. Triggering of TLR9 initiates a cascade of signaling events that leads to NF-kB and activator protein 1 (AP-1) activation, and the subsequent induction of a pro-inflammatory response characterized by the release of cytokines and chemokines, e.g. type I interferons (IFNs), interleukin (IL)-6, IL-12 and tumor necrosis factor (TNF)-a (83). In early work, the inclusion of additional CpG motifs within the plasmid backbone was shown to improve DNA vaccine efficiency after ID vaccination in a murine melanoma model (39). As TLR9 is differentially expressed between mice (all dendritic cell subsets) and men (only plasmacytoid dendritic cells) (84), it has been suggested that a reduced ability to initiate a CpG-dependent danger response could explain the poor track record of DNA vaccines in humans. However, several studies have shown that both the induction of cellular as well as humoral immune responses is unaffected in TLR9-deficient mice (85;86). Assuming that TLR9 forms the sole receptor for CpG, these data suggest that danger in DNA vaccination must (also) be sensed by other means.

Recently, evidence has been provided indicating that double stranded DNA (dsDNA) in the B form (right-handed helical structure) functions as an intrinsic adjuvant in DNA vaccines (reviewed

Table 1. Selection of methods to enhance DNA vaccine potency.

Type of optimization	Method	Proposed mode of action*	Ref.
improvement of the vector	gene optimization	stabilization of RNA; more efficient translation of RNA	(15;16)
	addition of viral post-transcriptional regulatory elements	increased cytoplasmic mRNA levels	(17) (18)
improvement of vaccine formulation	formulation of naked DNA into nano/micro particles	increased cellular uptake of DNA	(19;20) (21;22)
improvement of antigen immunogenicity	TTFC fusion	provision of CD4+ help	(23)
	HSP-70 fusion	improved cross-presentation of antigen	(24)
	Calreticulin fusion	targeting of antigen for antigen processing and presentation	(25)
	Invariant chain fusion	enhanced stability/changed subcellular localisation	(26)
	E. coli β-glucoronidase fusion	changed subcellular localization of antigen, provision of CD4+ help	(27)
	HSV VP 22 fusion	improved antigen spreading	(28;29)
enhancement of immune activation by addition of adjuvants	co-delivery of pro-inflamatory cytokines (GM-CSF, IL-2, IL-12)	recruitment, expansion and activation of APCs	(30-32)
	co-delivery of chemokines (CCL-21, CCL27, CCL-28, CCL-5)	attraction of immune cells to the site of vaccination	(33-35)
	co-delivery of co-stimulatory genes (CD80, CD86)	improvement of co-stimulation	(35;35;36)
	HMGB-1 co-delivery	recruitment, expansion and activation of APCs	(37)
	TLR agonists (imiquimod,CpG)	activation of APCs	(38;39)

***** For most of these methods, evidence that the increase in vaccine immunogenicity is indeed due to the proposed mechanism is at best circumstantial. Furthermore, only for selected strategies their added value has been confirmed in independent studies.

in (83) and (87)). Two dsDNA sensors have been identified thus far, namely DAI (DNA-dependent activator of IFN-regulatory factors) and AIM 2 (absent in melanoma-2). Contrary to TLR9, these dsDNA sensors are expressed within the cytosol, providing transfected cells with the ability to detect incoming DNA. DAI-induced immune activation is mediated through the activation of IFN regulatory factor 3 (IRF3) and NF-kB and results in the production of type I IFNs (88). AIM 2 has recently been described as the cytosolic DNA sensor that is responsible for activation of the

inflammasome, thereby resulting in the production of active IL-1β, IL-18 and IL-33 (89). However, as optimal DNA vaccine immunogenicity requires type I IFNs (90) and AIM2 is not required for type I IFN production, it is considered to have a secondary role in the DNA-induced adjuvant response (83). An important study by Ishii et al. has demonstrated a pivotal role for TANK-binding kinase 1 (TBK-1), a non-canonical IkB kinase, in mediating the adjuvant effect of DNA vaccines. In the presence of dsDNA, TBK-1 activates IRF3 and IRF7, leading to the production of type I IFNs. Notably, TBK-1 deficient mice were unable to generate antigen-specific humoral and cellular immune responses upon vaccination with a DNA vaccine delivered by IM injection followed by electroporation (90). In constrast, DNA vaccine-induced immune responses were not affected by DAI deficiency and from this observation it was concluded that TBK-1 but not DAI is essential to the DNA vaccine mediated adjuvant response. Recently evidence was provided for the involvement of another signaling component named stimulator of IFN genes (STING) in TBK-1 mediated dsDNA sensing (91). STING assembles with TBK-1 after dsDNA stimulation (92) and TBK-1 trafficking is blocked in the absence of STING (91). Morever STING is essential for intracellular DNA-mediated type I IFN production and STING deficient mice showed an almost complete inhibition of both humoral and cellular immune responses upon DNA vaccination. Notably, despite the increasing knowledge on the signaling route that controls cellular responses upon cytosolic DNA encounter, the critical element recognizing dsDNA in this pathway still needs to be identified. Our current knowledge on intracellular DNA sensors is summarized in Table 2.

4.2 Administration-Induced Danger

While recognition of the introduced DNA forms one route through which DNA vaccination results in a danger response, the physical damage induced by the administration procedure itself is likely to be a second factor. Sensing of physical damage seems likely to be of particular importance for ID delivered DNA vaccines, as the skin has an important barrier function in host defense and is densely populated with immune cells. Therefore, administration procedure-induced local skin injury is likely to result in an inflammatory response that can boost vaccine immunogenicity (93). This notion is supported by a recent report demonstrating that epidermal injury during poxvirus immunization is crucial for the generation of protective T-cell mediated immunity (94). Furthermore, delivery-induced damage has also been suggested to play a role following electroporation mediated IM delivery (95;96) and even following simple IM injection in mice, as the injection volume used (usually about 50 μl) exceeds the fluid capacity of the muscle resulting in local tissue damage (41;62).

Table 2. Cellular DNA sensing elements and their importance in DNA vaccination-induced immune responses.

Pattern recognized	DNA recognizing element	Signaling components involved	Mediators released	Relevance for DNA vaccination	Ref.
CpG motifs	TLR9	MyD88	IL-6, IL-12, TNF-α, Type 1 IFN	little/moderate	(58;85;86)
dsDNA	AIM2	Inflammasome	IL-1β, IL-18, IL-33	little	(89)
	DAI	TBK-1/IRF3	Type 1 IFN	little	(88)
	unknown	TBK-1/STING/IRF3	Type 1 IFN	high	(90;91)

What are the molecular mediators of the inflammatory response that is induced by physical damage? First, cell death that occurs during vaccination may lead to the release of intracellular molecules (with HMGB-1 as a prototype) that can be recognized by neighboring cells, or can result in the formation of uric acid crystals. This class of endogenous indicators of danger, sometimes referred to as alarmins (reviewed in references (81;97)) is likely to grow further in coming years, and it seems plausible that the role of individual alarmins as indicators of danger will depend on the strategy used for DNA vaccine delivery. In the case of ID DNA vaccine delivery, the vaccination-induced damage may also result in a danger response through an indirect mechanism. Specifically, the disruption of the skin barrier will create opportunities for pathogens/skin-resident microorganisms to locally invade the epidermal or dermal layer. As a consequence, immune activation can be expected to occur via the sensing of one of the many identified PAMPs, such as LPS, peptidoglycans, flagellin etc (98).

While there is increasing interest in the role of adjuvant signals provided by the DNA itself, little attention has thus far been given to the contribution of the DNA vaccination procedure induced damage to vaccine immunogenicity. Furthermore, our understanding of the contribution of different danger signals (be they either DNA- or damage-induced) to different types of adaptive immune responses (humoral, Th1, Th2, Th17, cytotoxic) is still limited.

5. OPTIMIZING DNA VACCINATION BY INTRADERMAL TATTOOING

Given the poor performance of DNA vaccines (mostly IM delivered) in non-human primates and early clinical trials we set out to develop an improved strategy for DNA vaccine delivery. First, we postulated that a strategy in which DNA vaccines are introduced into the skin by a multitude of needle injections rather than a single injection would be superior. This method, in which DNA is delivered to the epidermal skin layer by many thousands of injections using a permanent make-up or tattoo device has been named DNA tattooing (99). Secondly, by measuring DNA vaccination-induced antigen expression *in vivo* using a firefly luciferase-encoding DNA, the kinetics of antigen expression could be followed. Notably, despite the fact that antigen expression after ID tattoo was approximately 10-100 fold lower and of much shorter duration than after IM injection, presentation of the vaccine-encoded epitope to $CD8^+$ T cells was shown to be markedly better. Based on the observation that DNA tattoo-induced antigen expression was restricted to approximately 96 hours, a vaccination schedule was developed in which DNA is applied three times with 2 days intervals. Using this short-interval ID DNA delivery schedule, robust $CD8^+$ T-cell responses that can readily be measured directly *ex vivo* could be induced within two weeks. In contrast, IM vaccination with this short interval regimen did not lead to detectable T-cell responses. Furthermore, in comparison to IM DNA vaccination, DNA tattooing was shown to mediate substantially better protection in mouse models of influenza A infection and HPV16-associated cancer. A likely explanation for the higher immunogenicity of DNA tattoo vaccination is that skin is a better equipped for the induction of immune responses. In contrast to muscle, skin is rich in APCs (100) and is the body's first line of defense against many pathogens (93). Also, since the tattoo procedure inflicts thousands of skin perforations it is likely to result in the release of many more danger signals than simple IM or ID injection, thereby serving as a potent adjuvant (see below).

Interestingly, ID tattoo vaccination has also been applied to other vaccine modalities. For peptide-based vaccines it was shown that ID tattooing was more efficient than a subcutaneous

(SC) injection (101). Also adenoviral vectors have been administered via ID tattoo in a side-by-side comparison with SC injection. In contrast to the results obtained with DNA vaccines, delivery of adenovirus via ID tattoo immunization did not provide any obvious advantage over delivery via ID injection (102). A possible explanation for this lack of superiority of ID tattoo vaccination is that the uptake of the adenovirus into host cells is much more efficient obviating the need for a more sophisticated delivery procedure and/or that viral particles themselves serve as a strong adjuvant, thereby making the tattoo procedure redundant.

6. MECHANISM OF IMMUNE INDUCTION UPON DNA TATTOOING

6.1 Antigen Expression and Priming

How does ID DNA tattooing induce $CD8^+$ T cell responses? Upon ID DNA tattoo vaccination, antigen expression is largely confined to cells within the epidermal layer, as revealed by beta-Gal staining (99). Furthermore, by flow cytometric analysis of single cell suspensions of tattooed *ex vivo* human skin (see below for more details on this model) it was shown that the vast majority of transfected cells consists of keratinocytes. Sporadic transfection of Langerhans cells (LCs) in the epidermis could also be observed (approximately 1% of transfected cells, more or less proportional to their frequency in human skin cell preparations) (103). Notably, the fact that only few antigen-expressing LCs could be recovered from human skin could not be explained by rapid migration of these cells after DNA administration. Does the fact that antigen expression upon DNA tattoo is largely restricted to keratinocytes indicate a dominant role for this cell type in the induction of immune responses? When vaccination-induced antigen expression is restricted to keratinocytes by the use of the K14 promoter, $CD8^+$ T cell responses could still be induced by this strategy for DNA vaccination in a murine model (75). As there is no evidence for migration of keratinocytes to the skin-draining lymph nodes, nor for naïve T cell priming at the site of vaccination, these data strongly suggest that the induction of a vaccine-specific $CD8^+$ T cell response upon DNA tattooing is at least partially due to cross-priming. A schematic representation of the different possibilities that lead to CD8+ T cell priming upon DNA tattooing is provided in figure 1

6.2 Provision of Danger Signals

As discussed above, recognition of danger signals upon DNA vaccination may either involve the direct recognition of the introduced DNA, or the detection of physical damage caused by DNA introduction. Thus far, only the role of TLR9 in sensing unmethylated CpG motifs upon DNA tattoo has been evaluated. Consistent with data from studies that have evaluated the role of TLR9 in other DNA vaccination modalities, the magnitude of $CD8^+$ T cell responses induced by DNA tattoo in wild type and in $TLR9^{-/-}$ mice were identical, demonstrating that – at least in mice – TLR9 mediated signaling is not essential for the induction of immune responses by DNA tattoo vaccination (99). Evidence for or against a role for different cytosolic dsDNA sensing systems in DNA tattoo vaccination induced immunity is at present lacking. However, as this DNA vaccination strategy relies on the generation of thousands of skin perforations, a contribution of vaccination-induced skin damage to the immunogenicity of DNA tattooing therefore seems plausible. Support for the notion that inflammatory signals inflicted by tissue damage contribute to the immunogenicity of DNA tattoo is provided by a study in which we

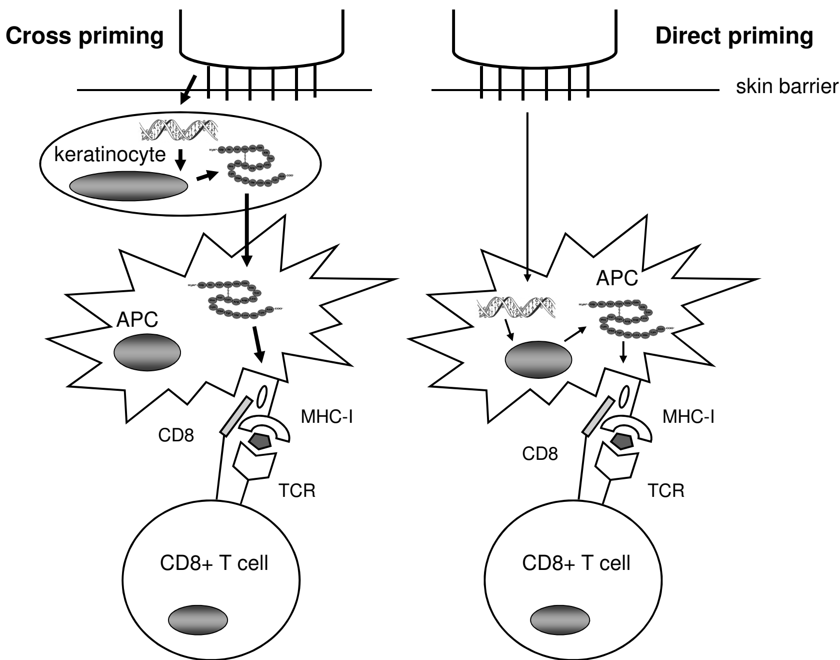


Figure 1. Possible routes for the priming of CD8+ cytotoxic T cells upon ID DNA tattoo vaccination. Upon DNA tattooing keratinocytes are transfected and produce antigen that is acquired by professional APCs, leading to cross-presentation to $CD8^+$ cytotoxic T cells. As an alternative, APCs can become directly transfected, leading to direct priming of $CD8^+$ cytotoxic T cells. Based on available evidence, cross-presentation is considered the predominant route for priming (see text).

measured serum IL-6 levels upon tattoo application of either DNA or water-for-injections (WFI). Notably, systemic IL-6 levels were increased to the same extent in both groups of mice and exceeded those seen upon intraperitoneal delivery of 100 IU of LPS (a known inducer of IL-6 (104)). These data suggest that administration-induced danger signals form a major factor in the immunogenicity of DNA tattoo (105). A schematic representation of the different routes by which danger can be sensed upon DNA tattooing is provided in figure 2.

7. DNA TATTOO VERSUS OTHER DNA DELIVERY TECHNIQUES

To date, a large number of different delivery methods for DNA vaccines have been developed. In the following section a selection of these methods is discussed and their pros and cons relative to DNA tattooing are evaluated.

7.1 Intramuscular Injection

IM injection is one of the first routes of administration used for the delivery of naked plasmid DNA (1) and the ease and simplicity of the method are particularly attractive for large scale

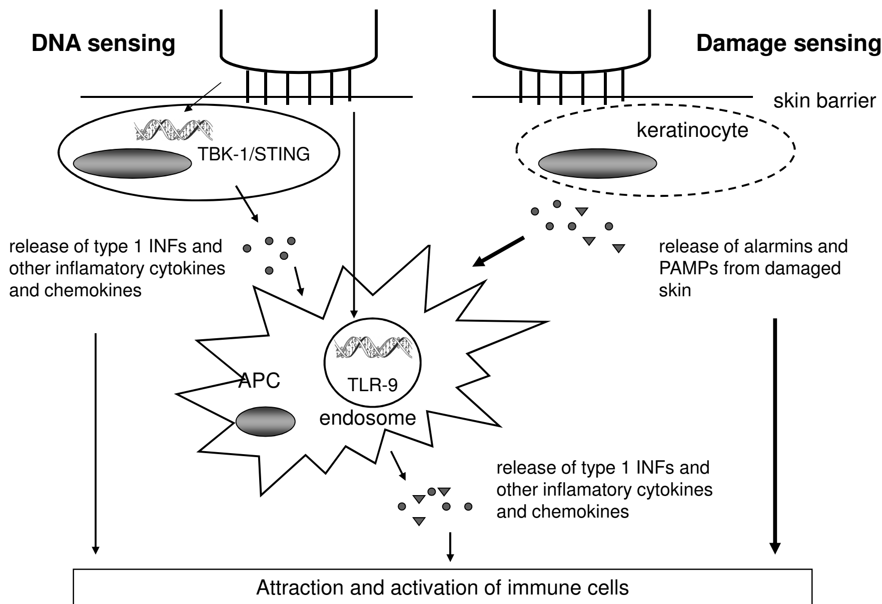


Figure 2. Routes by which danger can be sensed upon intradermal DNA tattoo vaccination. Danger can be sensed via detection of dsDNA by cytosolic DNA sensors and signaling via TBK-1/STING, or via detection of CpG motifs by endosomal TLR9 (although the latter route is not critical). On the other hand, the tattoo procedure can induce damage to the skin leading to the release of PAMPs and alarmins. The thus released mediators can activate skin resident APCs and attract/activate other immune cells. The fact that tattooing without any DNA already results in strong immune activation as measured by serum IL-6 levels (see text) suggests that damage induced danger signals may play a dominant role.

use. Although the method has proven effective in small animal models, the results obtained in studies in non-human primates and clinical trials have been disappointing even when doses up to 5 mg plasmid were used (11;106;107). This translational block has been referred to as the "simian barrier" (107) and is possibly explained by the impossibility to scale-up the injection volume used in mice (50 µl) to non-human primates and humans. Based on the difference in body weight (20 gr versus 80 kg) an injection volume of about 200 ml would be needed for a linear scale-up. Inability to perform such scale-up may be a particularly important factor as it has been suggested that the tissue damage inflicted by injection of a large volume of DNA relative to the volume of the injection site contributes to the immunogenicity of IM DNA vaccination in mice (41;62).

As described above we have performed an extensive comparison of the efficiency of ID tattoo vaccination and classical IM DNA vaccination in murine models. These data demonstrated that DNA tattoo outperformed IM DNA vaccination both with respect to the speed of $CD8^+$ T cell induction and with respect to the magnitude of this immune response. The superiority of DNA tattoo in mice has since then been confirmed in a study by Pokorna et al., in which previously described strategies to enhance the potency of IM DNA vaccination (cardiotoxin pretreatment or GM-CSF DNA co-delivery) were also included. In this study, ID DNA tattooing

elicited significantly higher L1 specific humoral and cellular immune responses as compared to all IM conditions evaluated, even if the number of IM injections exceeded the number of tattoo administrations (108).

Having demonstrated the superiority of DNA tattoo in small animal models we set out to determine if the translational block described for IM delivery would also apply to DNA tattoo. To this end we performed a study in rhesus macaques, in which we delivered an HIV clade C DNA vaccine via ID DNA tattooing and compared the results with a prior study using exactly the same vaccine and vaccine dose but delivered by IM injection. This study demonstrated a 10- to 100-fold increase in the magnitude of vaccine-specific T cell responses in peripheral blood from rhesus macaques vaccinated by DNA tattoo, as compared to T cell responses in animals immunized via the IM route. Furthermore an increase in the fraction of animals responding to the immunogens was also observed. In conclusion, DNA tattoo outperforms IM DNA vaccination in both small and large animal models, warranting its further testing in humans (109).

7.2 Particle-mediated epidermal delivery

Particle-mediated epidermal delivery (PMED) comprises the bombardment of the skin with gold particles coated with DNA and is often referred to as 'gene gun' vaccination. Gene gun-mediated gene transfer is the first method that was successfully used for DNA vaccination in murine models (2). The method, which was originally developed for the transfection of cells *in vitro*, has been extensively studied in human subjects. Within these studies, both cellular and humoral vaccine-specific responses have been demonstrated and – even though a side by side comparison has to our knowledge not been performed in clinical trials – the method is generally considered more efficient than IM injection (106;110). A comparison of the efficiency of gene gun and ID tattoo vaccination (using the same short interval administration schedule) has demonstrated that the two methods are equipotent in $CD8^+$ T cell activation in a murine model.

The doses of DNA required to induce immune responses by gene gun administration are surprisingly low, about 1 mg/dose, being approximately 100-1000 fold lower than that used for IM injection, and this holds true for both murine and larger animal models (106;110). Moreover, also in human clinical trials, immune responses have been detected with doses below 10 mg (110). A possible explanation for this high efficiency is that gene gun is believed to directly deliver the DNA into the intracellular environment, in contrast to any other DNA delivery method (106). However, as the capacity of the current delivery devices is also low (1-2 µg of DNA per 'shot') the scaling of gun vaccination from mice to human application may still form an issue (40;110). Specifically, taking into account the difference in body surface (0.0075 vs 1.85 m^2), approximately 250 vaccinations would be required to achieve the same dose per body surface. A second drawback is that the costs per immunization may be substantial (in particular when such scaling is performed), because of the need for formulating the DNA onto gold particles (40).

7.3 Electroporation-Mediated Gene Transfer

Electroporation (EP) is successfully used as a strategy for the transfection of cells *in vitro* (111). EP uses short electrical pulses to destabilize cell membranes. While the precise mechanism is unclear, EP is thought to promote cellular uptake of DNA through permeabilizing cell membranes and driving DNA entry via an electrophoretic process (112). As it is believed that the poor performance of DNA vaccines in larger animals and humans can at least in part be

explained by the low transfection efficiency upon needle mediated delivery of naked DNA (113;114), EP has been extensively evaluated for its potential to increase *in vivo* transfection. Several devices for EP-assisted DNA vaccination have been developed and EP has been shown to result in an increase in antigen expression and vaccine immunogenicity in murine models when combined with either IM (62;115) or ID DNA injection (116;117). In a direct comparison, the combination of IM injection and EP was shown to be more efficient than gene gun-mediated DNA administration (118). EP mediated DNA vaccination has also been shown to increase antigen expression levels and vaccine immunogenicity in large animals (113;119). Based on these highly promising preclinical data EP is now also being evaluated in clinical trials (reviewed in ref (111)).

A slight complication of this technique is that there are many variables such as pulse duration, pulse strength and the number of pulses that need to be optimized. It has been demonstrated that EP settings that result in high expression levels are not necessarily those that induce the highest immune responses (116). Furthermore, the optimal settings may also differ depending on the array used and the targeted tissue (120;121).

A safety concern that has been associated with the improved transfection efficiency upon EP is a possible increase of the number of chromosomal integrations (50;60). In one study by Wang et al. it was observed that EP markedly increased the amount of plasmid associated with high molecular weight (i.e. genomic) DNA. Furthermore, using a newly developed PCR method, four independent integration events were detected in electroporated muscle, providing direct proof for genomic integration upon DNA vaccination (54). However, other studies have shown no increase in the amount of plasmid DNA associated with high-molecular-weight DNA after EP in combination with IM delivery (122). More importantly, it seems plausible that the risk of genomic integration will scale proportionally with any improvement in DNA vaccine delivery strategies and this risk is therefore unlikely to be unique to EP.

7.4 Jet Injection

Jet injection is a needle free technique in which fluid is injected under high pressure and this technique is suitable for both IM and ID administration. Jet injectors have successfully been used for immunizing humans with live attenuated vaccines against measles and smallpox, as well as inactivated life vaccines against cholera, hepatitis B, influenza and polio (123). Advantages of the method are that it avoids the use of sharps and its compatibility with existing vaccine formulations that have been developed for needle-based administration. Disadvantages of the method include higher levels of pain and more frequent side reactions than observed with needle-based vaccine delivery (123). In a report by Trimble et al., $CD8^+$ T cell responses and antitumor effects generated by a DNA vaccine administered ID via gene gun or Biojector® (a jet injector suitable for ID delivery) and IM via needle injection were directly compared in a murine model (124). In this comparison, gene gun vaccination formed the most potent method of immunization. Furthermore, in non-human primates IM jet injection with the Biojector® or Mini-Ject™ was not more efficient than simple IM injection with respect to the induction of both cellular and humoral immune responses (125). Based on these data it can be concluded that jet injection does not significantly improve the immunogenicity of IM administered DNA vaccines.

7.5 Microneedle-Assisted Gene Transfer

Microneedles are small needles with a size between 200 and 400 μm that have been designed to deliver drugs to the epidermal layer of the skin, without stimulating the pain receptors that populate the underlying dermis (126). Microneedles have been shown to be useful for the

delivery of protein-based vaccines in clinical trials as delivery of the seasonal influenza vaccine via microneedles has been shown to be more effective than simple IM delivery (127;128). A recent study by Zhou et al. demonstrated that microneedle-based delivery of a hepatitis B virus DNA vaccine in mice resulted in higher levels of humoral and cellular immune responses when compared to IM injection with the same DNA construct (129). However, protein expression levels upon DNA application with microneedles are also reported to be unpredictable and difficult to control, and because of this, further optimization is likely to be required for the future development of this strategy into a robust DNA vaccination platform (126).

7.6 Concluding Remarks on the Different DNA Vaccine Delivery Methods

Compared to the above-mentioned administration techniques (summarized in Table 3), DNA tattoo stands out by its simplicity. There is no need for formulation of the DNA as is the case for PMED. As compared to jet injection, EP and PMED, the required equipment is relatively simple and cheap. More importantly, linear scale up from mice to man can simply be done by vaccination of larger skin areas. This notion is supported by the promising results of DNA tattoo in non-human primates (109). Furthermore, as the tattoo procedure causes substantial damage to the skin requirement for the inclusion of adjuvants may not be needed, making clinical translation relatively straightforward. By the same token, it is noted however that the invasiveness of the method and also the requirement for repeated administration are likely to limit the current DNA tattoo strategy to high-risk diseases.

Table 3: Advantages and limitations of different DNA delivery methods.

Gene transfer method	Advantages	Limitations	Ref.
IM injection	Ease of the method, low costs of equipment	Poor track record in larger animals and human subjects	(1;107;109;130)
PMED ('gene gun')	High potency in relation to dose, extensive preclinical and clinical experience	Complex gold particle-based formulation/high cost of the equipment, dose limitation to microgram range	(40;110;123)
Electroporation	Extensive preclinical and clinical experience, can be combined with other delivery methods	High cost of the equipment,	(54;111)
Jet injection	Needle-free method, ability to work with existing formulations, and success with many forms of vaccines	High cost of the device, higher levels of pain than with needles No evidence for superior performance relative to IM DNA vaccination	(123;131)
Microneedle-based application	Favorable patient acceptability, possibility for self administration	Limited experience in DNA vaccination, low protein expression levels	(126)
DNA tattoo	Linear scale up from mice to men possible, relatively cheap/portable instrument	Invasiveness of the method may limit patient acceptability	(99;109)

8. CLINICAL TRANSLATION OF INTRADERMAL DNA TATTOOING

8.1 Ex Vivo Human Skin Model

On the basis of the promising preclinical data both in mice and in non-human primates an effort has been made to translate DNA tattoo vaccination into clinical application. However, before initiation of studies in humans it was important to determine the optimal settings for DNA tattoo in human skin, in particular because the physiology of human skin has obvious differences compared to that of furred murine and non-human primate skin (132). To address this issue we have analyzed the parameters that result in optimal expression of vaccine-encoded antigens applied to human skin by DNA tattoo (103). For this purpose, we have developed an *ex vivo* human skin, in which DNA vaccines encoding reporter proteins are applied via ID tattoo. These studies revealed that gene expression upon ID DNA tattoo of human skin is almost exclusively restricted to the epidermal layer. Furthermore, consistent with the data obtained in mice, the vast majority of transfected cells consisted of keratinocytes. In order to optimize variables that we considered likely to influence the efficiency of DNA vaccination we have tattooed a total of 428 skin areas with luciferase-encoding DNA, thereby examining the effect of variations in 1) DNA concentration, 2) the duration of tattooing, 3) needle depth and 4) the type of tattoo machine. From these experiments, analyzed in a linear mixed effects model, it was concluded that DNA concentration is the most important factor influencing antigen expression in human skin. Furthermore, it was shown that also tattoo time and tattoo depth had significant effects on antigen expression. These data have been instrumental for the design of the first clinical trial of DNA tattoo and we speculate that this *in vitro* model will also be of value for the preclinical optimization of other DNA vaccine delivery strategies.

8.2 Ongoing and Planned Clinical Trials

Currently a first phase I clinical trial is ongoing to evaluate the safety and tolerability of ID DNA tattoo for the treatment of HLA-A2 positive advanced stage melanoma patients. Melanoma forms an interesting target for therapeutic vaccination as there is evidence to suggest that cellular immune responses contribute to the spontaneous regressions that are sporadically observed (133). Furthermore, a large number of melanoma-associated antigens (such as MART-1, tyrosinase and gp100) has been identified and a substantial number of cytotoxic T cell epitopes from these antigens have been mapped. The DNA vaccine that is being used within this first trial encodes a modified (affinity-enhanced) MART-1 epitope fused to tetanus toxin fragment C. The plasmid DNA for this trial was manufactured in our in-house GMP production facility (134), illustrating that clinical translation of DNA vaccines is relatively straightforward as compared to most other vaccine formats. Thus far, the tattoo procedure is well tolerated and no obvious toxicity has been observed. MART-1 specific T cell immunity will be assayed in peripheral blood samples and on skin biopsies from the vaccination site, using both MHC-tetramer staining and IFN-g ELISPOT. Furthermore, serum anti-tetanus toxin antibody titers will be measured in order to monitor the induction of humoral immune responses by the vaccine.

In the near future we will also initiate a phase I clinical trial to evaluate DNA tattoo for the treatment of HPV-16 positive penile- and cervical cancer. HPV-induced malignancies form an excellent target for immunotherapy as the transformed cells express viral proteins, thereby enabling recognition of malignant cells without the danger of targeting healthy cells (135). For this trial we have developed two DNA vaccines directed against the HPV16 E6 and E7 oncogenes and clinical grade production of these plasmids is currently ongoing.

9. OPINION ON USEFULNESS OF INTRADERMAL DNA VACCINATION, LARGE-SCALE USE OF DNA TATTOO, AND FUTURE PERSPECTIVES

As mentioned above, disadvantages of DNA tattoo are the invasiveness of the method, the fact that the tattoo procedure is more time consuming than simple injection and that multiple administrations are required in order to induce high-level immune responses. Because of this we consider the method currently well suitable for the development of therapeutic vaccines for high risk diseases, but not for (prophylactic) mass vaccination. However, when tattoo time can be shortened and/ or the number of administrations can be reduced, for example by improving transfection efficiency and construct immunogenicity, the method may become more suitable for large-scale application. One of the strategies to further improve the efficiency of DNA tattoo vaccination that is currently under evaluation is briefly discussed below.

Thus far, most of our studies have focused on the use of naked DNA. However, from studies in the *ex vivo* human skin model it has been calculated that the transfection efficiency of naked DNA upon tattooing is extremely low: estimated between 1 out of $1x10^{-6}$ to $1x10^{-9}$ copies applied (103). Therefore, there is ample room for improving the transfection efficiency of DNA vaccines applied by DNA tattoo. In a recent study we have evaluated the use of cationic nanoparticles as synthetic delivery vehicles for DNA vaccines (19). Interestingly, these studies revealed that the positive charge of such particles that dramatically enhances transfection efficiency in cell culture systems essentially prevents transfection in human skin. Only when the cationic surface charge of these particles was shielded with polyethylene glycol (PEG), transfection in human skin was apparent. Delivery of a model vaccine using these PEGylated nanoparticles resulted in an increase in transfection efficiency as compared to naked DNA both in *ex vivo* human skin and in mice (in the latter about 2-5 fold depending on the type of nanoparticle). Unfortunately, for this first variant DNA formulation that we have analyzed, no significant increase in immunogenicity was observed in spite of these higher expression levels. These data do however illustrate the value of the combined use of these two models for preclinical DNA vaccine delivery optimization. First, the human skin model allows one to rapidly identify vaccine formulations that yield substantial antigen expression (going from the reasonable assumption that in the absence of substantial antigen expression immunogenicity will be poor). Second, those selected formulations that yield substantial antigen expression in human skin can subsequently be analyzed for immunogenicity within the murine model. As an example, skin electroporation-mediated DNA delivery has shown great promise in both small and large animal models (117), and the combined use of DNA tattoo and electroporation may well be evaluated by combining these two models.

10. CONCLUSION

DNA tattoo has progressed from the first preclinical evaluation to clinical testing in a period of approximately 5 years, and based on its preclinical track record ID DNA tattoo can be considered a promising strategy for DNA vaccination. At present the two main priorities will be to evaluate the current strategy for DNA tattoo in clinical trials, while at the same time developing optimized strategies in preclinical models that can be evaluated in follow-up trials.

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

REFERENCE LIST

1. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A et al. Direct gene transfer into mouse muscle in vivo. *Science* 1990;247:1465-8.
2. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature* 1992;356:152-4.
3. Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y et al. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* 1993;90:4156-60.
4. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993;259:1745-9.
5. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. *Annu Rev Immunol* 1997;15:617-48.
6. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. *Annu Rev Immunol* 2000;18: 927-74.
7. Donnelly J, Berry K, Ulmer JB. Technical and regulatory hurdles for DNA vaccines. *Int J Parasitol* 2003;33:457-67.
8. Liu MA, Ulmer JB. Human clinical trials of plasmid DNA vaccines. *Adv Genet* 2005;55: 25-40.
9. Lu S, Wang S, Grimes-Serrano JM. Current progress of DNA vaccine studies in humans. *Expert Rev Vaccines* 2008;7:175-91.
10. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med* 2003;9: 729-35.
11. Lu S. Immunogenicity of DNA vaccines in humans: it takes two to tango. *Hum Vaccin* 2008;4:449-52.
12. Redding L, Weiner DB. DNA vaccines in veterinary use. *Expert Rev Vaccines* 2009;8:1251-76.
13. Jones S, Evans K, McElwaine-Johnn H, Sharpe M, Oxford J, Lambkin-Williams R et al. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. *Vaccine* 2009;27:2506-12.
14. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet* 2008;9:776-88.
15. Liu WJ, Gao F, Zhao KN, Zhao W, Fernando GJ, Thomas R et al. Codon modified human papillomavirus type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. *Virology* 2002;301:43-52.
16. Lin CT, Tsai YC, He L, Calizo R, Chou HH, Chang TC et al. A DNA vaccine encoding a codon-optimized human papillomavirus type 16 E6 gene enhances CTL response and anti-tumor activity. *J Biomed Sci* 2006;13:481-8.
17. Sun J, Li D, Hao Y, Zhang Y, Fan W, Fu J et al. Posttranscriptional regulatory elements enhance antigen expression and DNA vaccine efficacy. *DNA Cell Biol* 2009;28:233-40.
18. Garg S, Oran AE, Hon H, Jacob J. The hybrid cytomegalovirus enhancer/chicken beta-actin promoter along with woodchuck hepatitis virus posttranscriptional regulatory element enhances the protective efficacy of DNA vaccines. *J Immunol* 2004;173:550-8.
19. van den Berg JH, Oosterhuis K, Hennink WE, Storm G, van der Aa LJ, Engbersen JF et al. Shielding the cationic charge of nanoparticle-formulated dermal DNA vaccines is essential for antigen expression and immunogenicity. *J Control Release* 2009.
20. van den Berg JH, Nuijen B, Schumacher TN, Haanen JB, Storm G, Beijnen JH et al. Synthetic vehicles for DNA vaccination. *J Drug Target* 2009.
21. Hedley ML, Curley J, Urban R. Microspheres containing plasmid-encoded antigens elicit cytotoxic T-cell responses. *Nat Med* 1998;4:365-8.
22. Klencke B, Matijevic M, Urban RG, Lathey JL, Hedley ML, Berry M et al. Encapsulated plasmid DNA treatment for human papillomavirus 16-associated anal dysplasia: a Phase I study of ZYC101. *Clin Cancer Res* 2002;8:1028-37.
23. Stevenson FK, Rice J, Ottensmeier CH, Thirdborough SM, Zhu D. DNA fusion gene vaccines against cancer: from the laboratory to the clinic. *Immunol Rev* 2004;199:156-80.
24. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM et al. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res* 2000;60:1035-42.
25. Cheng WF, Hung CF, Chai CY, Hsu KF, He L, Ling M et al. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J Clin Invest* 2001;108:669-78.
26. Brulet JM, Maudoux F, Thomas S, Thielemans K, Burny A, Leo O et al. DNA vaccine encoding endosome-targeted human papillomavirus type 16 E7 protein generates CD4+ T cell-dependent protection. *Eur J Immunol* 2007;37:376-84.
27. Smahel M, Pokorna D, Mackova J, Vlasak J. Enhancement of immunogenicity of HPV16 E7 oncogene by fusion with E. coli beta-glucuronidase. *J Gene Med* 2004;6:1092-101.
28. Kim TW, Hung CF, Kim JW, Juang J, Chen PJ, He L et al. Vaccination with a DNA vaccine encoding

herpes simplex virus type 1 VP22 linked to antigen generates long-term antigen-specific CD8-positive memory T cells and protective immunity. *Hum Gene Ther* 2004;15:167-77.

29. Michel N, Osen W, Gissmann L, Schumacher TN, Zentgraf H, Muller M. Enhanced immunogenicity of HPV16 E7 fusion proteins in DNA vaccination. *Virology* 2002;294:47-59.
30. Scheerlinck JY. Genetic adjuvants for DNA vaccines. *Vaccine* 2001;19:2647-56.
31. Barouch DH, Letvin NL, Seder RA. The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses. *Immunol Rev* 2004;202:266-74.
32. Abdulhaqq SA, Weiner DB. DNA vaccines: developing new strategies to enhance immune responses. *Immunol Res* 2008;42:219-32.
33. Kutzler MA, Kraynyak KA, Nagle SJ, Parkinson RM, Zharikova D, Chattergoon M et al. Plasmids encoding the mucosal chemokines CCL27 and CCL28 are effective adjuvants in eliciting antigen-specific immunity in vivo. *Gene Ther* 2010;17:72-82.
34. Yamano T, Kaneda Y, Huang S, Hiramatsu SH, Hoon DS. Enhancement of immunity by a DNA melanoma vaccine against TRP2 with CCL21 as an adjuvant. *Mol Ther* 2006;13:194-202.
35. Calarota SA, Weiner DB. Enhancement of human immunodeficiency virus type 1-DNA vaccine potency through incorporation of T-helper 1 molecular adjuvants. *Immunol Rev* 2004;199:84-99.
36. Iwasaki A, Stiernholm BJ, Chan AK, Berinstein NL, Barber BH. Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J Immunol* 1997;158:4591-601.
37. Muthumani G, Laddy DJ, Sundaram SG, Fagone P, Shedlock DJ, Kannan S et al. Co-immunization with an optimized plasmid-encoded immune stimulatory interleukin, high-mobility group box 1 protein, results in enhanced interferon-gamma secretion by antigen-specific CD8 T cells. *Immunology* 2009;128:e612-e620.
38. Thomsen LL, Topley P, Daly MG, Brett SJ, Tite JP. Imiquimod and resiquimod in a mouse model: adjuvants for DNA vaccination by particle-mediated immunotherapeutic delivery. *Vaccine* 2004;22:1799-809.
39. Schneeberger A, Wagner C, Zemann A, Luhrs P, Kutil R, Goos M et al. CpG motifs are efficient adjuvants for DNA cancer vaccines. *J Invest Dermatol* 2004;123:371-9.
40. Jechlinger W. Optimization and delivery of plasmid DNA for vaccination. *Expert Rev Vaccines* 2006;5:803-25.
41. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. *Nat Rev Cancer* 2008;8:108-20.
42. Nagata T, Aoshi T, Uchijima M, Suzuki M, Koide Y. Cytotoxic T-lymphocyte-, and helper T-lymphocyte-oriented DNA vaccination. *DNA Cell Biol* 2004;23:93-106.
43. Liu MA. The immunologist's grail: vaccines that generate cellular immunity. *Proc Natl Acad Sci U S A* 1997;94:10496-8.
44. Li M, Davey GM, Sutherland RM, Kurts C, Lew AM, Hirst C et al. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J Immunol* 2001;166:6099-103.
45. Draper SJ, Heeney JL. Viruses as vaccine vectors for infectious diseases and cancer. *Nat Rev Microbiol* 2009.
46. Robert-Guroff M. Replicating and non-replicating viral vectors for vaccine development. *Curr Opin Biotechnol* 2007;18:546-56.
47. Limbach KJ, Richie TL. Viral vectors in malaria vaccine development. *Parasite Immunol* 2009;31:501-19.
48. Wilson CB, Marcuse EK. Vaccine safety--vaccine benefits: science and the public's perception. *Nat Rev Immunol* 2001;1:160-5.
49. O'Hagan DT, Rappuoli R. The safety of vaccines. *Drug Discov Today* 2004;9:846-54.
50. Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgonne H, Kimman TG. Preclinical and clinical safety studies on DNA vaccines. *Hum Vaccin* 2006;2:45-53.
51. Parker SE, Monteith D, Horton H, Hof R, Hernandez P, Vilalta A et al. Safety of a GM-CSF adjuvant-plasmid DNA malaria vaccine. *Gene Ther* 2001;8:1011-23.
52. Parker SE, Borellini F, Wenk ML, Hobart P, Hoffman SL, Hedstrom R et al. Plasmid DNA malaria vaccine: tissue distribution and safety studies in mice and rabbits. *Hum Gene Ther* 1999;10:741-58.
53. Ledwith BJ, Manam S, Troilo PJ, Barnum AB, Pauley CJ, Griffiths TG et al. Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. *Intervirology* 2000;43:258-72.
54. Wang Z, Troilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB et al. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Ther* 2004;11:711-21.
55. Gao YS, Huang TH, Wang D, Xie QD, Kang XJ. In vivo study on vertical transmission of the HIV-1 gag gene via mouse oocytes. *Curr HIV Res* 2009;7:562-8.
56. Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB et al. Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirology* 2000;43:273-81.

57. Jeon S, Allen-Hoffmann BL, Lambert PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J Virol* 1995;69:2989-97.
58. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002;20:709-60.
59. Isenberg DA, Manson JJ, Ehrenstein MR, Rahman A. Fifty years of anti-ds DNA antibodies: are we approaching journey's end? *Rheumatology (Oxford)* 2007;46:1052-6.
60. Smith HA, Klinman DM. The regulation of DNA vaccines. *Curr Opin Biotechnol* 2001;12:299-303.
61. Iwasaki A, Torres CA, Ohashi PS, Robinson HL, Barber BH. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J Immunol* 1997;159:11-4.
62. Dupuis M, Denis-Mize K, Woo C, Goldbeck C, Selby MJ, Chen M et al. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. *J Immunol* 2000;165:2850-8.
63. Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med* 1998;188:1075-82.
64. Norbury CC, Basta S, Donohue KB, Tscharke DC, Princiotta MF, Berglund P et al. CD8+ T cell cross-priming via transfer of proteasome substrates. *Science* 2004;304:1318-21.
65. Wolkers MC, Brouwenstijn N, Bakker AH, Toebes M, Schumacher TN. Antigen bias in T cell cross-priming. *Science* 2004;304:1314-7.
66. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD, Jr. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* 1996;2:1122-8.
67. Chattergoon MA, Robinson TM, Boyer JD, Weiner DB. Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells. *J Immunol* 1998;160:5707-18.
68. Torres CA, Iwasaki A, Barber BH, Robinson HL. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J Immunol* 1997;158:4529-32.
69. Cho JH, Youn JW, Sung YC. Cross-priming as a predominant mechanism for inducing CD8(+) T cell responses in gene gun DNA immunization. *J Immunol* 2001;167:5549-57.
70. Ulmer JB, Deck RR, DeWitt CM, Donnhly JI, Liu MA. Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunology* 1996;89:59-67.
71. Shedlock DJ, Weiner DB. DNA vaccination: antigen presentation and the induction of immunity. *J Leukoc Biol* 2000;68:793-806.
72. Laddy DJ, Weiner DB. From plasmids to protection: a review of DNA vaccines against infectious diseases. *Int Rev Immunol* 2006;25:99-123.
73. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. *Annu Rev Immunol* 2001;19:47-64.
74. Maecker HT, Umetsu DT, DeKruyff RH, Levy S. Cytotoxic T cell responses to DNA vaccination: dependence on antigen presentation via class II MHC. *J Immunol* 1998;161:6532-6.
75. Bins AD, Wolkers MC, van den Boom MD, Haanen JB, Schumacher TN. In vivo antigen stability affects DNA vaccine immunogenicity. *J Immunol* 2007;179:2126-33.
76. Wolkers MC, Toebes M, Okabe M, Haanen JB, Schumacher TN. Optimizing the efficacy of epitope-directed DNA vaccination. *J Immunol* 2002;168:4998-5004.
77. Wu TC. Therapeutic human papillomavirus DNA vaccination strategies to control cervical cancer. *Eur J Immunol* 2007;37:310-4.
78. Boyle JS, Koniaras C, Lew AM. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int Immunol* 1997;9:1897-906.
79. Kim SA, Liang CM, Cheng IC, Cheng YC, Chiao MT, Tseng CJ et al. DNA vaccination against foot-and-mouth disease via electroporation: study of molecular approaches for enhancing VP1 antigenicity. *J Gene Med* 2006;8:1182-91.
80. Rice J, King CA, Spellerberg MB, Fairweather N, Stevenson FK. Manipulation of pathogen-derived genes to influence antigen presentation via DNA vaccines. *Vaccine* 1999;17:3030-8.
81. Matzinger P. The danger model: a renewed sense of self. *Science* 2002;296:301-5.
82. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006;124:849-63.
83. Tang CK, Pietersz GA. Intracellular detection and immune signaling pathways of DNA vaccines. *Expert Rev Vaccines* 2009;8:1161-70.
84. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004;5:987-95.
85. Babiuk S, Mookherjee N, Pontarollo R, Griebel P, van Drunen Littel-van den Hurk, Hecker R et al. TLR9-/- and TLR9+/+ mice display similar immune responses to a DNA vaccine. *Immunology* 2004;113:114-20.

86. Spies B, Hochrein H, Vabulas M, Huster K, Busch DH, Schmitz F et al. Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J Immunol* 2003;171:5908-12.
87. Coban C, Koyama S, Takeshita F, Akira S, Ishii KJ. Molecular and cellular mechanisms of DNA vaccines. *Hum Vaccin* 2008;4:453-6.
88. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* 2007;448:501-5.
89. Schroder K, Muruve DA, Tschopp J. Innate immunity: cytoplasmic DNA sensing by the AIM2 inflammasome. *Curr Biol* 2009;19:R262-R265.
90. Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T et al. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* 2008;451:725-9.
91. Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 2009;461:788-92.
92. Saitoh T, Fujita N, Hayashi T, Takahara K, Satoh T, Lee H et al. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc Natl Acad Sci U S A* 2009.
93. Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol* 2009;9:679-91.
94. Liu L, Zhong Q, Tian T, Dubin K, Athale SK, Kupper TS. Epidermal injury and infection during poxvirus immunization is crucial for the generation of highly protective T cell-mediated immunity. *Nat Med* 2010.
95. Chiarella P, Massi E, De Robertis M, Sibilio A, Parrella P, Fazio VM et al. Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration. *Expert Opin Biol Ther* 2008;8:1645-57.
96. Peng B, Zhao Y, Xu L, Xu Y. Electric pulses applied prior to intramuscular DNA vaccination greatly improve the vaccine immunogenicity. *Vaccine* 2007;25:2064-73.
97. Pulendran B. Immune activation: death, danger and dendritic cells. *Curr Biol* 2004;14:R30-R32.
98. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006;124:849-63.
99. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med* 2005;11:899-904.
100. Zaba LC, Krueger JG, Lowes MA. Resident and “inflammatory” dendritic cells in human skin. *J Invest Dermatol* 2009;129:302-8.
101. Pokorna D, Polakova I, Kindlova M, Duskova M, Ludvikova V, Gabriel P et al. Vaccination with human papillomavirus type 16-derived peptides using a tattoo device. *Vaccine* 2009;27:3519-29.
102. Potthoff A, Schwannecke S, Nabi G, Hoffmann D, Grunwald T, Wildner O et al. Immunogenicity and efficacy of intradermal tattoo immunization with adenoviral vector vaccines. *Vaccine* 2009;27:2768-74.
103. van den Berg JH, Nujien B, Beijnen JH, Vincent A, van Tinteren H, Kluge J et al. Optimization of intradermal vaccination by DNA tattooing in human skin. *Hum Gene Ther* 2009;20:181-9.
104. Harden LM, du Plessis I, Poole S, Laburn HP. Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behavior. *Physiol Behav* 2006;89:146-55.
105. van den Berg JH, Quaak SG, Beijnen JH, Hennink WE, Storm G, Schumacher TN et al. Lipopolysaccharide contamination in intradermal DNA vaccination: Toxic impurity or adjuvant? *Int J Pharm* 2009.
106. Dean HJ, Fuller D, Osorio JE. Powder and particle-mediated approaches for delivery of DNA and protein vaccines into the epidermis. *Comp Immunol Microbiol Infect Dis* 2003;26:373-88.
107. Johnston SA, Talaat AM, McGuire MJ. Genetic immunization: what’s in a name? *Arch Med Res* 2002;33:325-9.
108. Pokorna D, Rubio I, Muller M. DNA-vaccination via tattooing induces stronger humoral and cellular immune responses than intramuscular delivery supported by molecular adjuvants. *Genet Vaccines Ther* 2008;6:4.
109. Verstrepen BE, Bins AD, Rollier CS, Mooij P, Koopman G, Sheppard NC et al. Improved HIV-1 specific T-cell responses by short-interval DNA tattooing as compared to intramuscular immunization in non-human primates. *Vaccine* 2008;26:3346-51.
110. Dean HJ, Haynes J, Schmaljohn C. The role of particle-mediated DNA vaccines in biodefense preparedness. *Adv Drug Deliv Rev* 2005;57:1315-42.
111. Bodles-Brakhop AM, Heller R, Draghia-Akli R. Electroporation for the delivery of DNA-based vaccines and immunotherapeutics: current clinical developments. *Mol Ther* 2009;17:585-92.
112. Mir LM. Application of electroporation gene therapy: past, current, and future. *Methods Mol Biol* 2008;423:3-17.
113. Babiuk S, Baca-Estrada ME, Foldvari M, Storms M, Rabussay D, Widera G et al. Electroporation improves the efficacy of DNA vaccines in large animals. *Vaccine* 2002;20:3399-408.

114. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum Gene Ther* 2001;12:861-70.
115. Widera G, Austin M, Rabussay D, Goldbeck C, Barnett SW, Chen M et al. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. *J Immunol* 2000;164:4635-40.
116. Roos AK, Moreno S, Leder C, Pavlenko M, King A, Pisa P. Enhancement of cellular immune response to a prostate cancer DNA vaccine by intradermal electroporation. *Mol Ther* 2006;13:320-7.
117. Roos AK, Eriksson F, Timmons JA, Gerhardt J, Nyman U, Gudmundsdotter L et al. Skin electroporation: effects on transgene expression, DNA persistence and local tissue environment. *PLoS One* 2009;4:e7226.
118. Best SR, Peng S, Juang CM, Hung CF, Hannaman D, Saunders JR et al. Administration of HPV DNA vaccine via electroporation elicits the strongest CD8+ T cell immune responses compared to intramuscular injection and intradermal gene gun delivery. *Vaccine* 2009;27:5450-9.
119. Hirao LA, Wu L, Khan AS, Satishchandran A, Draghia-Akli R, Weiner DB. Intradermal/ subcutaneous immunization by electroporation improves plasmid vaccine delivery and potency in pigs and rhesus macaques. *Vaccine* 2008;26:440-8.
120. Mir LM. Application of electroporation gene therapy: past, current, and future. *Methods Mol Biol* 2008;423:3-17.
121. Rabussay D. Applicator and electrode design for in vivo DNA delivery by electroporation. *Methods Mol Biol* 2008;423:35-59.
122. Luckay A, Sidhu MK, Kjeken R, Megati S, Chong SY, Roopchand V et al. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *J Virol* 2007;81:5257-69.
123. Mitragotri S. Immunization without needles. *Nat Rev Immunol* 2005;5:905-16.
124. Trimble C, Lin CT, Hung CF, Pai S, Juang J, He L et al. Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. *Vaccine* 2003;21:4036-42.
125. Rao SS, Gomez P, Mascola JR, Dang V, Krivulka GR, Yu F et al. Comparative evaluation of three different intramuscular delivery methods for DNA immunization in a nonhuman primate animal model. *Vaccine* 2006;24:367-73.
126. Birchall J, Coulman S, Pearton M, Allender C, Brain K, Anstey A et al. Cutaneous DNA delivery and gene expression in ex vivo human skin explants via wet-etch micro-fabricated micro-needles. *J Drug Target* 2005;13:415-21.
127. Arnou R, Icardi G, De Decker M, Ambrozaitis A, Kazek MP, Weber F et al. Intradermal influenza vaccine for older adults: a randomized controlled multicenter phase III study. *Vaccine* 2009;27:7304-12.
128. Van Damme P, Oosterhuis-Kafeja F, Van der Wielen M, Almagor Y, Sharon O, Levin Y. Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults. *Vaccine* 2009;27:454-9.
129. Zhou Q, Wang F, Yang F, Wang Y, Zhang X, Sun S. Augmented humoral and cellular immune response of hepatitis B virus DNA vaccine by micro-needle vaccination using Flt3L as an adjuvant. *Vaccine* 2009.
130. McCluskie MJ, Brazolot Millan CL, Gramzinski RA, Robinson HL, Santoro JC, Fuller JT et al. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. *Mol Med* 1999;5:287-300.
131. Ren S, Li M, Smith JM, DeTolla LJ, Furth PA. Low-volume jet injection for intradermal immunization in rabbits. *BMC Biotechnol* 2002;2:10.
132. Godin B, Touitou E. Transdermal skin delivery: predictions for humans from in vivo, ex vivo and animal models. *Adv Drug Deliv Rev* 2007;59:1152-61.
133. van Oijen M, Bins A, Elias S, Sein J, Weder P, de Gast G et al. On the role of melanoma-specific CD8+ T-cell immunity in disease progression of advanced-stage melanoma patients. *Clin Cancer Res* 2004;10:4754-60.
134. Quaak SG, van den Berg JH, Toebes M, Schumacher TN, Haanen JB, Beijnen JH et al. GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial. *Eur J Pharm Biopharm* 2008;70:429-38.
135. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nat Rev Immunol* 2004;4:46-54.

CHAPTER 3

PRECLINICAL DEVELOPMENT OF HIGHLY EFFECTIVE AND SAFE DNA VACCINES DIRECTED AGAINST HPV16 E6 AND E7

3

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.

Koen Oosterhuis[1], Peter Öhlschläger[2], Joost van den Berg[1,3], Mireille Toebes[1], Raquel Gomez[1], Ton N. Schumacher[1] and John B. Haanen[1,#]

[1] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands; [2] Department of Immunology, University of Constance, Universitätsstrasse 10, 78457 Konstanz, Germany; [3] Department of Pharmacy & Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC, Amsterdam, The Netherlands

Int. J. Cancer: 129, 397–406 (2011)

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-$E7_{49-57}$ has been described elsewhere (26). GFP-$E6_{48-57}$ encodes the immunodominant H-$2K^b$–restricted epitope EVYDFAFRDL as a genetic fusion with GFP and was constructed in an analogous manner as GFP-E7 $_{49-57}$, 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 NH_4Cl, 10mM $KHCO_3$, 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-$2D^b$ $E7_{49–57}$ or H-$2K^b$ $E6_{48-57}$ 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 $E7_{49-57}$ 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 $2 * 10^6$ cells per mouse. After 20 h, spleen cells were isolated and the ratio of $CFSE_{high}$/ $CFSE_{low}$ cells was determined by flow cytometry. The percentage antigen-specific cytotoxicity was determined as follows: 100 - ([% $CFSE_{high}$ in vaccinated/ % $CFSE_{low}$ in vaccinated) / (% $CFSE_{high}$ in naive / % $CFSE_{low}$ in naive)] * 100).

TC-1 tumor challenge

C57BL/6 mice were injected subcutaneously with $1*10^5$ 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 = ($width^2$ x length)/2 (30) Mice were sacrificed when the tumor diameter reached 15 mm or when the tumor volume exceeded $1000mm^3$.

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.

3

RESULTS

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

We first compared the immunogenicity of the immunodominant HPV16 $E7_{49\text{-}57}$ 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), *ex vivo* MHC tetramer staining of peripheral blood of mice that had received a DNA vaccine encoding GFP-$E7_{49\text{-}57}$ 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-$E7_{49\text{-}57}$). 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-$E7_{49\text{-}57}$). 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-$E6_{48\text{-}57}$ 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 $E7_{49\text{-}57}$ 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 *ex vivo*.

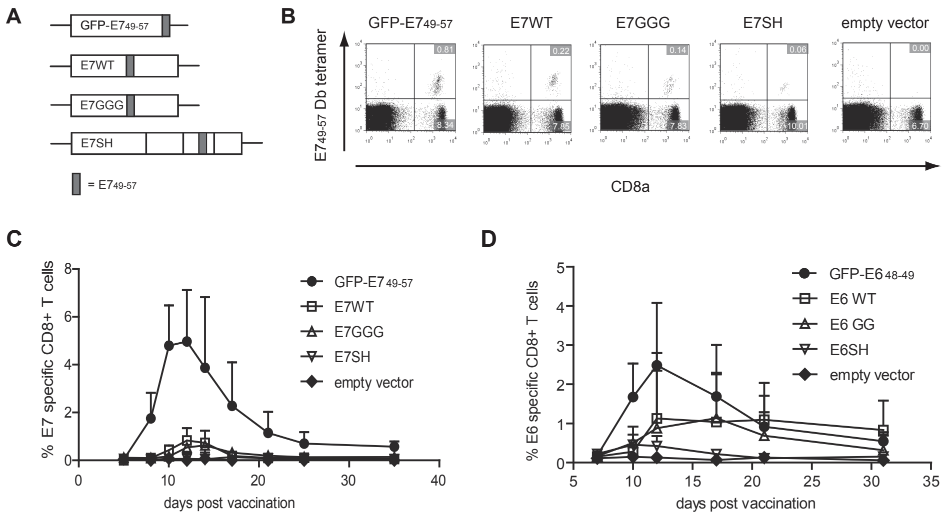


Figure 1. Transgene design and immunogenicity of HPV16 E7 and E6 encoding DNA vaccines. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific CD8$^+$ T cells by MHC tetramer staining. **A)** Schematic representation of the E7 directed DNA vaccines. GFP-E7$_{49-57}$ encodes the immunodominant H-2 D^b–restricted epitope of HPV16 E7$_{49-57}$ as a C-terminal fusion with eGFP. E7GGG encodes an E7 variant with 3 point mutations in the pRb binding domain: D21G, C24G and E26G (11). E7SH encodes a 'gene-shuffled' variant of E7 (23). HPV E6 directed DNA vaccines with corresponding names were constructed in an identical fashion (not shown). **B)** Representative dot plots of MHC tetramer stainings at the peak of the response for the different E7 directed constructs **C)** Plot depicting the mean percentage +/- S.D. of H-2D^b E7$_{49-57}$-specific CD8$^+$ T cells for the indicated groups over time. **D)** Plot depicting the mean percentage +/- S.D. of H-2K^b E6$_{48-57}$-specific CD8$^+$ T cells for the indicated groups over time.

Functional characterization of TTFC E7 induced long-term persisting cells

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 E7$_{49-57}$ 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$^+$

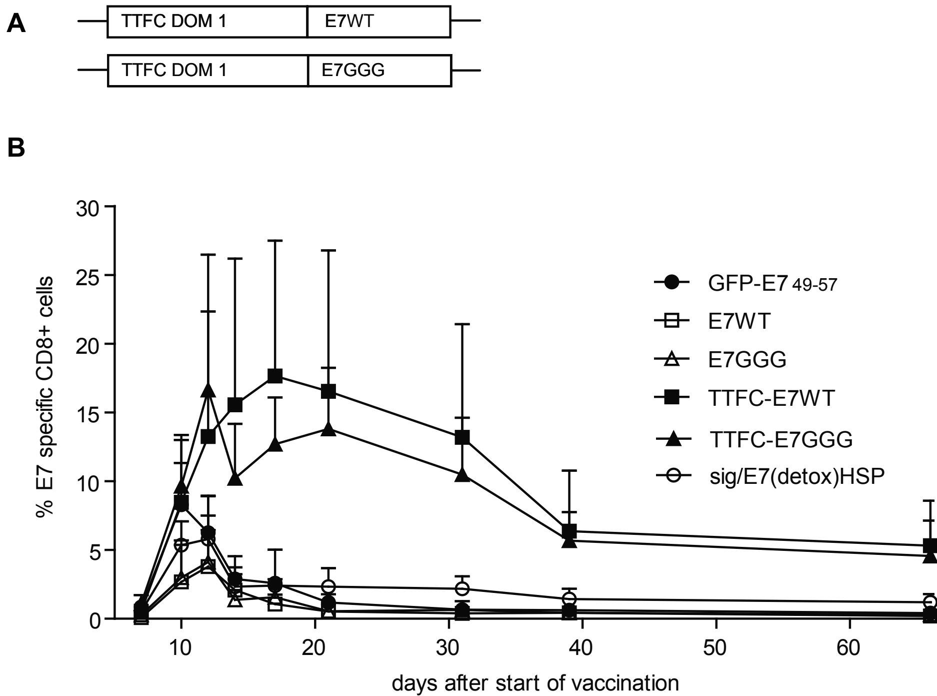


Figure 2. TTFC fusion enhances DNA vaccine immunogenicity. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific $CD8^+$ T cells by MHC tetramer staining **A)** Schematic representation of E7 variants that were expressed as C-terminal fusions with Tetanus Toxin Fragment C domain 1 (TTFC) (31). **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.

T cells, the ability to kill peptide loaded target cells was tested in an *in vivo* cytolytic assay 6 weeks after priming with either TTFC-E7GGG or E7GGG (Fig. 4). In TTFC-E7GGG vaccinated mice, 59,1 +/-13.7% specific lysis of target cells was observed compared to only 15,9 +/-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 TTCF 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

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, $p< 0.01$).

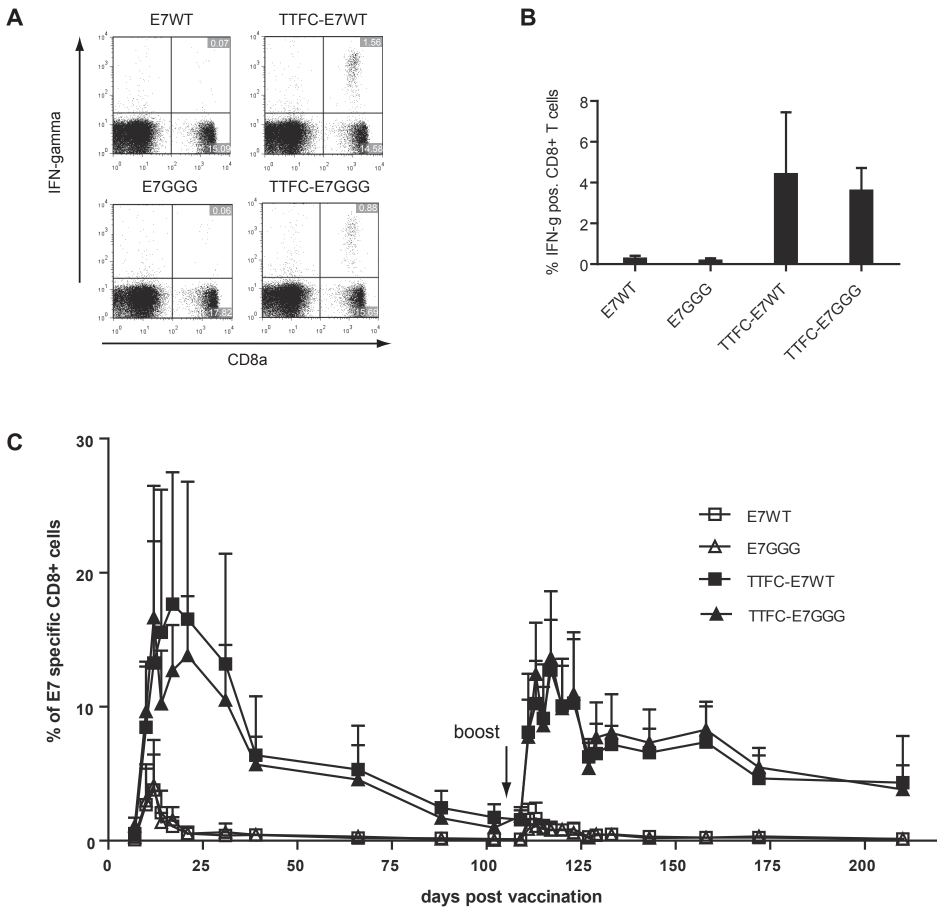


Figure 3. Long-term persisting $E7_{49-57}$-specific CD8⁺ T cells are functional in terms of interferon-γ production and secondary expansion. Interferon-γ production of peripheral blood CD8⁺ cells from indicated groups of the experiment displayed in fig. 2 was assayed 4 weeks after start of vaccination. **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 $E7_{49-57}$ 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 $E7_{49-57}$-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,

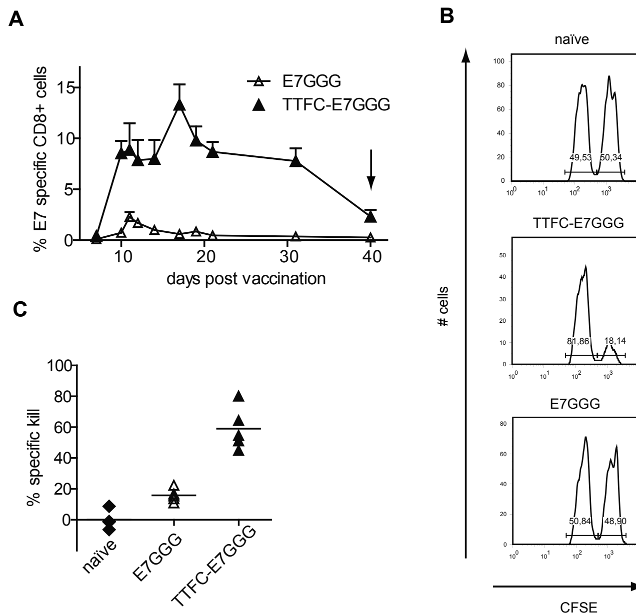


Figure 4. Cytolytic activity of long-term persisting $E7_{49-57}$-specific $CD8^+$ T cells. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific $CD8^+$ T cells by MHC tetramer staining. **A)** Plot depicting the percentage +/-S.D. of $H\text{-}2D^b$ $E7_{49-57}$-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.

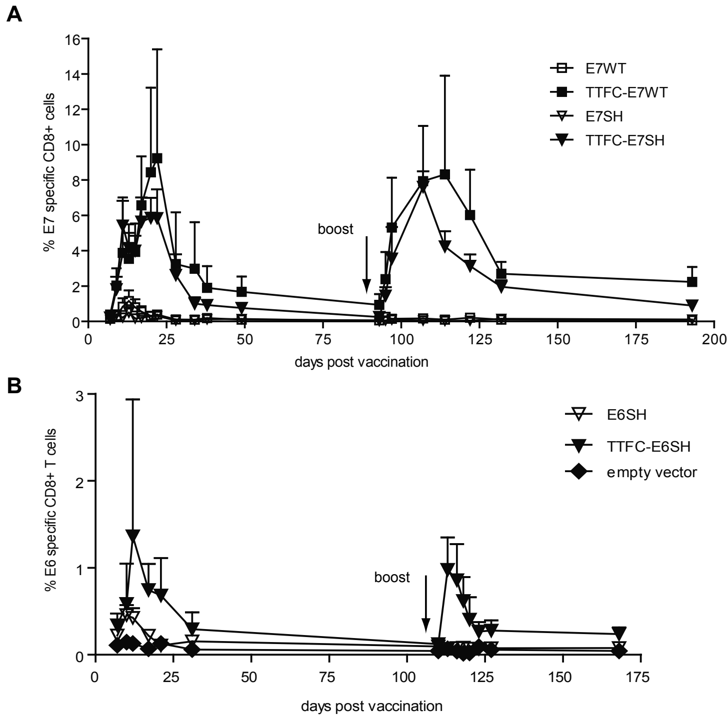


Figure 5. TTFC fusion enhances the immunogenicity of E7SH and E6SH. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and boosted with a single homologous tattoo vaccination at the indicated time point. Peripheral blood was analyzed for antigen-specific $CD8^+$ T cells by MHC tetramer staining. **A)** Plot depicting the mean percentage +/- S.D. of H-$2D^b$ $E7_{49-57}$ specific $CD8^+$ T cells for the indicated groups is displayed over time. **B)** Plot depicting the mean percentage +/- S.D. of H-$2K^b$ $E6_{48-57}$-specific $CD8^+$ T cells for the indicated groups over time.

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

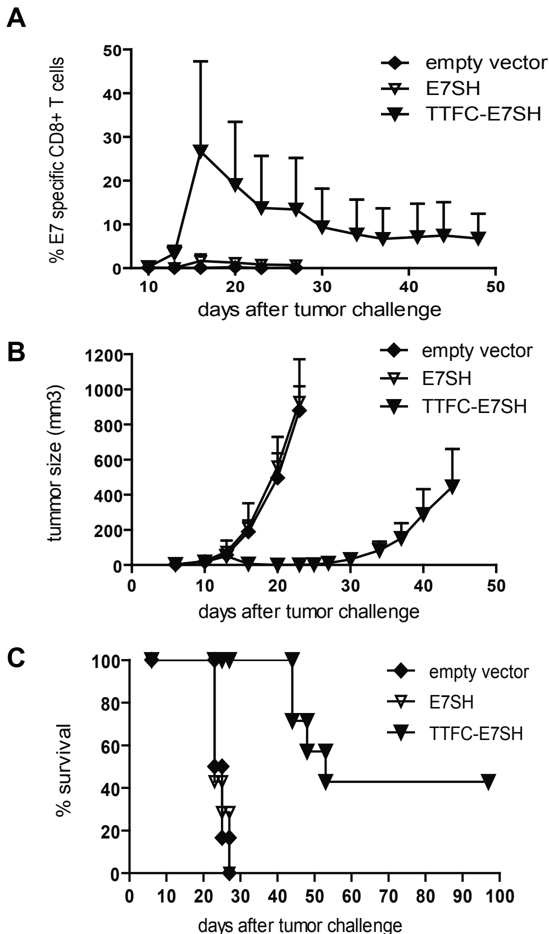


Figure 6. Tumor regression by vaccination with the TTFC-E7SH fusion vaccine. C57BL/6 mice (n=5-7 per group) were injected with $1*10^5$ TC-1 tumor cells on day 0. Subsequently, mice were immunized by DNA tattoo vaccination on day 3, 6 and 9 after tumor challenge with the indicated vaccines. Tumor sizes 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-$2D^b$ $E7_{49-57}$-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.

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).

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 1 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-$E7_{49-57}$ 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 $CD8^+$ 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.

REFERENCE LIST

1. Scheurer ME, Tortolero-Luna G, Adler-Storthz K. Human papillomavirus infection: biology, epidemiology, and prevention. *Int J Gynecol Cancer* 2005;15:727-46.
2. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* 2002;55:244-65.
3. Roden R, Wu TC. How will HPV vaccines affect cervical cancer? *Nat Rev Cancer* 2006;6:753-63.
4. Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;2:342-50.
5. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet* 2008;9:776-88.
6. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. *Nat Rev Cancer* 2008;8:108-20.
7. Hung CF, Monie A, Alvarez RD, Wu TC. DNA vaccines for cervical cancer: from bench to bedside. *Exp Mol Med* 2007;39:679-89.
8. Michel N, Osen W, Gissmann L, Schumacher TN, Zentgraf H, Muller M. Enhanced immunogenicity of HPV16 E7 fusion proteins in DNA vaccination. *Virology* 2002;294:47-59.
9. Osen W, Peiler T, Ohlschlager P, Caldeira S, Faath S, Michel N et al. A DNA vaccine based on a shuffled E7 oncogene of the human papillomavirus type 16 (HPV16) induces E7-specific cytotoxic T cells but lacks transforming activity. *Vaccine* 2001;19:4276-86.
10. Peng S, Ji H, Trimble C, He L, Tsai YC, Yeatermeyer J et al. Development of a DNA vaccine targeting human papillomavirus type 16 oncoprotein E6. *J Virol* 2004;78:8468-76.
11. Smahel M, Sima P, Ludvikova V, Vonka V. Modified HPV16 E7 Genes as DNA Vaccine against E7-Containing Oncogenic Cells. *Virology* 2001;281:231-8.
12. Smahel M, Pokorna D, Mackova J, Vlasak J. Enhancement of immunogenicity of HPV16 E7 oncogene by fusion with E. coli beta-glucuronidase. *J Gene Med* 2004;6:1092-101.
13. Haigh O, Kattenbelt J, Cochrane M, Thomson S, Gould A, Tindle R. Hepatitis B surface antigen fusions delivered by DNA vaccination elicit CTL responses to human papillomavirus

oncoproteins associated with tumor protection. *Cancer Gene Ther* 2010.

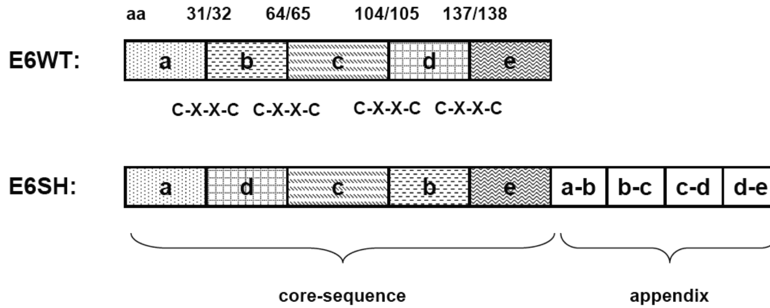
14. Garcia F, Petry KU, Muderspach L, Gold MA, Braly P, Crum CP et al. ZYC101a for treatment of high-grade cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet Gynecol* 2004;103:317-26.
15. Trimble CL, Peng S, Kos F, Gravitt P, Viscidi R, Sugar E et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clin Cancer Res* 2009;15:361-7.
16. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med* 2005;11:899-904.
17. Verstrepen BE, Bins AD, Rollier CS, Mooij P, Koopman G, Sheppard NC et al. Improved HIV-1 specific T-cell responses by short-interval DNA tattooing as compared to intramuscular immunization in non-human primates. *Vaccine* 2008;26:3346-51.
18. Quaak SG, van den Berg JH, Toebes M, Schumacher TN, Haanen JB, Beijnen JH et al. GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial. *Eur J Pharm Biopharm* 2008;70:429-38.
19. Wolkers MC, Toebes M, Okabe M, Haanen JB, Schumacher TN. Optimizing the efficacy of epitope-directed DNA vaccination. *J Immunol* 2002;168:4998-5004.
20. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. *Virology* 2009;384:324-34.
21. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. *Virology* 2009;384:335-44.
22. Cassetti MC, McElhiney SP, Shahabi V, Pullen JK, Le Poole IC, Eiben GL et al. Antitumor efficacy of Venezuelan equine encephalitis virus replicon particles encoding mutated HPV16 E6 and E7 genes. *Vaccine* 2004;22:520-7.
23. Ohlschlager P, Pes M, Osen W, Durst M, Schneider A, Gissmann L et al. An improved rearranged Human Papillomavirus Type 16 E7 DNA vaccine candidate (HPV-16 E7SH) induces an E7 wildtype-specific T cell response. *Vaccine* 2006;24:2880-93.
24. Munger K, Howley PM. Human papillomavirus immortalization and transformation functions. *Virus Res* 2002;89:213-28.
25. Yugawa T, Kiyono T. Molecular mechanisms of cervical carcinogenesis by high-risk human papillomaviruses: novel functions of E6 and E7 oncoproteins. *Rev Med Virol* 2009;19:97-113.
26. Wolkers MC, Stoetter G, Vyth-Dreese FA, Schumacher TN. Redundancy of direct priming and cross-priming in tumor-specific CD8+ T cell responses. *J Immunol* 2001;167:3577-84.
27. Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ et al. Design and use of conditional MHC class I ligands. *Nat Med* 2006;12:246-51.
28. Coccoris M, Swart E, de Witte MA, van Heijst JW, Haanen JB, Schepers K et al. Long-term functionality of TCR-transduced T cells in vivo. *J Immunol* 2008;180:6536-43.
29. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;56:21-6.
30. Scardino A, Alimandi M, Correale P, Smith SG, Bei R, Firat H et al. A polyepitope DNA vaccine targeted to Her-2/ErbB-2 elicits a broad range of human and murine CTL effectors to protect against tumor challenge. *Cancer Res* 2007;67:7028-36.
31. Rice J, Elliott T, Buchan S, Stevenson FK. DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines. *J Immunol* 2001;167:1558-65.
32. Smahel M, Tejklova P, Smahelova J, Polakova I, Mackova J. Mutation in the immunodominant epitope of the HPV16 E7 oncoprotein as a mechanism of tumor escape. *Cancer Immunol Immunother* 2008;57:823-31.
33. Wu TC. The role of vascular cell adhesion molecule-1 in tumor immune evasion. *Cancer Res* 2007;67:6003-6.
34. Boursnell ME, Rutherford E, Hickling JK, Rollinson EA, Munro AJ, Rolley N et al. Construction and characterisation of a recombinant vaccinia virus expressing human papillomavirus proteins for immunotherapy of cervical cancer. *Vaccine* 1996;14:1485-94.
35. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M et al. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* 2004;78:11451-60.
36. Wolkers MC, Brouwenstijn N, Bakker AH, Toebes M, Schumacher TN. Antigen bias in T cell cross-priming. *Science* 2004;304:1314-7.
37. Norbury CC, Basta S, Donohue KB, Tscharke DC, Princiotta MF, Berglund P et al. CD8+ T cell cross-priming via transfer of proteasome substrates. *Science* 2004;304:1318-21.
38. Bins AD, Wolkers MC, van den Boom MD, Haanen JB, Schumacher TN. In vivo antigen stability affects DNA vaccine immunogenicity. *J Immunol* 2007;179:2126-33.
39. Stevenson FK, Rice J, Ottensmeier CH, Thirdborough SM, Zhu D. DNA fusion gene vaccines against cancer: from the laboratory to the clinic. *Immunol Rev* 2004;199:156-80.
40. Chaise C, Buchan SL, Rice J, Marquet J, Rouard H, Kuentz M et al. DNA vaccination induces

WT1-specific T-cell responses with potential clinical relevance. *Blood* 2008;112:2956-64.

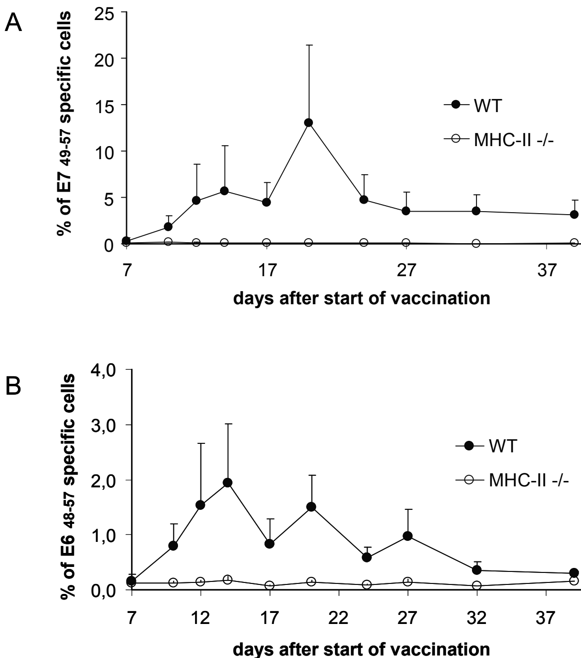
41. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* 2002;2:251-62.

42. Hand TW, Kaech SM. Intrinsic and extrinsic control of effector T cell survival and memory T cell development. *Immunol Res* 2008.

SUPPLEMENTARY FIGURES

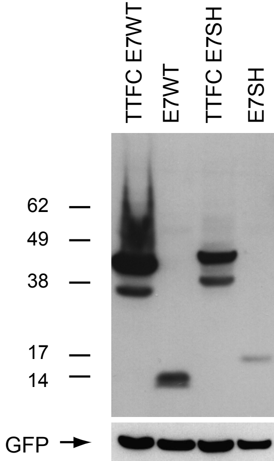


Supplementary figure 1. Schematic representation of E6SH. HPV16 E6 was taken apart at the amino acids positions indicated in the figure, thereby dissecting the C-X-X-C- motifs crucial for interaction with p53 (Crook et al, Cell, 1991). The resulting domains were reassembled in the indicated order, resulting 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'.

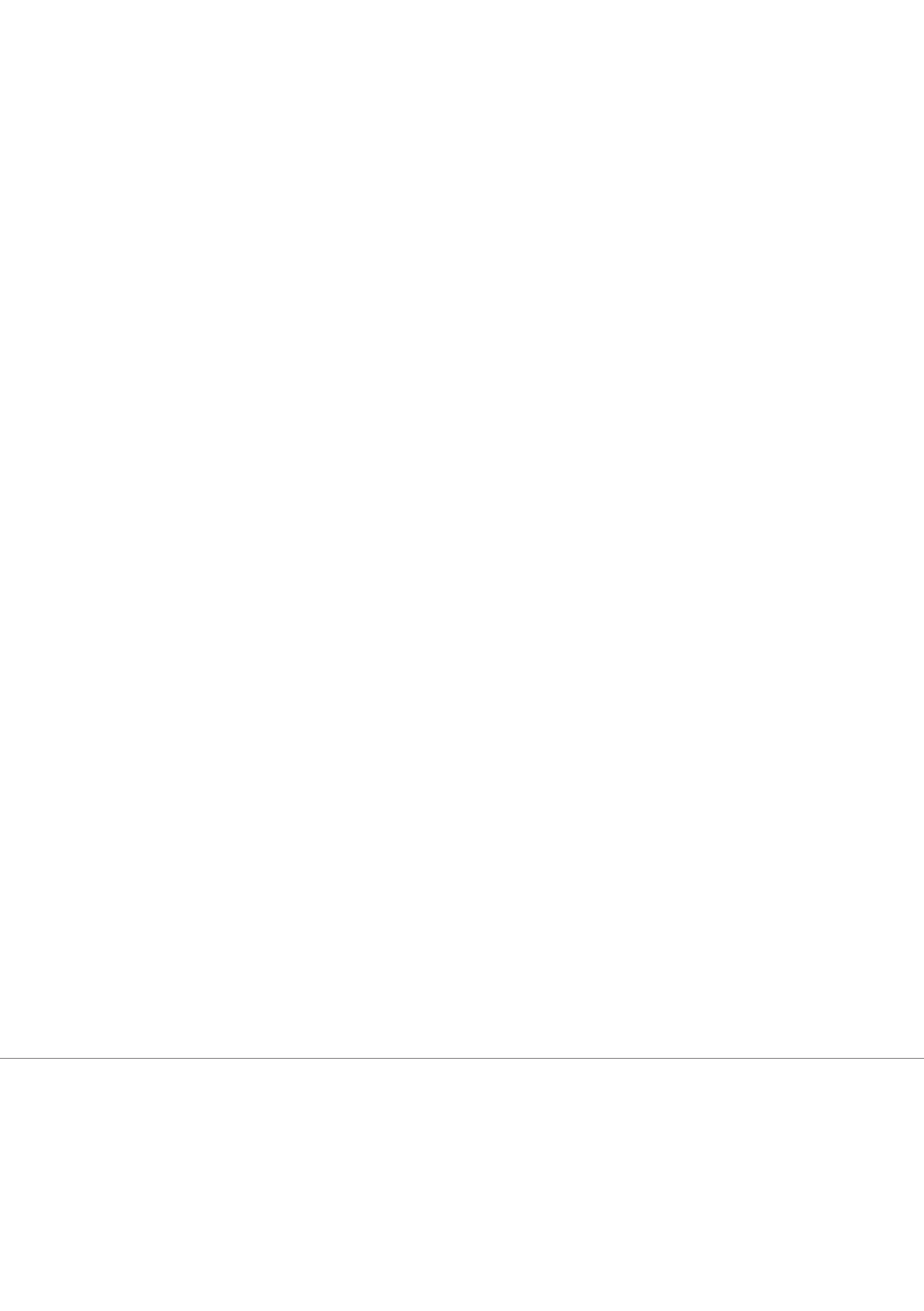


Supplementary figure 2. TTFC-E6SH and TTFC-E7SH specific $CD8^+$ T cell responses are dependent on MHC-II-restricted antigen presentation. WT C57BL/6 mice (n=5 per group) or MHC-$II^{-/-}$ mice (n=4 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific $CD8^+$ T cells by MHC-I tetramer staining. **A)** Plot depicting the percentage +/-S.D. of H-$2D^b$ $E7_{49-57}$-specific $CD8^+$ T cells of the TTFC-E7SH vaccinated mice over time. **B)** Plot depicting the percentage +/-S.D. of H-$2K^b$ $E6_{48-57}$-specific $CD8^+$ T cells of the TTFC-E6SH vaccinated mice over time.

3



Supplementary 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.



CHAPTER 4

PRECLINICAL SAFETY EVALUATION OF DNA VACCINES ENCODING MODIFIED HPV16 E6 AND E7

4

ABSTRACT

Persistent infection with high-risk human papillomaviruses (hrHPV) can result in the formation of anogenital cancers. As hrHPV proteins E6 and E7 are required for cancer initiation and maintenance, they are ideal targets for immunotherapeutic interventions. Previously, we have described the development of DNA vaccines for the induction of HPV16 E6 and E7 specific T cell immunity. These vaccines consist of 'gene-shuffled' (SH) versions of HPV16 E6 and E7 that were fused to Tetanus Toxin Fragment C domain 1 (TTFC) and were named TTFC-E6SH and TTFC-E7SH. Gene-shuffling was performed to avoid the risk of inducing malignant transformation at the vaccination site. Here, we describe the preclinical safety evaluation of these candidate vaccines by analysis of their transforming capacity *in vitro* using established murine fibroblasts (NIH 3T3 cells) and primary human foreskin keratinocytes (HFKs). We demonstrate that neither ectopic expression of TTFC-E6SH and TTFC-E7SH alone or in combination enabled NIH 3T3 cells to form colonies in soft agar. In contrast, expression of HPV16 E6WT and E7WT alone or in combination resulted in effective transformation. Similarly, retroviral transduction of HFKs from three independent donors with both TTFC-E6SH and TTFC-E7SH alone or in combination did not show any signs of immortalization. In contrast, the combined expression of E6WT and E7WT induced immortalization in HFKs from all donors. Based on these results we consider it justified to proceed to clinical evaluation of DNA vaccines encoding TTFC-E6SH and TTFC-E7SH in patients with HPV16 associated (pre) malignancies.

F.E. Henken[1#], K. Oosterhuis[2#], P. Öhlschläger[3], L. Bosch[1], E. Hooijberg[1], J.B.A.G Haanen[2], R.D.M. Steenbergen[1]

[1] Department of Pathology, Unit of Molecular Pathology, VU University Medical Center, PO Box 7057, 1007 MB, Amsterdam, The Netherlands; [2] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands; [3] Department of Chemistry and Biotechnology, University of Applied Sciences Aachen, Heinrich-Mußmann-Straße 1, 52428 Jülich, Germany

[#] These authors contributed equally to this work

Accepted for publication in Vaccine

INTRODUCTION

It has been well established that persistent infection with high-risk human papillomavirus (hrHPV) is causally related to the development of cervical cancer, as well as a subset of other anogenital and head and neck cancers (1-5). Continuous expression of the viral oncogenes E6 and E7 is required for the induction and maintenance of a malignant phenotype (6). The most well studied cellular targets of E6 and E7 are the tumour suppressor proteins p53 and pRb, respectively. HPV16 E6 binds to p53 via the cellular ubiquitin-protein ligase E6AP, resulting in rapid ubiquitin-dependent proteolytic degradation of p53 (7, 8), thereby interfering with the regulatory function of p53. High-risk HPV E7 interacts with pRb (9) and its family members, p107 and p130 (10, 11), resulting in their inactivation and thereby interfering with their control on the G1/S cell cycle transition. The continuous presence of viral proteins in hrHPV-induced malignancies provides a strong rationale for the immunotherapeutic treatment of these diseases (12, 13), as the recognition of these tumour specific antigens would enable the immune system to eradicate the malignant cells without the risk of inducing autoimmunity.

For safety reasons the oncogenic potential of E6 and E7 should be abolished before their use as an antigen in human application. This is particularly important when they are applied as DNA vaccines as low-level integration of introduced plasmid DNA into the host genome may theoretically occur upon DNA vaccination (14, 15). Most commonly, detoxification is achieved by the introduction of point mutations that have been shown to prevent the interaction of E6 and E7 with p53 and pRb, respectively (16-20). A more drastic approach to detoxify E6 and E7 is termed 'gene-shuffling' (21, 22). This method is based on the rearrangement of the primary gene sequences in such a way that the known ligand binding domains are disrupted. The original sequence junctions that are destroyed by the gene-shuffling are added as an appendix to prevent loss of possible T-cell epitopes (see Supplementary figure 1). It has already been demonstrated that a gene-shuffled version of E7 (E7SH) has lost its transforming potential, as determined by the inability of E7SH transduced murine fibroblasts to form colonies in soft agar (21, 23). We have previously reported on the construction and evaluation of the immunogenicity of plasmid DNA vaccines encoding gene-shuffled versions of HPV-16 E6 and E7 that are genetically fused to Tetanus Toxin Fragment C domain 1 (TTFC): TTFC-E6SH and TTFC-E7SH (22) (see also Supplementary figure 1). The fusion with TTFC was shown to be critical to overcome the observed loss in immunogenicity resulting from the gene-shuffling procedure. When applied via DNA tattooing (24), TTFC-E6SH and TTFC-E7SH encoding DNA vaccines induced strong E6 and E7 specific T-cell immunity in mice. Because of these promising pre-clinical data, we are now planning to evaluate these vaccines in patients suffering from HPV16 induced malignancies. In the present study we aimed to confirm that these candidate vaccines indeed have no oncogenic potential, before moving to clinical application. To this end two different assay systems were used as outlined below.

First we compared the ability of TTFC-E6SH and TTFC-E7SH with HPV16 E6 and E7 wild-type to induce colony formation in an established rodent fibroblast cell line (NIH 3T3 cells) in soft agar. Secondly, we wanted to demonstrate the loss of transforming potential in a physiologically more relevant cell type. As we are planning to administer our candidate vaccines via intradermal DNA tattooing, keratinocytes will be primarily transfected upon vaccination (25). Hence, we consider primary human keratinocytes the most relevant cell type to examine the safety of our candidate vaccines. Primary human keratinocytes like established rodent cell lines are extensively used to study the transforming potential of hrHPV types and genes (26-28).

Immortalization of keratinocytes has been considered a point of no return in terms of malignant transformation by hrHPV types (4). HPV-mediated immortalization has been recognized as a two-step process, involving the bypass of two replicative lifespan barriers, senescence and crisis, also referred to as M1 and M2, respectively. It has been well established that the host cell alterations leading to the immortalization of cultured primary human keratinocytes, including the inactivation of p53 and pRb and the activation of telomerase, closely reflect those observed in cervical carcinogenesis *in vivo* (4). To demonstrate the safety of our candidate vaccines in this system, primary human foreskin keratinocytes (HFKs) from 3 independent donors were transduced with retroviral constructs encoding the DNA vaccine candidates and grown under selection. Transduction with retroviral constructs was chosen in order to mimic the 'worst-case scenario' of chromosomal integration. Therefore, these studies will provide an answer to the question what would happen if our candidate DNA vaccines would stably integrate into the host cell genome, leading to constitutive expression at the vaccination site. The transduced keratinocytes were monitored for their ability to overcome replicative senescence, degradation of p53 and pRb, and the upregulation of hTERT as a marker for telomerase activity and immortalization. In the current study the results obtained in both assay systems are presented and the implications for the application of the candidate vaccines are discussed.

MATERIALS AND METHODS

DNA and Viruses

pVAX-TTFC-E6SH and pVAX- TTFC-E7SH fusion vaccines were developed in a previous study (22). These vaccines consist of 'gene-shuffled versions of HPV16 E6 and E7 (E6SH and E7SH) that are C-terminally fused with Tetanus Toxin fragment C (see also Supplementary figure 1 for a schematic representation) and are inserted into pVAX1 (Invitrogen Life Technologies, Breda, The Netherlands) via 5´HindIII/3´XbaI. Plasmids were expressed and amplified in *E. Coli* DH5alpha and were purified using an endotoxin free DNA purification kit (Qiagen, Hilden, Germany). Retroviral vectors were constructed by the insertion of these candidate vaccines in the LZRS backbone (29, 30). To allow for co-selection, TTFC-E6SH and TTFC-E7SH were inserted in LZRS containing different selection markers, resulting in LZRS-TTFC-E6SH-MS-IRES-blasticidin and LZRS-TTFC-E7SH-MS-IRES-neomycin, respectively. LZRS-E6WT-MS-IRES-blasticidin and LZRS-E7WT-MS-IRES-neomycin were constructed as positive controls. Empty vectors (selection marker only) were used as negative controls. Helper virus–free recombinant retroviruses were produced after transfection of the retroviral constructs into the 293T-based Phoenix amphotropic packaging cell line and selection on the selectable marker puromycin, as described before (29).

Soft agar transformation assays

NIH 3T3 cells were co-transfected with the CD4 expression vector (pMACS-hCD4, Miltenyi) together with either empty vector pTHamp (31), HPV16 E6WT or HPV16 E7WT or HPV16 E6WT + HPV16 E7WT (inserted via 5´HindIII/3´XbaI cloning sites into pTHamp), or TTFC-E6SH or TTFC-E7SH, using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Correct transgene expression from the different plasmids was verified by RT-PCR (Supplementary Figure 2). The cells were analyzed by flow cytometry on a FACScan™ flow cytometer (BD Biosciences) one day after transfection and 5000 CD4 positive cells were seeded (suspended in the same medium used for the base layer) onto a precast base layer consisting of

5 ml DMEM/10% FCS with 1% soft agar in 50 mm petri dishes. Soft agar cultures were incubated at 37°C / 5% CO2 adding 0.25 ml complete DMEM / 10% FCS once per week. After four weeks all colonies (> 8 cells) located within a 9 cm^2 field of the soft agar plate were counted. The percentage of transformed cells was calculated by dividing the number of colonies by the number of cells seeded in the field.

Primary human foreskin keratinocyte cultures

HFKs were isolated from neonatal foreskin as previously described (28). In short, epidermal neonatal foreskin cleared of fibrous tissue, fat and dermis upon dispase treatment was incubated with trypsin and filtered to obtain a cell suspension. Cells were seeded on collagen-coated plates and cultured in serum-free keratinocyte growth medium (Invitrogen), supplemented with bovine pituitary extract (50 μg/ml), epidermal growth factor (5 ng/ml), penicillin (100U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). After initial passaging keratinocytes isolated from three independent donors were transduced with amphotrophic retrovirus expressing HPV16 oncogenes or the candidate vaccines using 3μg/ml polybrene and after 48hours selected using 2μg/ml blasticidin (Invitrogen) and 80μg/ml neomycin (G418, Invitrogen). The different cell cultures were split 1:5 at subconfluency and splitting dates were noted to generate culture characteristics to compare the different created cell lines. Cells were harvested every few passages for stocks, RNA and protein. Transgene expression was verified using RT-PCR (Supplementary Figure 3).

Beta-galactosidase staining

Beta-galactosidase staining (adapted from (32)) was used to assess expression of this senescence associated marker. Cells were washed, fixed and incubated at 37°C for 16 hours (no CO2) with 5-bromo-4-chloro-3-indoyl b-D-galactopyranoside (X-gal) which yields a blue compound when cleaved by beta-galactosidase. By using a citric acid and sodium phosphate buffer at pH6, senescent associated beta-galastosidase is distinguished from acidic enzymatic activity.

Western blot

Whole cell extracts were prepared by lysing cells for 15min on ice in a buffer containing 150mM NaCl, 50mM Hepes, 5mM EDTA and 0.1% NP40 supplemented with protease and phosphatase inhibitors. Protein concentration was determined using the BCA protein assay (Pierce Biotechenology Inc, Rockford, IL, USA). Typically 20μg of protein lysates were separated by SPS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 3% dry milk in PBS containing 0.05% Tween20. Antibodies against pRb (clone 1F8, Novocastra, Leica Microsystems, Milton Keynes, UK), p53 (clone D07, Dako, Glostrup, Denmark), and loading control beta-actin (Cell Signalling Technology, Beverly, MA, USA) were used according to manufacturer’s instructions. Membranes were incubated with the appropriate horseradish peroxidise-conjugated secondary antibodies and the levels of corresponding proteins were visualized using SuperSignal West Dura Extended Duration Substrate (Pierce Biotechenology Inc).

Quantitative RT-PCR

mRNA from cell cultures was isolated using RNA-B reagent (Tel-Test, Friendswood, TX, USA). Isolated RNA was DNase treated (Promega, Madison, WI, USA) and used for cDNA synthesis using a specific reverse primer (see below). Quantitative RT-PCR for hTERT

was performed as described previously (33) using the following primers hTERT-forward 5'-CACGCGAAAACCTTCCTCA-'3, hTERT-reverse 5'-CAAGTTCACCACGCAGCC-'3 and the probe FAM-5'-CTCAGGGACACCTCGGACCAGGGT-3'-TAMRA. For quantification purposes a standard curve was used of serial dilutions of cell line (SIHa) cDNA synthesized with specific reverse primers. To correct for RNA quality and input, we performed RT-PCR for the housekeeping gene snRNP U1A using the following primers snRNP forward 5'- TCCTCACCAACCTGCCAGA-'3 and reverse 5'-TGAAGCCAGGGAACTGATTGA-'3 with probe 5'-AGACCAACGAGCTCATGCTGTCCATG-'3.

RESULTS

Transformation of established rodent cells

Murine NIH 3T3 cells have been extensively used to study the transforming potential of HPV types. Both HPV16 E7 wilde-type (E7WT) and E6WT are known to independently induce transformation in NIH 3T3 cells, as can be shown by anchorage independent growth in soft agar (27). Because of the relative simplicity and short duration, we first used this assay to test the transforming potential of the TTFC-E6SH and TTFC-E7SH encoding candidate vaccines. To this purpose NIH 3T3 fibroblasts were transiently transfected with either TTFC-E6SH or TTFC-E7SH alone or in combination. The empty vector pTHamp was used as a negative control, and HPV16 E6WT and E7WT were used either alone or in combination as positive controls. All vectors were co-transfected with an expression construct for human CD4, which allowed the purification of cells that had been successfully transfected. Selection of transfected cells has been shown to be important to increase the sensitivity of the assay (unpublished observation). As shown in Figure 1, neither transfection with TTFC-E6SH nor with TTFC-E7SH alone or in combination enabled NIH 3T3 cells to form colonies. Expression of HPV16 E6WT or E7WT on the other hand, as well as the combination of E6WT and E7WT, resulted in colony formation indicating transformation. In addition, when transfection of TTFC-E6SH or TTFC-E7SH was combined with their wild-type counterparts the number of colonies was not increased compared to cells transfected with E6WT or E7WT alone. Importantly in all cases mRNA expression in the transfected cells was proven by RT-PCR (Supplementary figure 2). Therefore we conclude that our candidate DNA vaccines did not display transforming potential in this assay.

Retroviral transduction and culture of primary human keratinocytes

The transforming potential of shuffled E6 and E7 constructs was further analyzed using HFKs from three independent donors. This cell type is a well established system to analyse the oncogenic potential of HPV types and oncogenes (26, 27). HFKs were transduced with combinations of retroviruses (LZRS) containing the different constructs listed in Figure 2A. In the HFKs from the first donor we tested all combinations listed, in HFKs from the additional two donors we only tested the most critical conditions. The combination of the shuffled vaccine candidates with LZRS empty vector (TTFC-E6SH/LZRS, TTFC-E7SH/LZRS) best represents the situation in the planned clinical trial, as we will apply the vaccines on distinct areas on the skin. To maximize the chance of detecting residual oncogenic activity, we also tested the combination of both shuffled vaccine candidates (TTFC-E6SH/TTFC-E7SH), or the combinations with their wild-type counterparts (TTFC-E6SH/E7WTand TTFC-E7SH/E6WT). We considered this important as E6 and E7 are known to cooperatively transform epithelial cells (34). As a positive control for immortalization we introduced the wild-type oncogenes together (E6WT/E7WT). Finally double empty vector transductions (LZRS/LZRS) served as negative controls next to untransduced

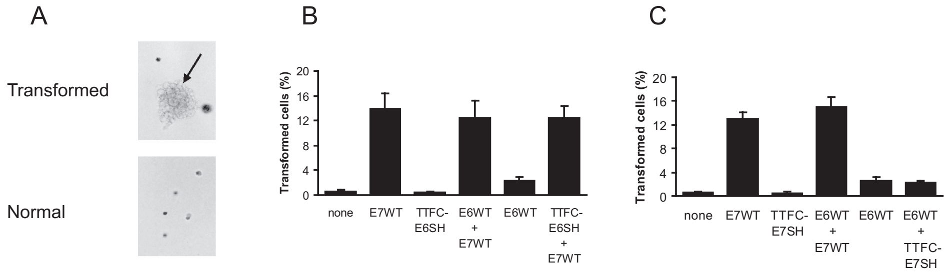


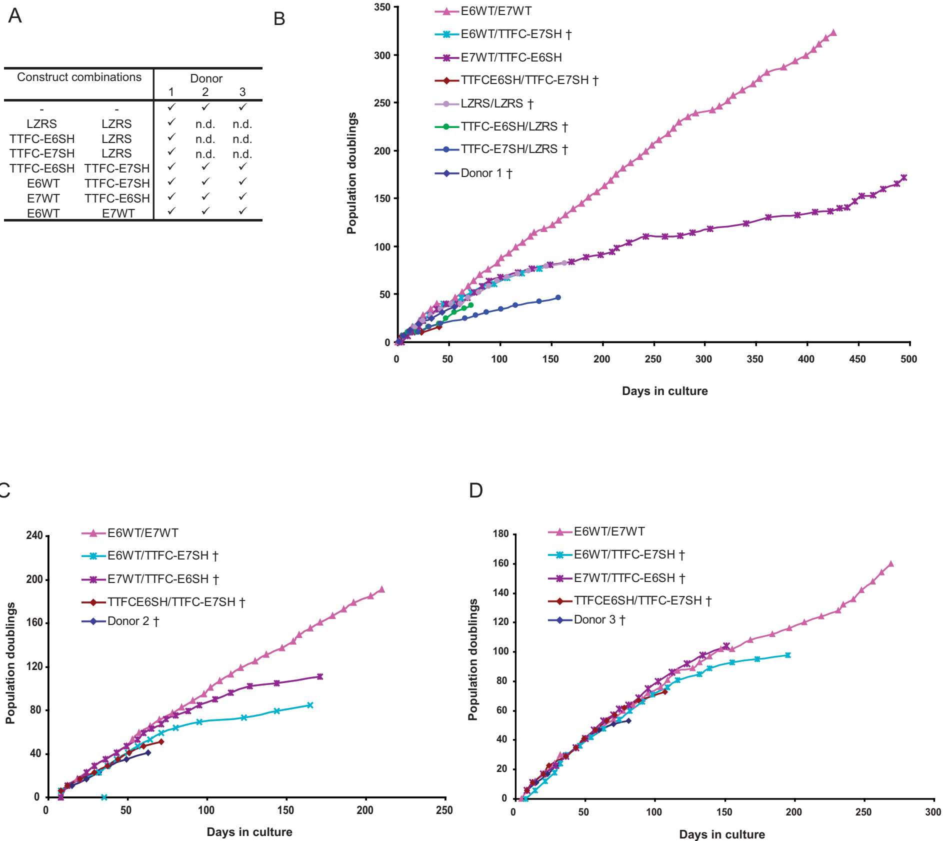
Figure 1. Colony formation of NIH 3T3 cells in soft agar. NIH 3T3 cells were co-transfected with the indicated constructs and a CD4 expressing vector to enable selection of transfected cells. A total of 5000 CD4 positive cells were plated, and after 4 weeks all colonies (>8 cells) located within a 9 cm^2 field of the soft agar plate were counted. The number of transformed cells is expressed as a percentage of total number of cells seeded in the field. **A)** Photographs showing transformed NIH 3T3 cells and the normal appearance, the arrow highlights a single focus. **B)** Bar diagram summarizing the results of three independent experiments for the TTFC-E6SH containing combinations and controls. **C)** Bar diagram summarizing the results of three independent experiments for the TTFC-E7SH containing combinations and controls.

HFKs. After initial passaging, transgene expression was confirmed by RT-PCR using specific reverse primers for the shuffled sequences (Supplementary figure 3). Upon culturing, cells were harvested at different time-points for further analysis.

Primary keratinocyte cell culture characteristics

Untransduced HFKs (from donor 1-3) ceased to proliferate after 37-53 population doublings (PDs) (Figure 2 and Table 1). In the HFKs from the first donor, double empty vector combinations (LZRS/LZRS) as well as cells transduced with TTFC-E6SH alone (TTFC-E6SH/LZRS) and TTFC-E7SH alone (TTFC-E7SH/LZRS) and the cells transduced with both vaccine candidates (TTFC-E6SH/TTFC-E7SH) showed a similar growth pattern. Whereas the transductants carrying the candidate vaccines ceased dividing at around the same number of PDs as untransduced cells (20-46 PD), only LZRS/LZRS transduced cells showed an increase in PDs (82PD). Also HFKs of donor 2 and 3 transduced with the combination TTFC-E6SH/TTFC-E7SH had a similar, though slightly increased, lifespan as their parental cells. All cell cultures containing the above combinations contained larger flattened cells at later passages, comparable to the untreated donor cells, which corresponded to senescence associated beta-galactosidase staining (SA-beta-gal) (see Figure 3 and Table 1).

In HFKs from all three donors, cells containing either E6WT or E7WT constructs combined with its shuffled counterpart (E6WT/TTFC-E7SH, E7WT/TTFC-E6SH), proliferated longer, ranging from 81 to 104 PDs after which they ceased to divide and eventually died. These cells also displayed a senescent morphology upon reaching their final number of cell divisions. One exception, namely the E7WT/TTFC-E6SH combination in HFKs from donor 1, continued to grow at a low rate and was still in culture at the time of writing at 177 population doublings, most probably due to the expression of HPV16 E7WT (see discussion). Finally, the combined introduction of the wild-type genes (E6WT/E7WT) induced the most pronounced increase in the lifespan of HFKs from all donors and in all cases the cells were still in culture at 160 to 323 PDs at the time of writing.



Construct combinations		Donor 1	Donor 2	Donor 3
-	-	✓	✓	✓
LZRS	LZRS	✓	n.d.	n.d.
TTFC-E6SH	LZRS	✓	n.d.	n.d.
TTFC-E7SH	LZRS	✓	n.d.	n.d.
TTFC-E6SH	TTFC-E7SH	✓	✓	✓
E6WT	TTFC-E7SH	✓	✓	✓
E7WT	TTFC-E6SH	✓	✓	✓
E6WT	E7WT	✓	✓	✓

Figure 2. Growth characteristics of HFKs from three independent donors transduced with the candidate vaccines and controls. A) Overview of the construct combinations used for the transduction of the HFKs from the different donors. **B)** Plot depicting the growth characteristics of the HFKs from donor 1 after transduction with the indicated combinations. **C)** Growth characteristics of HFKs from donor 2. **D)** Growth characteristics from of HFKs from donor 3. The cross symbol in the legend indicates that the culture stopped growing and the cells died.

In summary these growth characteristics indicate that introduction of the candidate vaccines either alone or in combination did not induce an extended growth potential in primary keratinocytes. The only conditions in which an extended or even infinite lifespan was observed were those conditions where one of the wild-type genes was present.

Protein degradation p53 and pRb in primary keratinocytes

Previous studies have demonstrated that the bypass of the first barrier in the finite lifespan of keratinocytes, referred to as M1 or senescence, upon HPV E6 and E7 expression results from inactivation of p53 (9, 35) and pRb (36). Osen et al. already demonstrated that gene-shuffling of E7 resulted in loss of its ability to induce degradation of pRb (23). To demonstrate that the inability of the TTFC fusions of E7SH and E6SH to extend the number of PDs is reflected by

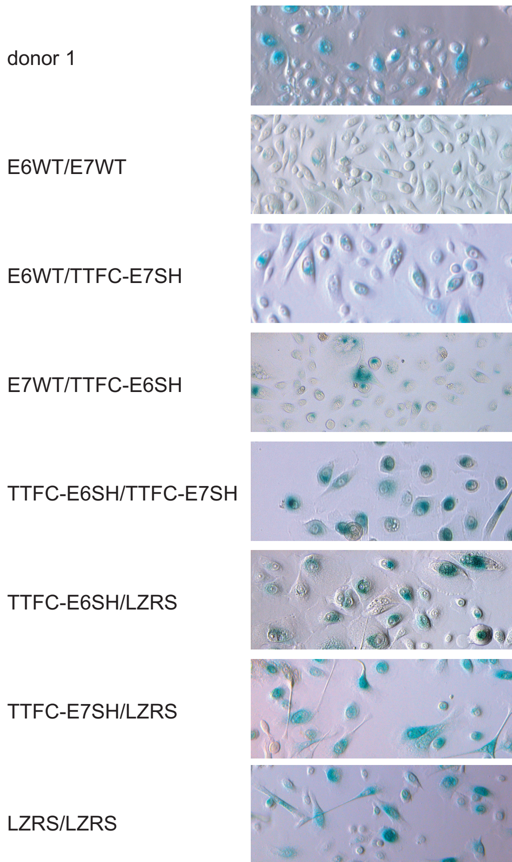


Figure 3. Senescence associated beta-galactosidase activity. Representative pictures after staining for senescence-associated beta-galactosidase activity of the HFKs from donor 1 after transduction with the various construct combinations. Pictures were taken around passage 10 corresponding to about 40 PDs. Similar results were obtained in HFKs from donor 2 and 3 (data not shown).

their inability to target pRb and p53, we examined the status of p53 and pRb protein expression in HFKs transduced with the various combinations. Both p53 and pRb remained detectable at the protein level in donor cells transduced with either TTFC-E7SH alone (TTFC-E7SH/LZRS), TTFC-E6SH alone (TTFC-E6SH/LZRS) or in combination (TTFC-E7SH/TTFC-E6SH) (Figure 4A/B and Table 1). As expected, cells containing E6WT (E6WT/E7WT and E6WT/TTFC-E7SH) showed a strongly reduced p53 expression, and E7WT containing cells (E6WT/E7WT and E7WT/TTFC-E7SH) showed reduced pRb levels compared to conditions in which the mutated variant was present. Cells containing E6WT/E7WT seemed less efficient in targeting p53 for degradation compared to E6WT/TTFC-E7SH containing cells, which might be explained by the fact that intact E7WT indirectly leads to an increase in p53 expression whereas TTFC-E7SH would not be expected to increase p53 levels. The finding that pRb was hardly detectable in untreated donor cells most likely resulted from the fact that cells were already near senescence at the time of protein isolation. Importantly, pRb is clearly detectable in cells expressing TTFC-E7SH (E6WT/TTFC-E7SH and TTFC-E6SH/TTFC-E7SH) in contrast to cells expressing E7WT (E6WT/E7WT and E7WT/TTFC-E6SH). Taken together, these data show that both vaccine candidates do not display their wild-type function of targeting p53 and pRb for degradation in all three donors tested.

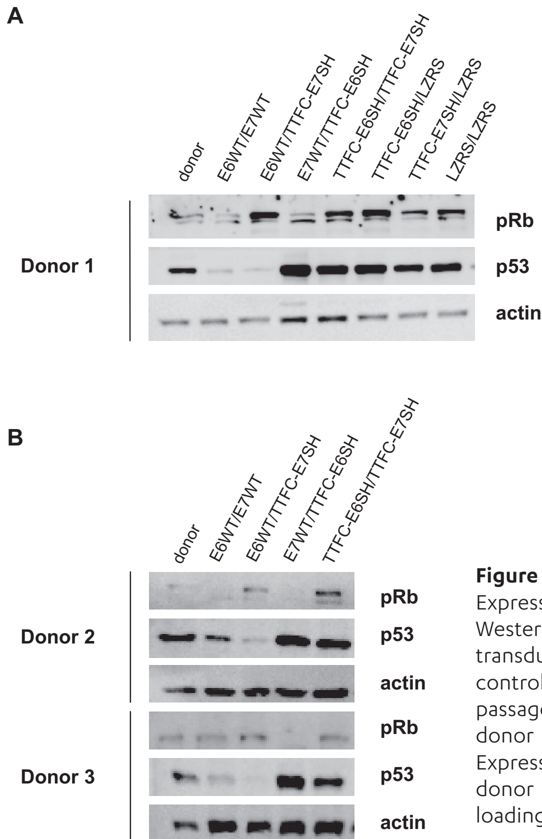


Figure 4. Expression levels of p53 and pRb. Expression of p53 and pRb was determined by Western blotting in HFKs from all donors after transduction with the indicated constructs and controls. Cellular lysates were prepared around passage 10 corresponding to about 40 PDs in donor 1 and about 60 PDs in donor 2 and 3. **A)** Expression levels of p53 and pRb for in HFKs from donor 1, actin was used as a control for equal loading. **B)** The same data for donor 2 and 3.

hTERT expression in primary keratinocytes

In primary keratinocytes, bypass of the second barrier towards immortalization (M2/crisis) has been associated with re-activation of telomerase, resulting at least in part from the upregulation of its catalytic subunit hTERT (4). In HFKs expressing E6WT either in combination with E7WT or E7SH displayed an upregulation of hTERT mRNA expression (Figure 5 and Table 1), consistent with the fact that HPV16 E6 can activate telomerase (37). The observed increase in hTERT mRNA upon co-expression of E6WT and E7WT is in line with previous findings (38). Importantly messenger RNA levels of hTERT were undetectable in all passages of HFKs containing shuffled oncogene constructs without wild-type constructs. Unexpectedly cells from donor 1 transduced with E7WT/TTFC-E6SH, which reached >177 PD's, also displayed strongly elevated hTERT mRNA levels, consistent with the culture characteristics. At present it remains unclear whether the increase in hTERT mRNA expression and concomitant continued growth results from the expression of E7WT, TTFC-E6SH or otherwise, such as genetic host cell alterations resulting from retroviral integration or prolonged culturing. However, based on the fact that in conditions where the wild-type E6 and E7 genes are absent no hTERT could be detected it can be concluded that the shuffled candidate vaccines alone or in combination consistently failed to induce this key characteristic of immortalization in primary keratinocytes.

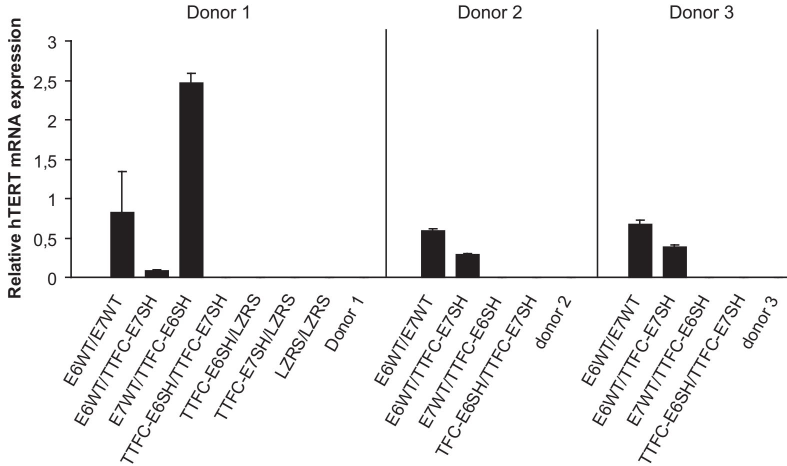


Figure 5. hTERT expression levels. hTERT expression levels were determined by RT-PCR in all HFKs from all donors after transduction with the indicated constructs. RNA was isolated at the latest passages that the cells were still in culture. Displayed are the hTERT mRNA levels relative to mRNA levels of the reference gene snRNP U1A.

Table 1. Summary of the results obtained in the different HFK donors.

Construct combination		Population-doublings			SA-Beta-gal			p53 expression			pRb expression			hTERT mRNA		
Donor		**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
-	-	37	41	53	+	+	+	+	+	+	±	-	±	-	-	-
LZRS	LZRS	82	n.d.	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	-	n.d.	n.d.
TTFC-E6SH	LZRS	43	n.d.	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	-	n.d.	n.d.
TTFC-E7SH	LZRS	46	n.d.	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	+	n.d.	n.d.	-	n.d.	n.d.
TTFC-E6SH	TTFC-E7SH	20	51	73	+	+	+	+	+	+	+	+	±	-	-	-
E6WT	TTFC-E7SH	81	85	98	+	+	+	-	-	-	+	±	±	+	+	+
E7WT	TTFC-E6SH	>177	111	104	+	+	+	++	++	++	±	-	-	++	-	-
E6WT	E7WT	>323	>191	>160	-	-	-	±	±	±	±	-	±	+	+	+

'+' positive, '-' negative, '++' highly positive, '±' intermediate, 'n.d.' not determined

DISCUSSION

The current study describes a comprehensive analysis using two cell culture systems which determines whether our candidate DNA vaccines TTFC-E6SH and TTFC-E7SH, intended for use in a phase I clinical trial, have lost the ability to induce cellular transformation that is associated with the wild-type HPV16 E6 and E7 proteins. In summary, both the assays performed in NIH 3T3 cells and the assays performed in three independent donors of HFKs demonstrate that our

candidate vaccines have lost their transforming and immortalizing potential, respectively. As outlined below we believe that the results we obtained in these assay systems justify clinical evaluation of these candidate vaccines.

Both NIH 3T3 cells and HFKs have been extensively used to study the transforming potential of HPV types (27). The ability of hrHPV types to induce carcinogenesis in humans is reflected by their ability to either transform NIH 3T3 (39) cells or immortalize HFKs (40), whereas HPV types that do not induce carcinogenesis in humans do not induce transformation in either cell type (27, 41). Subtle differences exist between the effects of E6 and E7 in NIH 3T3 cells and HFKs. For example, NIH 3T3 cells can be transformed by expression of either E6 or E7 alone, albeit E7 is more efficient (42, 43). Immortalization of HFKs, however is generally considered to require the expression of both E6 and E7 (34, 44, 45). Also E6 or E7 transformed NIH 3T3 cells are tumorigenic in nude mice (39), whereas E6 and E7 immortalized HFKs are not tumorigenic (46). These differences are likely to be explained by the general finding that established rodent cell lines are more easily transformed than primary human cells (47). We therefore argue that by examination of transforming potential in both assay systems we increased the likelihood of detecting residual transforming activity of the candidate vaccines. Moreover, the value of these *in vitro* systems over *in vivo* toxicity studies in vaccinated animals is that *in vitro* the cells are selected to express the vaccine candidates, thereby mimicking the worst-case scenario of stable expression. In case of our assays on HFKs we used integrating retroviral vectors and cultured the cells under selection in order to prevent loss of expression, thereby maximizing the chance of immortalization. In this respect it should also be noted that the event of stable integration upon DNA vaccination is estimated to be three orders of magnitude below the spontaneous mutation frequency (15) and therefore is negligibly low.

As mentioned above the combination of E6 and E7 is considered most effective in inducing cellular transformation, therefore we are planning to apply the E6SH and E7SH encoding vaccines on separate locations on the body in order to further minimize the risk for cellular transformation. In this respect the conditions where cells were transfected or transduced with TTFC-E6SH or TTFC-E7SH alone are the most relevant for our risk assessment. In none of these conditions we observed any signs of cellular transformation neither in NIH 3T3 cells nor in HFKs. The small increase in PDs that is observed in the case of TTFC-E6SH or TTFC-E7SH transduced HFKs compared to the untransduced control, is not more pronounced than the increase observed for the LZRS/LZRS combination (Figure 2 and Table 1) and is most likely due to the nature of the viral construct used and/or the effect of retroviral integration per se. In HFKs we also combined TTFC-E6SH and TTFC-E7SH arguing that if any residual transforming activity would still be present this would be more readily detected when the two constructs are introduced together. Also for this combination no signs of transformation could be detected and the number of PDs was in the same range as the untransduced controls. Finally, to increase the sensitivity of our assays even more we chose to also include combinations of one of the shuffled genes with its wild-type counterpart. In NIH 3T3 cells these wild-type/shuffled combinations did not show any increase in the percentage of transformed cells compared to usage of the wild-type sequence alone. In HFKs the combination of a wild-type and a shuffled gene resulted in an increase in the number of PDs in all cases compared to non-transduced cells. In some combinations also increased expression of hTERT could be observed (Figure 5 and Table 1), which can most likely be attributed to the wild-type gene introduced. Of note, immortalization of HFKs by E7 alone has been observed before when expressed from a retroviral vector (48). Importantly the Western-blot data show that in case of the wild-type/

shuffled combinations only the targets of the wild-type sequence are degraded, and the targets of the shuffled sequences are in all cases still present (Figure 4 and Table 1), thus suggesting that any sign of cellular immortalization for the wild-type/shuffled combinations is caused by the wild-type protein only. Nonetheless, we cannot completely rule out the remote possibility that the shuffled genes do still possess some residual transforming potential when combined with its wild-type counterpart. However, since we will DNA vaccinate normal skin and not the anogenital region or oropharynx, we consider the chance of the presence of hrHPV E6 or E7 genes negligible. Hence, we do consider it justified to proceed to clinical evaluation based on these results.

For several reasons we consider the assays in HFKs paramount in the preclinical safety evaluation of our candidate vaccines. An obvious reason is that human genital keratinocytes are the natural host cells of HPV16 infections in humans and HFKs more closely reflect the target cells of our vaccination procedure (skin keratinocytes) than NIH 3T3 cells. Furthermore, the HFK assays used allowed us to study the individual steps leading to immortalization separately, whereas the colony formation assay in NIH 3T3 cells only provides a simple yes or no answer. It is well established that the steps that lead to immortalization of HFKs closely reflect the steps that lead to HPV induced carcinogenesis in humans (4). Finally, because HFKs from different donors were used, different genetic backgrounds of the target cells are taken into account, whereas the NIH 3T3 cells comprise a single non-human genetic background. We therefore consider the colony formation assay in NIH 3T3 cells suitable for a ‘quick scan’ on transforming potential of modified E6 and E7 but not sufficient for a preclinical safety evaluation. Our study demonstrates that it is feasible to use HFKs for this purpose. This may be relevant for the safety evaluation of other vaccine candidates targeting E6 and/or E7 as many vaccination methods target the skin.

In conclusion, we show that our candidate DNA vaccines, TTFC-E6SH and TTFC-E7SH do no longer posses the transforming activity that is associated with HPV16 E6WT and E7WT. As a consequence the risk of inducing a squamous cell carcinoma (or precursor thereof) at the vaccination site is negligible. Therefore we consider our candidate DNA vaccines safe for use in a phase 1/2a clinical trial in patients suffering from recurrent HPV16 associated malignancies. At the same time we demonstrate that it is feasible to use primary human keratinocytes to evaluate the safety of HPV16 E6 and E7 directed vaccine candidates, thereby setting a new standard for future safety studies in this field.

ACKNOWLEDGEMENTS:

The authors like to thank Dr. Fahad N. Almajhdi from the King Saud University Riyadh, Kingdom of Saudi Arabia, for performing RT-PCRs to control for transgene expression in the NIH 3T3 cells.

4

REFERENCE LIST

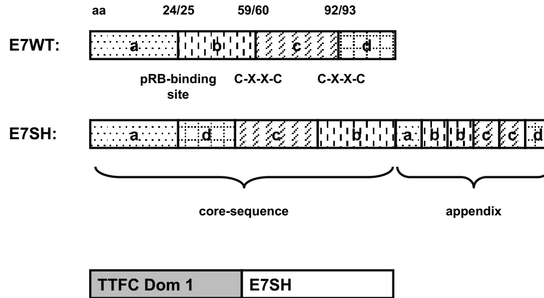
1. Scheurer ME, Tortolero-Luna G, Adler-Storthz K. Human papillomavirus infection: biology, epidemiology, and prevention. *Int J Gynecol Cancer* 2005;15:727-46.
2. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol* 2002;55:244-65.
3. Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;2:342-50.
4. Snijders PJ, Steenbergen RD, Heideman DA, Meijer CJ. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* 2006;208:152-64.
5. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* 2011;11:9-22.
6. Knebel Doeberitz M, Rittmuller C, Aengeneyndt F, Jansen-Durr P, Spitkovsky D. Reversible repression of papillomavirus oncogene expression in cervical carcinoma cells: consequences for the phenotype and E6-p53 and E7-pRB interactions. *J Virol* 1994;68:2811-21.
7. Huibregtse JM, Scheffner M, Howley PM. Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Mol Cell Biol* 1993;13:4918-27.
8. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. *Virology* 2009;384:324-34.
9. Dyson N, Guida P, Munger K, Harlow E. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *J Virol* 1992;66:6893-902.
10. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. *Virology* 2009;384:335-44.
11. Davies R, Hicks R, Crook T, Morris J, Vousden K. Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J Virol* 1993;67:2521-8.
12. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nat Rev Immunol* 2004;4:46-54.
13. Brinkman JA, Hughes SH, Stone P, Caffrey AS, Muderspach LI, Roman LD et al. Therapeutic vaccination for HPV induced cervical cancers. *Dis Markers* 2007;23:337-52.
14. Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgonne H, Kimman TG. Preclinical and clinical safety studies on DNA vaccines. *Hum Vaccin* 2006;2:45-53.
15. Nichols WW, Ledwith BJ, Manam SV, Troilo PJ. Potential DNA vaccine integration into host cell genome. *Ann N Y Acad Sci* 1995;772:30-9.
16. Cassetti MC, McElhiney SP, Shahabi V, Pullen JK, Le Poole IC, Eiben GL et al. Antitumor efficacy of Venezuelan equine encephalitis virus replicon particles encoding mutated HPV16 E6 and E7 genes. *Vaccine* 2004;22:520-7.
17. Smahel M, Sima P, Ludvikova V, Vonka V. Modified HPV16 E7 Genes as DNA Vaccine against E7-Containing Oncogenic Cells. *Virology* 2001;281:231-8.
18. Borysiewicz LK, Fiander A, Nimako M, Man S, Wilkinson GW, Westmoreland D et al. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* 1996;347:1523-7.
19. Polakova I, Pokorna D, Duskova M, Smahel M. DNA vaccine against human papillomavirus type 16: modifications of the E6 oncogene. *Vaccine* 2010;28:1506-13.
20. Wu TC. Therapeutic human papillomavirus DNA vaccination strategies to control cervical cancer. *Eur J Immunol* 2007;37:310-4.
21. Ohlschlager P, Pes M, Osen W, Durst M, Schneider A, Gissmann L et al. An improved rearranged Human Papillomavirus Type 16 E7 DNA vaccine candidate (HPV-16 E7SH) induces an E7 wildtype-specific T cell response. *Vaccine* 2006;24:2880-93.
22. Oosterhuis K, Ohlschlager P, van den Berg JH, Toebes M, Gomez R, Schumacher TN et al. Preclinical development of highly effective and safe DNA vaccines directed against HPV16 E6 and E7. *Int J Cancer* 2011.
23. Osen W, Peiler T, Ohlschlager P, Caldeira S, Faath S, Michel N et al. A DNA vaccine based on a shuffled E7 oncogene of the human papillomavirus type 16 (HPV16) induces E7-specific cytotoxic T cells but lacks transforming activity. *Vaccine* 2001;19:4276-86.
24. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med* 2005;11:899-904.
25. van den Berg JH, Nuijen B, Beijnen JH, Vincent A, van Tinteren H, Kluge J et al. Optimization of intradermal vaccination by DNA tattooing in human skin. *Hum Gene Ther* 2008.
26. Durst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE, Gissmann L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* 1987;1:251-6.

27. Massimi P, Banks L. Transformation assays for HPV oncoproteins. *Methods Mol Med* 2005;119:381-95.
28. Steenbergen RD, Walboomers JM, Meijer CJ, van der Raaij-Helmer EM, Parker JN, Chow LT et al. Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. *Oncogene* 1996;13:1249-57.
29. Kinsella TM, Nolan GP. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* 1996;7:1405-13.
30. Heemskerk MH, Blom B, Nolan G, Stegmann AP, Bakker AQ, Weijer K et al. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* 1997;186:1597-602.
31. Hanke T, Schneider J, Gilbert SC, Hill AV, McMichael A. DNA multi-CTL epitope vaccines for HIV and Plasmodium falciparum: immunogenicity in mice. *Vaccine* 1998;16:426-35.
32. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 1995;92:9363-7.
33. Henken FE, Banerjee NS, Snijders PJ, Meijer CJ, Castro Arce J, Rosl F et al. PIK3CA-mediated PI3-kinase signalling is essential for HPV-induced transformation in vitro. *Mol Cancer* 2011;10:71.
34. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989;63:4417-21.
35. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990;63:1129-36.
36. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res* 1996;56:4620-4.
37. Klingelhutz AJ, Foster SA, McDougall JK. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* 1996;380:79-82.
38. Liu X, Roberts J, Dakic A, Zhang Y, Schlegel R. HPV E7 contributes to the telomerase activity of immortalized and tumorigenic cells and augments E6-induced hTERT promoter function. *Virology* 2008;375:611-23.
39. Yasumoto S, Burkhardt AL, Doniger J, DiPaolo JA. Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. *J Virol* 1986;57:572-7.
40. Schlegel R, Phelps WC, Zhang YL, Barbosa M. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *EMBO J* 1988;7:3181-7.
41. Woodworth CD, Doniger J, DiPaolo JA. Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. *J Virol* 1989;63:159-64.
42. Sedman SA, Barbosa MS, Vass WC, Hubbert NL, Haas JA, Lowy DR et al. The full-length E6 protein of human papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. *J Virol* 1991;65:4860-6.
43. Vousden KH, Doniger J, DiPaolo JA, Lowy DR. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. *Oncogene Res* 1988;3:167-75.
44. Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *EMBO J* 1989;8:3905-10.
45. Hudson JB, Bedell MA, McCance DJ, Laiminis LA. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. *J Virol* 1990;64:519-26.
46. Kaur P, McDougall JK. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. *J Virol* 1988;62:1917-24.
47. Hahn WC. Immortalization and transformation of human cells. *Mol Cells* 2002;13:351-61.
48. Halbert CL, Demers GW, Galloway DA. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J Virol* 1991;65:473-8.

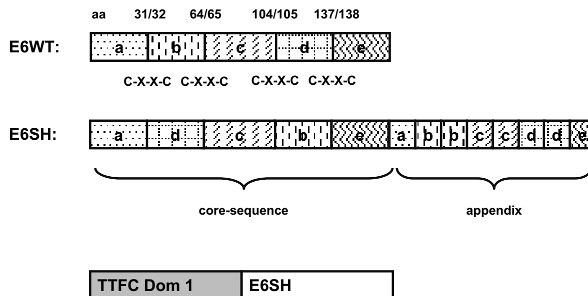
4

SUPPLEMENTARY FIGURES

A

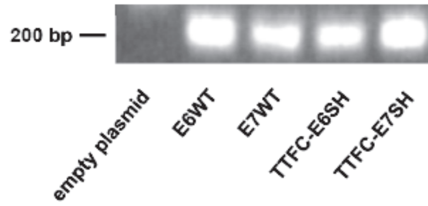


B



Supplementary figure 1. Schematic representation of the gene shuffling procedure and the construction of the TTFC-fusion vaccines. A) HPV16 E6 WT 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 loss of putative T cell epitopes at the junctions an appendix was added encoding sequences encoding the 14-18 amino acids surrounding the original junctions. In order to generate TTFC-E6SH, the E6SH sequence was fused towards the C terminus of tetanus toxin fragment C domain 1 (TTFC). **B)** The HPV16 E7 WT gene was dissected at the amino acid positions indicated in the figure corresponding to the pRb binding site (nt 72/37) and between the two C-X-X-C motifs (nt177/178 and nt 276/277). Similar to E6SH the 9 amino acids surrounding the original junctions were added as an appendix. TTFC-E7SH was generated by fusion of E7SH to the C terminus of TTFC. To minimize the potential risk of “back-to-wilde-type recombination” the codons of the core elements were optimized for expression in humans, but not the codons of the appendix. For both genes a Kozak sequence was added in front of the gene to enhance translation.

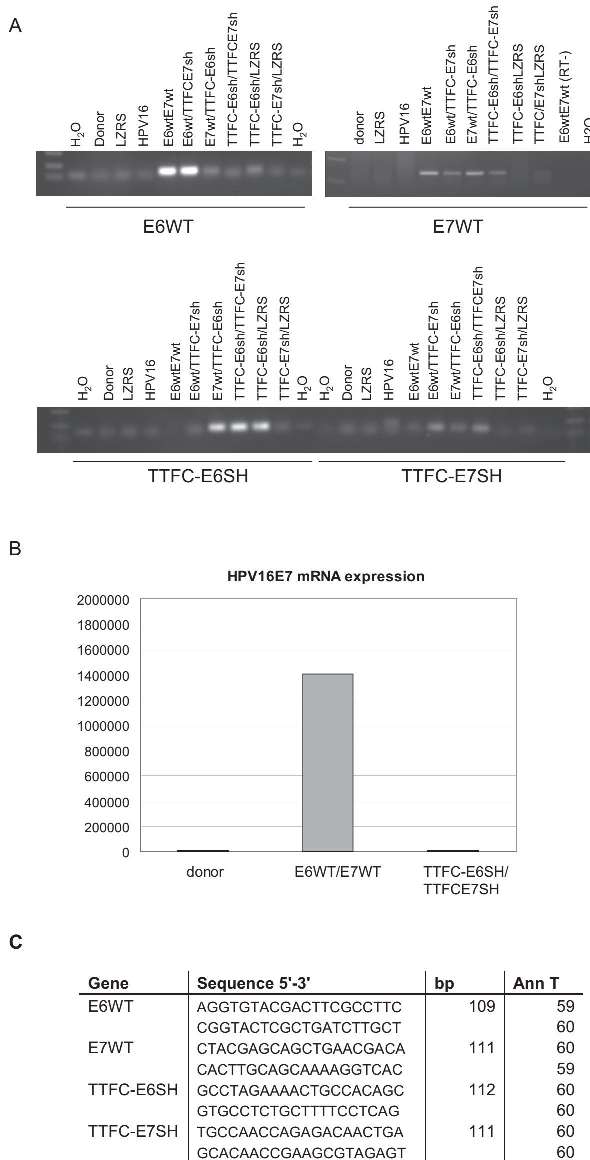
A



B

Gene	Sequence 5'-3'
E6WT	AAT GTT TCA GGA CCC ACA GGA
	TCA CTC ATA TCT GTA ATA ACA
E7WT	GAT TTG CAA CCA GAG ACA ACT
	CCT TAA CAC ACG GGG TAG ACA
TTFC-E6SH	ATG GCC GTG ATG ACG ATG TCG
	TTC CAG GAC CCC CAG GAG CGG
TTFC-E7SH	CTG GAC CTG CAG CCC GAG ACC
	CTC GGG CTG GCC CGG GTG ATG

Supplementary figure 2. Verification of correct transgene expression in NIH 3T3 cells. A) Transgene expression upon transfection was verified using different primer combinations resulting in 200bp fragments for each gene product. **B)** Sequences of the used primers.



Gene	Sequence 5'-3'	bp	Ann T
E6WT	AGGTGTACGACTTCGCCTTC	109	59
	CGGTACTCGCTGATCTTGCT		60
E7WT	CTACGAGCAGCTGAACGACA	111	60
	CACTTGCAGCAAAAGGTCAC		59
TTFC-E6SH	GCCTAGAAAACTGCCACAGC	112	60
	GTGCCTCTGCTTTTCCTCAG		60
TTFC-E7SH	TGCCAACCAGAGACAACTGA	111	60
	GCACAACCGAAGCGTAGAGT		60

Supplementary Figure 3. Verification of correct transgene expression in HFKs. A) Representative RT-PCR results for donor 1, using different primer combination to verify transgene expression. In all cases the combinations of constructs were correctly introduced and mRNA could be detected. A cross reaction was however observed between E7wt and E7sh, for which a qRT-PCR was added. **B)** qRT-PCR results, used to discriminate between E7wt and E7sh, confirmed E7wt expression in E7wt cells only. **C)** Sequences of the used primers.

SUPPLEMENTARY MATERIALS AND METHODS

RT-PCR on NIH 3T3 cells

Upon transfection with the indicated constructs RNA was isolated according using the Nucleospin® Total RNA Isolation Kit. For every transgene a 200bp fragment was amplified with gene-specific primers (see supplementary figure 2B for the primer sequences) using the QIAGEN® OneStep RT-PCR Kit. RT-PCR was performed in a total volume of 50µl, containing 10 µl 5x PCR buffer, 2 µl dNTPs and 2 µl of enzyme mix and 10 µl of Q-solution. The concentration of the primers varied from 0.5-1.2 µM, and the amount of template varied from 0.5 µg-2 µg. PCR conditions were 30 minutes at 50°C, 15 minutes at 95°C, followed by 30 cycles of 1 minute at 94°C , 1 minute at 60°C and 1,5 minute at 72°C and finally 10 minutes and 72°C. PCR products were separated on agarose gel.

RT-PCR on HFKs

Reverse transcriptase PCR was performed as described previously (van den Brule et al., Am J Pathol 1991) using primers and annealing temperatures as listed in Supplementary Table 1. PCR products were separated on agarose gel. Quantitative RT-PCR for HPV16 E7WT was used to discriminate between E7WT and TTFC-E7SH as described previously (Hesselink et al., J Clinical Microbiology 2005).

CHAPTER 5

RATIONAL DESIGN OF DNA VACCINES FOR THE INDUCTION OF HPV16 E6 AND E7 SPECIFIC CYTOTOXIC T CELL RESPONSES

5

ABSTRACT

Many DNA vaccine candidates have been developed for the treatment of HPV16 induced malignancies. Most of these vaccines consist of a fusion of E7 with a “carrier-protein” that functions to increase the potency of the vaccine. The nature of these carrier-proteins varies widely, and the mechanisms proposed to explain the enhanced immunogenicity of such fusions are often linked to the biological function of the carrier-protein. However, the potentiating effect of these carrier-proteins might also be explained by more general mechanisms such as the provision of CD4+ T cell help, increased antigen stability or altered subcellular localization of the antigen. To assess whether these more generic mechanisms could suffice to generate highly immunogenic DNA vaccines, we evaluated a series of modular HPV E7 DNA vaccines in which the presence of CD4+ T cell help, the presence of an endogenous carrier protein and the subcellular localization of the antigen could be systematically altered. Using this approach we demonstrate that the addition of an element that provides CD4+ T cell help and an element that enforces ER localization/retention are both necessary and sufficient to create markedly effective E7 directed DNA vaccines. Importantly, the resulting design rules also apply to an HPV16 E6 directed DNA vaccine. The thus developed “HELPER HPV DNA vaccines” encode only very limited additional sequences besides the antigen, thereby reducing the risk of antigenic competition and/or autoimmunity.

Koen Oosterhuis[1], Esil Aleyd[1*], Kim Vrijland[1*], Ton N. Schumacher[1] and John B. Haanen[1,#]

[1] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands; * These authors contributed equally to this work

Submitted for publication

INTRODUCTION

The E6 and E7 proteins of high risk HPV strains are ideal tumor associated antigens. First, the expression of these proteins is essential for cellular transformation (1), and as a consequence every HPV-induced (pre)malignancy expresses these proteins (2). Second, as E6 and E7 are foreign proteins, a high avidity T cell repertoire specific for these antigens is likely to be present and the activity of these cells should not affect healthy tissue (3). Importantly, the presence of E6 and E7 specific T cell responses is correlated with the clearance of HPV induced lesions (4, 5), and the boosting of this immune reactivity in women with premalignant vulvar lesions by peptide vaccination is associated with regression of those lesions (6).

As HPV type 16 is one of the most common subtypes associated with HPV induced malignancies, a substantial interest has grown in the development of therapeutic vaccination strategies that aim to enhance HPV16 E6 and E7 specific T cell responses (7, 8). Among these strategies DNA vaccination is attractive because of its relative simplicity, excellent safety record, and its ability to elicit strong cellular immunity (9, 10). The number of DNA vaccine candidates that have been developed for the induction of HPV16 E7 or E6-specific T cell responses is large (11). Most of these vaccine candidates consist of a genetic fusion of the E6 or E7 antigen and a so-called “carrier protein”. The nature of the carrier proteins used for this purpose varies widely and examples include heat shock proteins (HSPs) (12, 13), calreticulin (CRT) (14), E. coli Beta glucoronidase (GUS) (15), interferon gamma inducible protein 10 (IP10) (16), Herpes simplex viral protein 22 (HSV VP22) (17), and Tetanus toxin fragment C (TTFC) (18) (see also table 1). In most cases, the enhanced immunogenicity of these fusion proteins has been postulated to be directly related to the specific biological function of the carrier protein. To provide some examples: ER chaperones such as calreticulin have been suggested to deliver the antigen directly into the antigen presentation pathway thereby increasing the efficiency of antigen presentation (14, 19); linkage to HSPs or Flt3 ligand is thought to result in an enhanced uptake of the antigen by antigen presenting cells (12, 20); Fusion with HSV VP22 is believed to result in enhanced immunogenicity as a consequence of transfer of the coupled antigen to neighboring cells, thereby increasing antigen cross presentation (17); Fusion with IP-10 is thought to lead to enhanced immunogenicity by promoting the recruitment T cells to the vaccination site through its chemo-attractive function (16).

Noting that such a diverse group of carriers can apparently enhance HPV vaccine immunogenicity we considered the possibility that vaccine immunogenicity might primarily be determined by more general properties of the vaccine-encoded protein. For instance, in those cases in which the carrier is a foreign molecule (e.g. mycobacterial HSP-70, TTFC, HSV VP22 and Pseudomonas Aaeruginose exototoxin A), provision of CD4+ T cell help may at least partly explain the carrier effect (12, 21-23). Secondly, both HPV16 E6 and E7 proteins are known to have a short half life (24, 25) and for several of the carrier proteins that have been utilized, fusion has been shown to result in increased steady state levels of the antigen (15, 17, 18). As it has previously been demonstrated that the extent of *in vivo* accumulation of DNA vaccine-encoded antigens correlates with the magnitude of the CD8+ T cell response (26), such carrier-induced protein stabilization could contribute to vaccine immunogenicity. Finally, many of the fusions that have been utilized result in an altered subcellular localization of the antigen (14, 16, 20, 27, 28), and antigen localization – rather than the biological function of the carrier used – could conceivably alter immunogenicity. In line with this possibility, in some reports addition of only protein domains, or even signal sequences was shown to already improve vaccine immunogenicity (29, 30). To directly determine the potential contribution and

relative importance of these generic mechanisms, we have developed a modular DNA vaccine and utilized this to assess which elements are crucial for HPV E7 and also HPV E6 DNA vaccine immunogenicity.

Table 1. Selected examples of carrier proteins known to improve the immunogenicity of HPV16 E7 or E6 encoding DNA vaccines.

Carrier protein	Antigen	Proposed mode of action	Reference
Mycobacterium tuberculosis HSP-70	E7	Provision of CD4+ T cell help, increased antigen uptake by DC	(12)
Heat shock protein 60	E6, E7	Increased antigen uptake by DC	(13)
Calreticulin	E6, E7	Targeting of antigen into the antigen presentation pathway	(14, 27)
Extracellular domain of Flt3-ligand	E7	Altered subcellular localization/ increased antigen uptake by DC	(20)
HSV VP22	E7	Antigen spreading, improved antigen stability	(17)
E. coli β-glucoronidase	E7	Enhanced stability/ altered subcellular localisation	(15)
Pseudomonas aeruginosa exotoxin A (domain II)	E7	Enhanced cross presentation	(50)
Invariant chain with PADRE epitope insertion	E6	Provision of CD4+ T cell help	(45)
IP-10	E7	Enhanced antigen presentation, chemo-attraction	(16)
TTFC	E6, E7	Provision of $CD4^{+}$ help, increased antigen stability	(18, 21)

MATERIALS AND METHODS

Mice

C57BL/6 mice (6-10 weeks) were obtained from Jax® Mice (The Jackson Laboratory). All experiments 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). The generation of E7SH, TTFC-E7SH, E6SH and TTFC-E6SH has been described previously (18). FM4 consists of 4 moieties of a mutated human FK506 binding protein (97% homology to the mouse protein) with the signal peptide of hGH fused to the N-terminus (33). FM4-HELP-E7SH (see Fig. 1A for a schematic representation) was ordered from GeneArt with codon-optimization for expression in human cells and was cloned between the HindIII and XbaI sites of pVAX. FM4-E7SH and HELP-E7SH were generated by removal of either the BamHI flanked helper cassette or the SpeI flanked FM4 moiety. Histone

2B, endoplasmic reticulum protein 29, keratin 14 and cluster of differentiation antigen 8a (all mouse origin), were ordered from GeneArt with codon optimization for expression in human cells and flanked by SpeI sites. FM4(minus sig)-HELP-E7SH was generated by PCR using FM4-HELP-E7SH as a template. sig-HELP-E7SH-KDEL was constructed by replacing the complete FM4 sequence with only the signal peptide. The KDEL sequence was fused to E7SH and E6SH by PCR. The different E6SH encoding DNA vaccines were constructed by replacing E7SH with E6SH or E7SH-KDEL by E6SH-KDEL. Identity of all fusion genes was confirmed by sequence analysis. Plasmids were expressed in *E. coli* DH5a and were purified using an endotoxin free DNA purification kit (Qiagen). DNA vaccines for intradermal tattoo application were dissolved at a concentration of 2mg/ml in water for injections (Aqua B. Braun).

Transfection and immunoblotting

HEK 293 T cells were transfected with 10 μg of a mixture of a GFP encoding plasmid and the indicated constructs at a ratio of 3:7 by use of FuGENE 6 (Roche), according to the manufacturer's instructions. Cells were harvested 24 hours after transfection and equal transfection efficiency was confirmed by analyzing the percentage of GFP positive cells by flow cytometry. Subsequently, the remainder of the samples was lysed on ice in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Roche) and 0.1mM PMSF (Thermo Fisher Scientific). Cell lysates were subsequently cleared by centrifugation at 4 °C. Total cellular protein was determined using a Bradford assay (Bio-Rad Corporation) and proteins were separated at 30ug per lane on 4–12% NuPage Bis-Tris gradient gels (Invitrogen) in MES buffer, according to the manufacturer's instructions. Following immunoblotting, E7 expression was detected using a monoclonal mouse anti-HPV16 E7 antibody (Invitrogen, clone 8C9, 1:100 dilution) and actin expression was detected using a mouse anti-human actin antibody (Millipore, clone C4, 1:10000 dilution). In both cases HRP-rabbit anti-mouse antibody (DAKO, P 0161) was used as secondary antibody at a 1:7500 dilution and detection was performed by enhanced chemiluminescence (Pierce Biotechnology).

Tattoo vaccination

Intradermal DNA tattoo vaccination was performed on day 0, 3 and 6, as described previously (18). In brief, on day -1 the hair on the hind leg was removed using depilating cream (Veet®, Reckitt Benckiser). On day 0, mice were anesthetized and 10 μl of a 2 mg/ml DNA solution in water was applied to the hairless skin of the hind leg. The DNA vaccine was then applied with a Permanent Make Up (PMU) tattoo machine (kindly provided by MT Derm), 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. Unless indicated otherwise, vaccination was repeated on day 3 and 6. In cases in which mice received a single tattoo vaccination, 15μl of the DNA solution was used.

Detection of HPV-specific CD8+ T cells

Peripheral blood cells were obtained by tail bleeding, and erythrocytes were removed by incubation in erythrocyte lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA (pH 7.4)) on ice. 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) plus phycoerythrin (PE)-conjugated H-$2D^b$ $E7_{49–57}$ or H-$2K^b$ $E6_{48-57}$ 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 (31).

Detection of P30 and PADRE specific CD4+ T cells

Intracellular IFN-γ staining was performed using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. Peripheral blood cells were stimulated for 16 h with either the PADRE (AKFVAAWTLKAAA) or P30 (FNNFTVSFWLRVPKVSASHLE) peptide at a 1μg/ml concentration. Cells were subsequently stained using PE-conjugated anti-INF-γ mAb (BD Pharmingen), and APC-conjugated anti-CD8a mAb (BD Pharmingen). All samples were analysed on a FACScalibur (BD Biosciences), using Flow-Jo® software for data analysis.

TC-1 tumor challenge

C57BL/6 mice were injected subcutaneously with 10^5 TC-1 tumor cells that express both HPV16 E6 and E7 (32). DNA tattoo vaccination was subsequently performed on either day 4, or on day 4, 7 and 10 after tumor challenge, as indicated. Tumor growth was monitored by caliper measurements in two dimensions, tumor volume was calculated as ($width^2$ x length)/2. Mice were sacrificed when tumor length reached 15 mm or when the tumor volume exceeded 1000 mm^3.

Statistical analysis

Statistical analysis was performed using the student's t-test. A p-value <0.05 was considered to be significant (two-tailed). For evaluation of survival data a log-rank test was used.

RESULTS

Design of a modular DNA vaccine

In order to be able to separate the contribution of factors such as antigen localization, antigen stabilization and the presence of T-helper cell epitopes on vaccine immunogenicity, we designed a modular HPV E7 directed DNA vaccine that contains an endogenous 'carrier protein' and a separate minimal domain for the provision of CD4+ T cell help (see Fig. 1A). The use of this design minimizes the risk of antigenic competition at the level of CD8+ T cell response induction as only minimal foreign sequences are incorporated besides the antigen. As a first carrier protein we selected an engineered human protein called FM4. This protein contains four repeats of a point mutated version of the FK506 binding protein, FKBP12, and is targeted to the endoplasmic reticulum (ER) by the inclusion of a signal peptide. As a result of these modifications, FM4 forms stable aggregates inside the ER (33). To generate a minimal domain that would provide CD4+ T cell epitopes that can be presented by a large series of MHC class II alleles we created a 'helper-cassette' (Fig. 1B), consisting of the TTFC P30 pan DP epitope (34), the PADRE pan DR epitope (35) and the HIV NEF pan DQ epitope (36). Both P30 and PADRE are also recognized in the context of mouse MHC class II allowing evaluation of this strategy in mice (35, 37). In order to avoid the potential formation of CD8+ T cell epitopes at the junctions of these three sequences, all epitopes were linked by GPGPGPG spacers (38). To obtain insight in the value of the individual components used, we also generated HPV E7 encoding vaccines that only contained the FM4 carrier protein or only the helper-cassette (Fig. 1C). In all cases, a gene shuffled version of E7 (E7SH) was used as antigen, in order to avoid the risk of cellular transformation at the vaccination site (18, 39). All fusion constructs displayed the expected molecular weight when expressed in HEK 293 cells. Furthermore, the expression level of all E7 fusion vaccines was strongly increased compared to that of a non-fused E7SH vaccine (Fig. 1D).

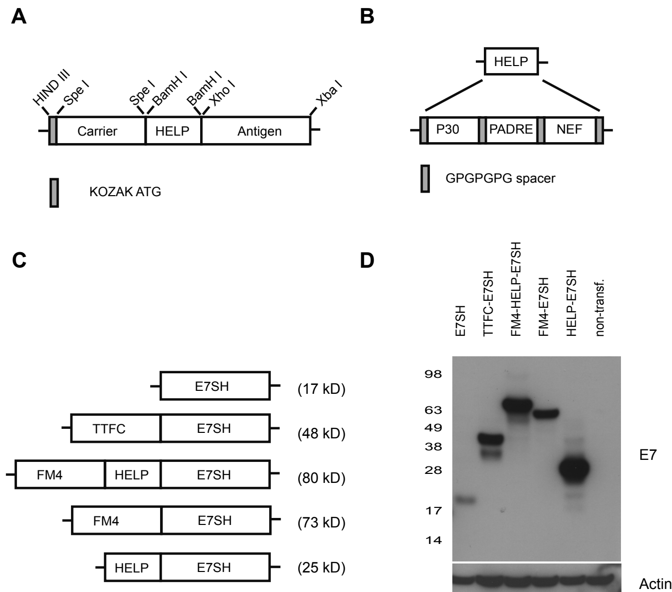


Figure 1. Design and validation of a modular DNA vaccine construct. A) Schematic representation of vaccine design. The carrier and the helper cassette are both placed between identical restriction sites to allow their removal. The antigen is placed between two different restriction sites to enable replacement. **B)** Design of the "helper-cassette". GPGPGPG spacers (see text) are included to avoid formation of CD8+ T cell epitopes at the junctions. **C)** Schematic representation of the constructs used for proof of principle experiments and their expected molecular weight. **D)** Western blot analysis of HEK 293 cells transfected with the constructs shown in C. In all cases a dominant band of the expected size (see C for expected molecular weights) was detected.

The combined inclusion of a carrier protein and helper-cassette results in superior vaccine immunogenicity.

In order to reveal possible differences in immunogenicity between the above-described vaccines, C57BL6/J mice (n=5 per group) were immunized by short interval DNA tattooing (40) and E7 specific CD8+ T cell responses in peripheral blood samples were analyzed over time. As shown in Fig. 2A-B, a clear pattern in vaccine immunogenicity is noted. First, HPV16 E7 specific CD8+ T cell responses are not above background in mice that received a vaccine that solely encodes E7SH, but clear CD8+ T cell responses are detected in groups that were treated with the fusion vaccines, thereby underlying the value of such genetic fusions. Second, when comparing the magnitude of the CD8+ T cell responses induced by the different fusion vaccines at the peak of the primary response (Fig. 2C) it is apparent that both the addition of the FM4 carrier (FM4-E7SH) and in particular the helper cassette (HELP-E7SH) results in a substantial improvement in immunogenicity of E7SH, but that the combination of the carrier and the helper-cassette (FM4-HELP-E7SH) is clearly superior (mean peak response of 19.05 ± 5.02% $E7_{49-57}$ specific CD8+ T cells directly ex vivo, $p < 0.05$ relative to HELP-E7SH the second best vaccine) FM4-HELP-E7SH also significantly ($p < 0.05$) outperformed a vaccine encoding a fusion of Tetanus toxin fragment C and E7SH (TTFC-E7SH) that was previously developed in

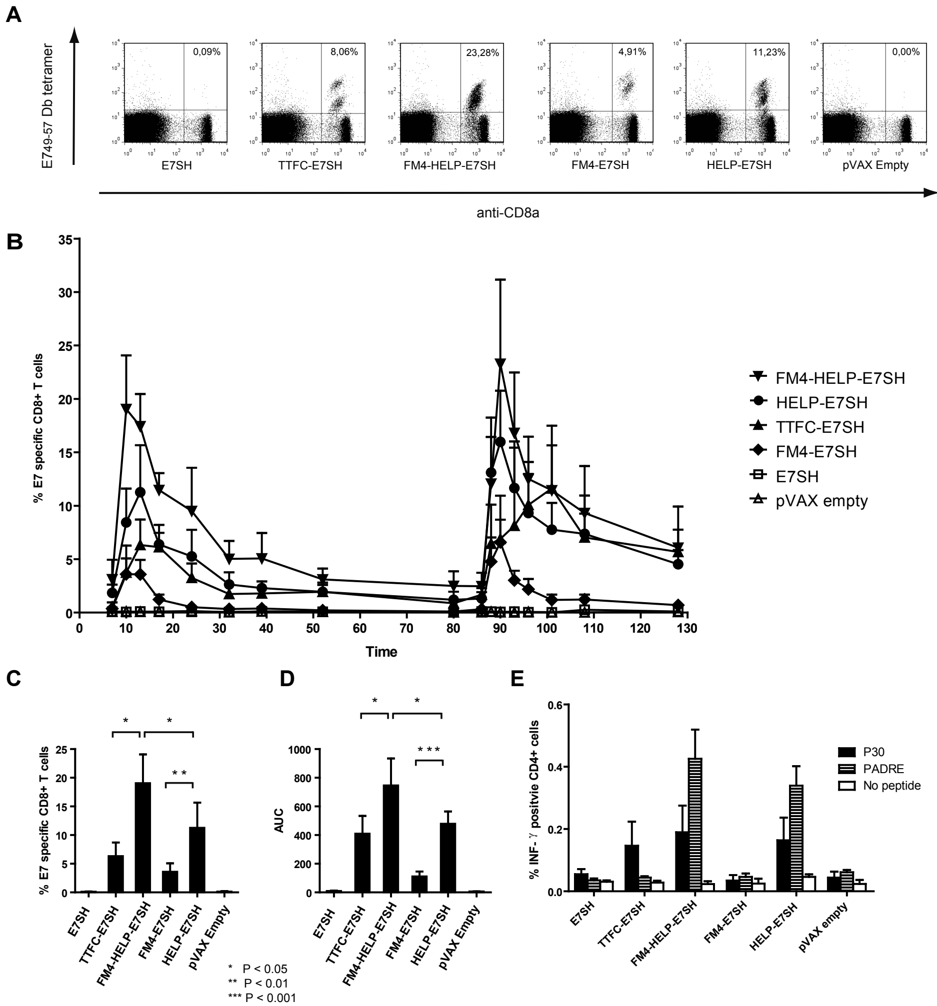


Figure 2. Synergistic effect of an endogenous carrier protein and a helper-cassette on vaccine immunogenicity. C57BL/6 mice (n=5 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6 with 20 μg the indicated vaccines, and peripheral blood was analyzed for antigen-specific CD8^{+} T cells by MHC tetramer staining. **A)** Representative dot plots of H-2D^{b} E7$_{49-57}$ MHC tetramer stainings at the peak of the response. The number depicts the % of E7 specific CD8+ T cells. **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. **C)** Bar graph depicting the mean percentage +/- S.D. of H-2D^{b} E7$_{49-57}$-specific CD8+ T cells for the indicated groups at the peak of the response. **D)** Bar graph depicting the area under the curve (AUC) +/- S.D. for recipients of the indicated vaccines as a measure that describes both T cell response magnitude and persistence. **E)** Bar graph showing the mean percentage +/- S.D. of interferon-g positive CD4^{+} T cells in peripheral blood samples (day 11 post-vaccination), following 16 h stimulation with the indicated peptides.

our lab (18). Differences in T cell response magnitude were not only observed at the peak of the primary CD8+ T cell response but remained visible over time and were maintained after a secondary challenge, as shown in Fig. 2D by area under the curve (AUC) values for each group.

In order to evaluate whether the inclusion of the helper cassette resulted in the expected CD4+ T cell responses towards the encoded epitopes, CD4+ T cell reactivity against P30 and PADRE epitopes was evaluated by intracellular IFN-g staining. In mice that received DNA vaccines encoding the helper-cassette, P30 and PADRE specific CD4+ T cell responses could readily be demonstrated. In mice vaccinated with TTFC-E7SH, which contains the P30 but not the PADRE epitope, P30 specific CD4+ T cell responses could also be observed (Fig. 2E).

Only ER-localized carriers enhance vaccine immunogenicity

Having demonstrated that the combination of a carrier molecule and the helper-cassette results in superior immunogenicity we wished to explore which aspects of the carrier molecule determined its effect on vaccine potency. To this purpose we selected 4 endogenous proteins - histone 2B (H2B, nuclear localization), endoplasmic reticulum protein 29 (ERP-29, ER localized), keratin 14 (KRT 14, cytosolic localization), cluster of differentiation antigen 8a (CD8a, plasma membrane localized) - for which there are no prior data that would suggest a specific role in antigen presentation. As shown in Fig. 3B, transfection of HEK 293 T cells with the different constructs leads to expression of proteins with the expected size (see Fig. 3A). Furthermore, while expression levels vary widely, in all cases the expression level of the fusion vaccine is strongly enhanced relative to that of unmodified E7SH.

In order to study possible differences in vaccine immunogenicity, mice were vaccinated and immune responses were monitored as described above. Of the 5 fusion vaccines tested, both FM4-HELP-E7SH and ERP29-HELP-E7SH were significantly ($p<0.05$) more immunogenic than HELP-E7SH, whereas the immunogenicity of H2B-HELP-E7SH and CD8a-HELP-E7SH was comparable to that of HELP-E7SH. Finally, KRT14-HELP-E7SH was significantly ($p<0.01$ based on AUC) less immunogenic than HELP-E7SH. These differences in vaccine immunogenicity did not directly correlate with differences in expression levels as displayed in Fig. 3B. For example, the expression level of CD8a-HELP-E7SH was extremely high but immunogenicity was mediocre. Vice versa, the expression level of FM4-HELP-E7SH was intermediate, but immunogenicity was high. Thus, when a series of different endogenous carriers is utilized (for which the the presence of CD4 T cell epitopes is therefore not a confounding factor), the enhancement of vaccine immunogenicity can only partially be ascribed to increased accumulation of the antigen. An interesting observation in this regard is that the two carrier proteins that results in a significant enhancement of E7 vaccine immunogenicity are both ER localized.

The carrier effect can be fully explained by ER targeting of the antigen

In order to determine whether the observed correlation between ER localization of the carrier and vaccine immunogenicity was due to chance or reflected a true effect of ER localization, we generated vaccine variants of FM4-HELP-E7SH in which either the signal peptide of FM4 was removed (FM4(minus-sig)-HELP-E7SH), or in which only the signal peptide of the FM4 moiety was retained. In the latter case (sig-HELP-E7SH-KDEL), a C-terminal KDEL sequence was included to achieve ER retention. As a control, a variant that encoded the antigen with the signal peptide and ER retention signal but that lacked the helper-cassette (sig-E7SH-KDEL) was also generated (see Fig. 4A for a schematic representation of the constructs). Expression

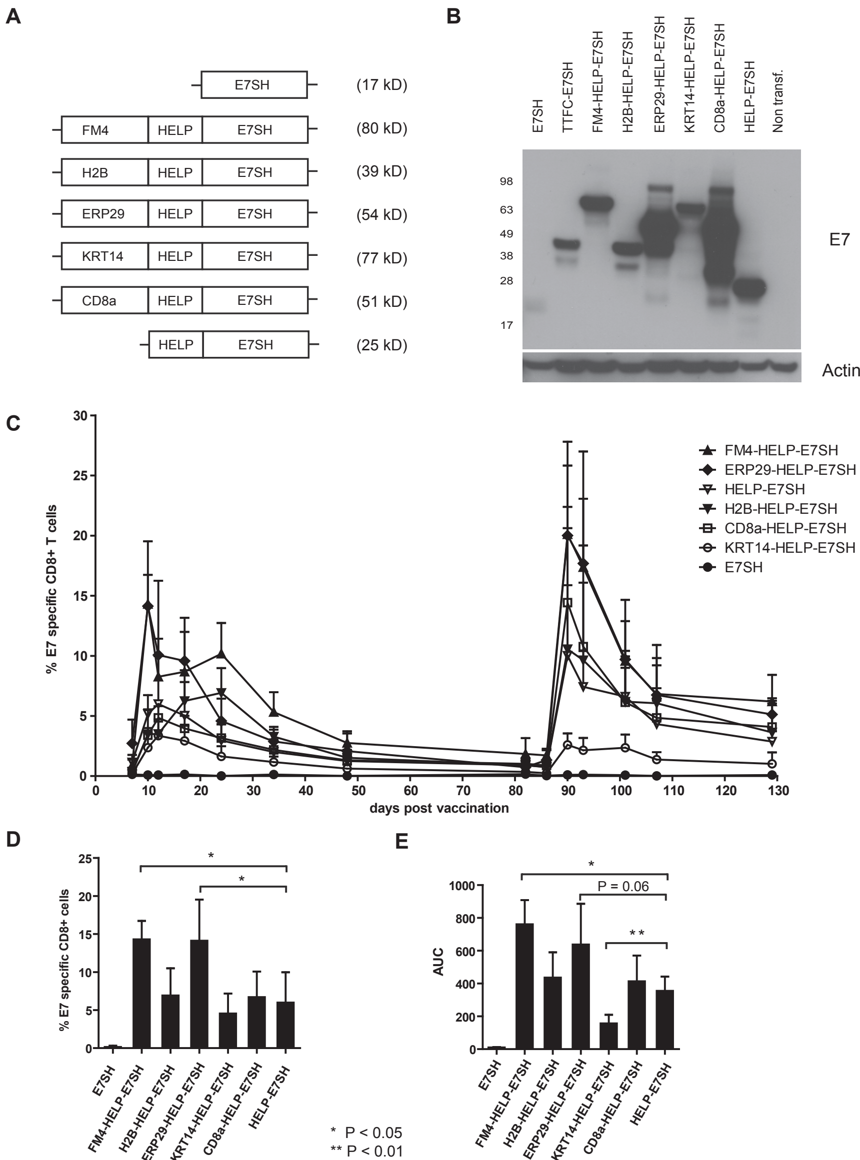


Figure 3. Only ER localized self-carriers provide an advantage over the addition of the helper-cassette alone. A) Schematic representation and molecular weights of DNA vaccines with different carrier proteins. **B)** Western blot analysis of HEK 293 cells transfected with the constructs shown in A. In all cases a dominant band of the expected size could be detected, demonstrating the correct expression of the DNA vaccine encoded antigens. **C)** Plot depicting the mean percentage +/- S.D. of peripheral blood H-2D^{b} E7$_{49-57}$-specific CD8+ T cells over time in C57BL6/J mice (n=5) DNA tattoo vaccinated on day 0, 3 and 6 with 20 µg of the indicated vaccines. **D)** Bar diagram depicting the mean percentage +/- S.D. of H-2D^{b} E7$_{49-57}$-specific CD8+ T cells for the indicated groups at the peak of the response. **E)** Bar diagram depicting the total area under the curve (AUC) +/- S.D. for each vaccine as measure for the total vaccine potency.

of protein products of the expected size was demonstrated by western blotting (Fig. 4B) as described above.

As shown in Fig. 4 C-E, removal of the signal peptide from FM4 resulted in a strong decrease in immunogenicity of FM4-HELP-E7SH. Furthermore, the same detrimental effect of signal peptide removal was observed for ERP29, the second ER localized carrier protein (data not shown). In stark contrast, the sole inclusion of an ER localization signal (i.e. signal peptide and KDEL) sufficed to significantly enhance the immunogenicity of HELP-E7SH ($p<0.05$). Together these results demonstrate that retention in the ER suffices to explain the potentiating effect of endogenous carriers on E7 vaccine-specific T cell responses. Consistent with the notion that such a 'minimal carrier' does not provide CD4+ T cell epitopes, inclusion of the helper-cassette was required for maximal immunogenicity as illustrated by the very moderate immunogenicity of sig-E7SH-KDEL (Fig. 4F-H).

Application of design rules to HPV E6SH encoding vaccines

The above data indicate that the combination of a helper-cassette and ER targeting/ retention signal suffices to create a highly immunogenic E7 DNA vaccine. In order to further validate this minimal "HELP^{ER}" design for HPV directed vaccines, we generated a set of HPV16 E6SH DNA vaccines (Fig. 5A) in which the value of the helper cassette or the combination of this cassette with ER localization were compared. Mice were tattoo vaccinated with these vaccines and E6 specific CD8+ T cell responses were monitored directly ex-vivo by MHC tetramer staining. Similar to what was observed for E7SH encoding vaccines, the addition of the helper cassette by itself strongly improved the immunogenicity of E6SH. In addition, the joint inclusion of the minimal ER localization/retention signal led to a further increase in immunogenicity, although the latter difference was not statistically significant (Fig. 5C-E). Importantly, the CD8+ T cell responses induced by sig-HELP-E6SH-KDEL significantly outperformed those elicited by a previously developed TTFC-E6SH fusion vaccine (18) ($p<0.01$ based on AUC). Superiority of the "HELP^{ER}" DNA vaccine design was also apparent when immunogenicity was assessed in HLA-A2 transgenic mice (41), demonstrating that these design rules also apply in the context of human MHC class I (Suppl. Fig. 1).

"HELP^{ER}" vaccine formats allow for dose reduction and show superior anti-tumor effects

As the CD8+ T cell responses observed after vaccination with sig-HELP-E7SH-KDEL were highly potent, we evaluated whether substantial CD8+ T cell responses could still be induced in case either the number of vaccinations or the vaccine dose was reduced. As shown in Fig. 6A, vaccination with a single dose of the control vaccines HELP-E7SH and TTFC-E7SH led to the induction of only very modest CD8+ T cell responses. In contrast vaccination with a single dose of the sig-HELP-E7SH-KDEL vaccine still induced CD8+ T cell responses of around 15% *ex vivo* (Fig. 6A, compare with Fig. 4F,G). Likewise, marked (>20% *ex vivo*) CD8+ T cell responses were still induced by sig-HELP-E7SH-KDEL when mice were vaccinated with a 5-fold reduced DNA dose (0.4 mg/ml instead of 2mg/ml) (Fig. 6B). The latter finding is of particular relevance for clinical translation as the inability to scale DNA doses used in mice to humans is considered one of the main explanations for the lower efficacy of DNA vaccination in humans as compared to small animals (42).

In order to evaluate whether the enhanced immunogenicity of the HELP^{ER} E6 and E7 DNA vaccines also translated into a superior anti-tumor effect, mice (n=10) were challenged

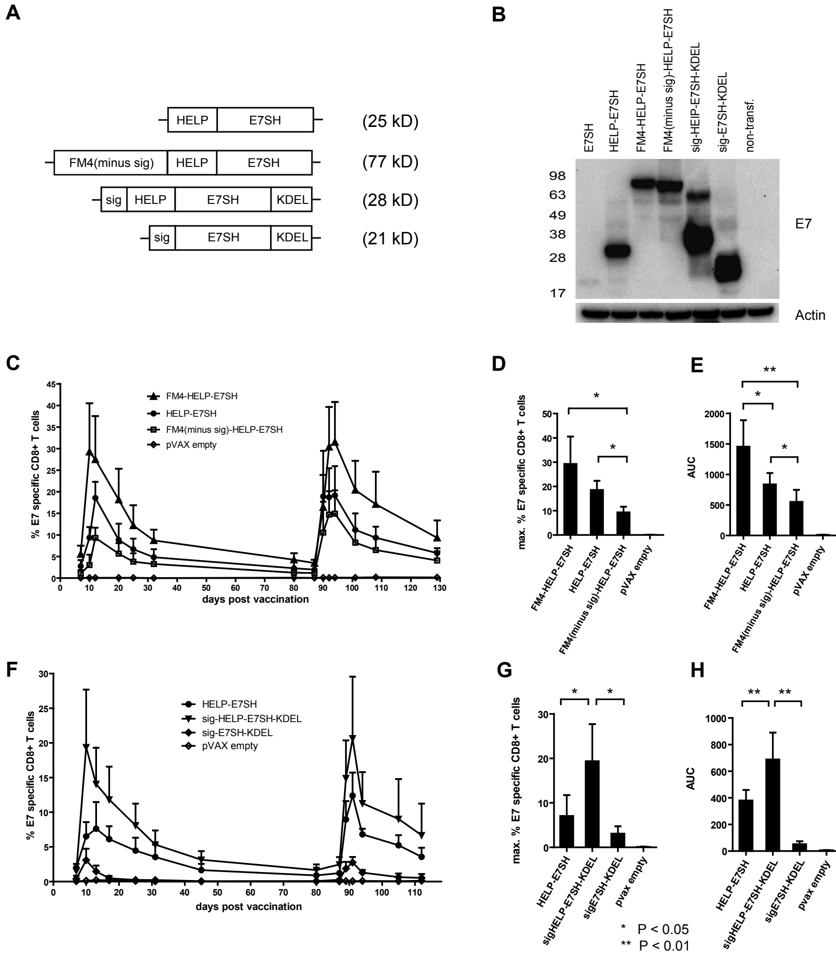


Figure 4. The 'carrier effect' can be solely explained by ER targeting of the antigen. A) Schematic representation and molecular weights of constructs used to determine if the carrier effect can be explained by ER targeting of the antigen. **B)** Western blot analysis of HEK 293 cells transfected with the constructs shown in A. In all cases a dominant band of the expected size could be detected, demonstrating the correct expression of the DNA vaccine encoded antigens. **C-E**. Data demonstrating that the removal of the signal peptide renders the carrier useless. **C)** Curve depicting the mean percentage +/- S.D. of peripheral blood H-2D^b E7$_{49-57}$-specific CD8+ T cells over time in C57BL6/J mice (n=5) DNA tattoo vaccinated on day 0, 3 and 6 with 20 µg of the indicated vaccines. **D)** Bar diagram depicting the mean percentage +/- S.D. of H-2D^b E7$_{49-57}$-specific CD8+ T cells for the indicated groups at the peak of the primary response. **E)** Bar diagram depicting the total area under the curve (AUC) +/- S.D. for each vaccine as measure for the total vaccine potency. **F-H)** Data demonstrating that the addition of only ER localization and retention signals is sufficient to increase the immunogenicity of HELP-E7SH. **F)** Time curve depicting the mean percentage +/- S.D. of peripheral blood H-2D^b E7$_{49-57}$-specific CD8+ T cells over time in C57BL6/J mice (n=5) DNA tattoo vaccinated on day 0, 3 and 6 with 20 µg of the indicated vaccines. **G)** Bar diagram depicting the mean percentage +/- S.D. of H-2D^b E7$_{49-57}$-specific CD8+ T cells for the indicated groups at the peak of the response. **H)** Bar diagram depicting the total area under the curve (AUC) +/- S.D. for each vaccine as measure for the total vaccine potency.

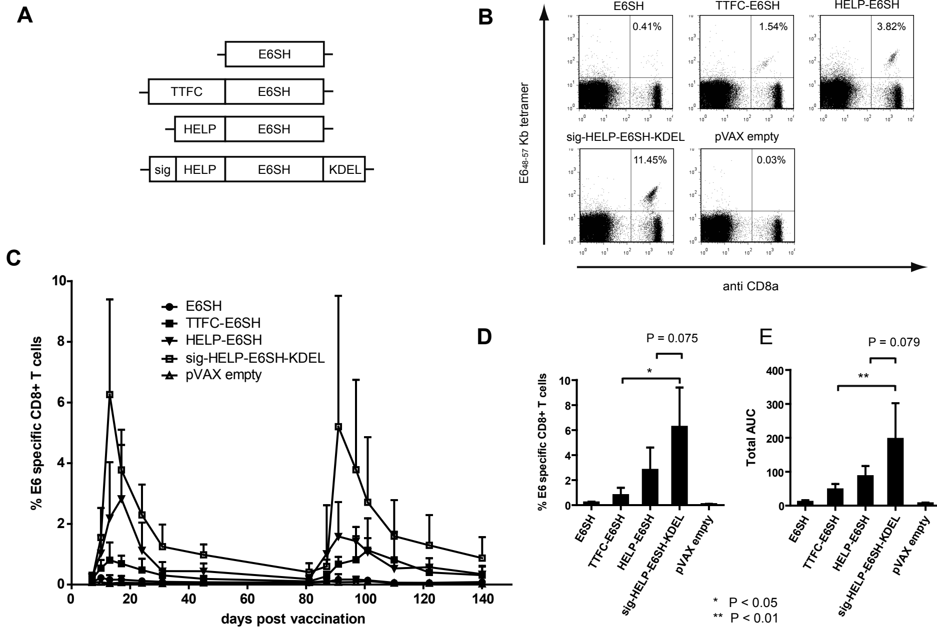


Figure 5. The combined addition of the helper-cassette and ,ER entry/retention signal also improves the immunogenicity of E6SH. A) Schematic representation and molecular weights of E6SH encoding DNA vaccines utilized. **B)** Representative dot plots at the peak of the response for the indicated vaccines. The number depicts the % of E6 specific CD8+ T cells. **C)** Curve depicting the mean percentage +/- S.D. of peripheral blood H-2K^{b} E6$_{48-57}$-specific CD8+ T cells over time in C57BL6/J mice (n=5) DNA tattoo vaccinated on day 0, 3 and 6 with 20 µg of the indicated vaccines. **D)** Bar diagram depicting the mean percentage +/- S.D. of H-2K^{b} E6$_{48-57}$-specific CD8+ T cells for the indicated groups at the peak of the response. **E)** Bar diagram depicting the total area under the curve (AUC) +/- S.D. for each vaccine as measure for the total vaccine potency.

with HPV E6/E7-expressing TC-1 cells and vaccination was started at day 4. Notably, a single vaccination with sig-HELP-E7SH-KDEL induced initial regression of tumors in 10 out of 10 mice, whereas a single vaccination with TTFC-E7SH only induced regression in 2 out of 10 mice (Fig. 6C). This difference also translated into a significant effect on survival (P=0.0019) (Fig. 6D). A similar pattern was observed in mice vaccinated with the E6SH encoding variants: sig-HELP-E6SH-KDEL significantly (p=0.0002) outperformed TTFC-E6SH in terms of tumor control and survival. These results show that both E6 and E7 HELP^{ER} DNA vaccines are superior with respect to CD8+ T cell induction and with respect to *in-vivo* tumor clearance.

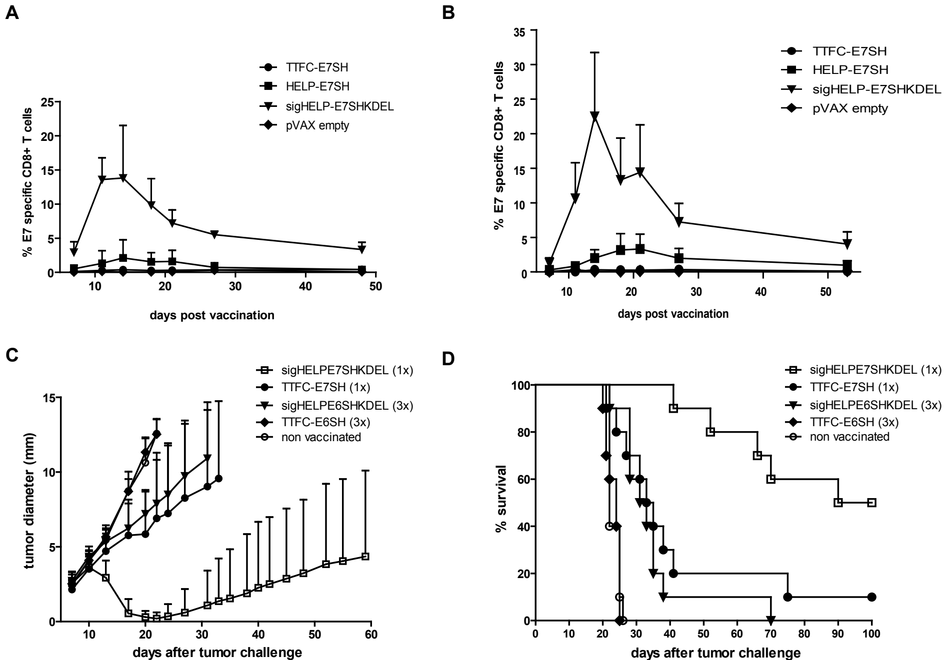


Figure 6. The novel design allows for dose sparing and shows superior functionality. A) Curve depicting the mean percentage +/- S.D. of peripheral blood H-2D^b E7$_{49-57}$-specific CD8+ T cells over time in C57BL6/J mice (n=5) after a single DNA tattoo vaccination with 30 μg of the indicated constructs. **B)** Curve depicting the mean percentage +/- S.D. of H-2D^b E7$_{49-57}$-specific CD8+ T cells over time in C57BL6/J mice (n=5) DNA tattoo vaccinated 3 times with the indicated vaccines, but using a 5 times lower DNA concentration (0.4 mg/ml) resulting in a dose of 4 μg DNA/tattoo. **C-D)** Tumor regression and prolonged survival after DNA tattoo vaccination with the sig-HELP-E7SH-KDEL and sig-HELP-E6SH-KDEL vaccine. C57BL/6 mice (n=10 per group) were injected with 1*10^5 TC-1 tumor cells on day 0. Subsequently, mice were immunized by a single DNA tattoo vaccination with 15μg DNA on day 4 (1x) after tumor challenge or with 20 μg DNA on day 4, 7, 10 (3x), as indicated in the legend. **C)** Plot depicting the mean tumor diameter (mm) +/- S.D. for the indicated groups over time. **D)** Plot depicting the percentage survival for the indicated groups over time.

DISCUSSION

In this study we aimed to rationally design DNA vaccines that induce immune reactivity against the HPV16 E6 and E7 oncoproteins. The resulting vaccine designs, sig-HELP-E6SH-KDEL and sig-HELP-E7SH-KDEL contain only minimal additional sequences apart from the antigen of interest, and induce extremely potent E6 and E7 specific CD8+ T cell responses. Our results underscore the importance of the addition of CD4+ T cell help in DNA vaccination and indicate that the enhanced immunogenicity that can be observed after addition of a carrier molecule can be achieved by the mere ER localization of the fused antigen.

The importance of CD4+ T cell help for the generation of effective CD8+ T cell responses is well established (43). In the field of DNA vaccination, multiple strategies have been developed to provide such CD4+ T cell help, and the most commonly used method is to fuse the antigen

of interest to an exogenous (e.g. bacterial) protein (21, 22). An extensively studied example of such as carrier protein is domain 1 of Tetanus Toxin fragment C that contains several CD4+ helper epitopes that can be presented by multiple MHC class II alleles (18, 21). A potential drawback of the use of foreign proteins as carrier molecules is that such proteins are likely to contain competing CD8+ T cell epitopes as well. This could result in skewing of the CD8+ T cell response towards the carrier molecule by the principle of immunodominance (44). A more elegant strategy is therefore to fuse the antigen of interest to one or multiple minimal CD4 T cell epitopes. Following this line of thought, prior work has shown the immunogenicity of DNA vaccines in which a modified version of the MHC class II invariant chain was used in which the CLIP peptide was replaced by CD4+ T cell epitopes such as P30 or PADRE (21, 45), although clinical application of this strategy may potentially be limited by the concern that vaccination could result in the induction of autoimmune reactivity towards the invariant chain. In the current study we show that robust CD4+ T cell help can simply be provided by fusing a set of promiscuous CD4+ helper epitopes that can be presented by a variety of common DP, DR and DQ alleles (see Fig. 2 and 5) to HPV E6 and E7 antigens. Because of the broad MHC class II coverage of those epitopes there may be little advantage in the inclusion of further CD4+ T cell epitopes, but if necessary this set can obviously be expanded. Importantly, as only minimal foreign sequences are added to the antigen of interest in this approach, the risk of antigenic competition should be small.

The observation that the immunogenicity of DNA vaccines can also be improved by the genetic fusion to carrier molecules that are of self origin indicates that mechanisms other than the provision of CD4+ T cell help must also play a role. Examples of endogenous carrier proteins that have been shown to improve the immunogenicity of HPV16 E6 and or E7 DNA vaccines include calreticulin, HSP 60 and IP-10 (13, 14, 16, 27). As discussed within the introduction, the enhanced immunogenicity of these self carriers is often attributed to the specific biological function of the carrier molecule (see also Table 1). However, noting that vaccine immunogenicity can be enhanced by such a variety of approaches, we speculated that more generic mechanisms may be involved, and such mechanisms could for instance involve the effect of fusion on antigen accumulation or subcellular localization (26). Comparison of the immunogenicity of E7 DNA vaccines utilizing 5 different endogenous carrier molecules indicated that immunogenicity was strongly enhanced by fusion to 2 different ER localized carrier proteins for which there is no prior data suggesting a specific role in antigen presentation. Direct support for the notion that antigen localization is the key parameter in these vaccines was provided by the demonstration that the mere addition of ER targeting and ER retention signals is sufficient to enhance immunogenicity, and this effect applied to E7SH, HELP-E7SH and HELP-E6SH. In this regard it is noteworthy that many of the strategies previously shown to improve the immunogenicity of E7 and or E6 also do result in ER localization (14, 20, 27, 28). A conceptual advantage of the use of a minimal ER localization/ retention system as developed here is the reduced risk of antigenic competition in case a foreign sequence is used, and the reduced risk of induction of autoimmune reactivity in case an endogenous protein is utilized.

At present it is unclear why ER localization of E6 and E7 benefits the induction of CD8+ T cell responses. It may be speculated that ER localization could increase the half-life of the (likely unfolded) vaccine-encoded antigens and thereby increase the size of the protein pool available for cross presentation (26). However, the observation that the correlation between the extent of antigen accumulation and immunogenicity is at best partial indicates that this can not form the sole explanation. Freigang et al (46) previously observed that modifications

that resulted in the ER localization of the LCMV glycoprotein also strongly enhanced immunogenicity in a mouse model system that was dependent on antigen cross-presentation, and several mechanisms have been proposed to explain such enhanced cross presentation. Firstly, upon cell death, ER localized antigens may be enclosed within ER-derived membranes/ vesicles that could protect them from degradation in the extracellular milieu. Secondly, defects in the folding of ER-localized proteins could lead to association with ER chaperones that could conceivably be involved in antigen delivery to APCs (47). Thirdly, the destruction of (misfolded) proteins in the ER via the endoplasmic reticulum associated protein degradation (ERAD) pathway could potentially lead in higher numbers or qualitatively different (48) antigenic peptides. Finally the accumulation of (misfolded) proteins in the ER may induce an ER-stress response, thereby triggering the cells that express the vaccine-encoded antigen to undergo apoptosis or by some other mechanism become 'visible' for APCs (49). To distinguish the first 3 from the latter hypothesis is of interest, as in the latter case the induction of ER stress could suffice to enhance immunogenicity even for cytosolic antigens. To evaluate the importance of ER presence of the antigen versus the induction of ER stress we inserted a 2A linker in between FM4 and HELP-E7SH. As a result, the FM4 moiety remains ER-resident, whereas the genetically fused HELP-E7SH (the antigen) will now remain cytosolic. Interestingly, the immunogenicity of this vaccine was not higher than that of a HELP-E7SH vaccine that lacks the ER-located FM4 moiety (Suppl. Fig. 2), thereby indicating that ER localization of the antigen itself is required. Future studies should reveal whether - and if so by which mechanism- the ER forms a dedicated compartment for antigen cross presentation.

In conclusion, here we have developed highly effective DNA vaccines for the treatment of HPV16 positive malignancies. The resulting candidate vaccines, HELP^{ER} E6 and HELP^{ER} E7, contain only minimal additional sequences apart from the antigen, thereby limiting potential safety risks and the risk of antigenic competition. The fact that these novel vaccine candidates strongly outperform two previously developed candidate vaccines both in terms of CD8+ T cell induction and tumor control warrants their clinical evaluation.

REFERENCE LIST

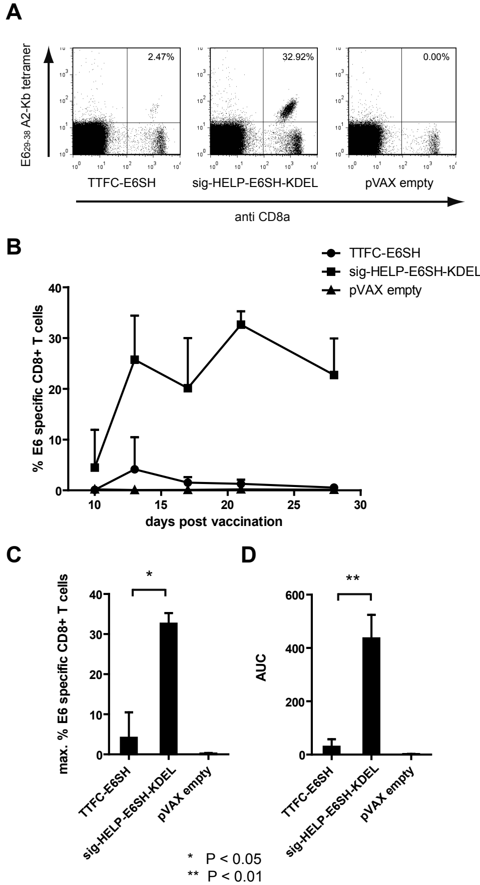
1. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol* 1989;63:4417-21.
2. Yugawa T, Kiyono T. Molecular mechanisms of cervical carcinogenesis by high-risk human papillomaviruses: novel functions of E6 and E7 oncoproteins. *Rev Med Virol* 2009;19:97-113.
3. Kessler JH, Melief CJ. Identification of T-cell epitopes for cancer immunotherapy. *Leukemia* 2007;21:1859-74.
4. Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res* 2007;67:354-61.
5. Nakagawa M, Stites DP, Farhat S, Sisler JR, Moss B, Kong F et al. Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia. *J Infect Dis* 1997;175:927-31.
6. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 2009;361:1838-47.
7. Brinkman JA, Hughes SH, Stone P, Caffrey AS, Muderspach LI, Roman LD et al. Therapeutic vaccination for HPV induced cervical cancers. *Dis Markers* 2007;23:337-52.
8. Hung CF, Ma B, Monie A, Tsen SW, Wu TC. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. *Expert Opin Biol Ther* 2008;8:421-39.

9. Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgonne H, Kimman TG. Preclinical and clinical safety studies on DNA vaccines. *Hum Vaccin* 2006;2:45-53.
10. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet* 2008;9:776-88.
11. Lin K, Roosinovich E, Ma B, Hung CF, Wu TC. Therapeutic HPV DNA vaccines. *Immunol Res* 2010;47:86-112.
12. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM et al. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res* 2000;60:1035-42.
13. Huang CY, Chen CA, Lee CN, Chang MC, Su YN, Lin YC et al. DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generates more potent immunotherapeutic effects than respective E6 or E7 tumor antigens. *Gynecol Oncol* 2007;107:404-12.
14. Cheng WF, Hung CF, Chai CY, Hsu KF, He L, Ling M et al. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J Clin Invest* 2001;108:669-78.
15. Smahel M, Pokorna D, Mackova J, Vlasak J. Enhancement of immunogenicity of HPV16 E7 oncogene by fusion with E. coli beta-glucuronidase. *J Gene Med* 2004;6:1092-101.
16. Kang TH, Kim KW, Bae HC, Seong SY, Kim TW. Enhancement of DNA vaccine potency by antigen linkage to IFN-gamma-inducible protein-10. *Int J Cancer* 2011;128:702-14.
17. Michel N, Osen W, Gissmann L, Schumacher TN, Zentgraf H, Muller M. Enhanced immunogenicity of HPV16 E7 fusion proteins in DNA vaccination. *Virology* 2002;294:47-59.
18. Oosterhuis K, Ohlschlager P, van den Berg JH, Toebes M, Gomez R, Schumacher TN et al. Preclinical development of highly effective and safe DNA vaccines directed against HPV16 E6 and E7. *Int J Cancer* 2011.
19. Lin CT, Chang TC, Chao A, Dzeng E, Soong YK, Hung CF et al. Enhancement of DNA vaccine potency through linkage of antigen gene to ER chaperone molecules, ER-60, tapasin, and calnexin. *J Biomed Sci* 2005;12:279-87.
20. Hung CF, Hsu KF, Cheng WF, Chai CY, He L, Ling M et al. Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. *Cancer Res* 2001;61:1080-8.
21. Stevenson FK, Rice J, Ottensmeier CH, Thirdborough SM, Zhu D. DNA fusion gene vaccines against cancer: from the laboratory to the clinic. *Immunol Rev* 2004;199:156-80.
22. Wolkers MC, Toebes M, Okabe M, Haanen JB, Schumacher TN. Optimizing the efficacy of epitope-directed DNA vaccination. *J Immunol* 2002;168:4998-5004.
23. Holst PJ, Bassi MR, Thomsen AR, Christensen JP. DNA fusion gene vaccines. *Curr Opin Mol Ther* 2010;12:47-54.
24. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. *Virology* 2009;384:335-44.
25. Tomaic V, Pim D, Banks L. The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. *Virology* 2009;393:7-10.
26. Bins AD, Wolkers MC, van den Boom MD, Haanen JB, Schumacher TN. In vivo antigen stability affects DNA vaccine immunogenicity. *J Immunol* 2007;179:2126-33.
27. Peng S, Ji H, Trimble C, He L, Tsai YC, Yeatermeyer J et al. Development of a DNA vaccine targeting human papillomavirus type 16 oncoprotein E6. *J Virol* 2004;78:8468-76.
28. Smahel M, Polakova I, Pokorna D, Ludvikova V, Duskova M, Vlasak J. Enhancement of T cell-mediated and humoral immunity of beta-glucuronidase-based DNA vaccines against HPV16 E7 oncoprotein. *Int J Oncol* 2008;33:93-101.
29. Brulet JM, Maudoux F, Thomas S, Thielemans K, Burny A, Leo O et al. DNA vaccine encoding endosome-targeted human papillomavirus type 16 E7 protein generates CD4+ T cell-dependent protection. *Eur J Immunol* 2007;37:376-84.
30. Ji H, Wang TL, Chen CH, Pai SI, Hung CF, Lin KY et al. Targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartment enhances the antitumor immunity of DNA vaccines against murine human papillomavirus type 16 E7-expressing tumors. *Hum Gene Ther* 1999;10:2727-40.
31. Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ et al. Design and use of conditional MHC class I ligands. *Nat Med* 2006;12:246-51.
32. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;56:21-6.
33. Rivera VM, Wang X, Wardwell S, Courage NL, Volchuk A, Keenan T et al. Regulation of protein secretion through controlled aggregation in the endoplasmic reticulum. *Science* 2000;287:826-30.
34. Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol* 1989;19:2237-42.
35. Alexander J, Sidney J, Southwood S, Ruppert J, Oseroff C, Maewal A et al. Development of high potency universal DR-restricted helper

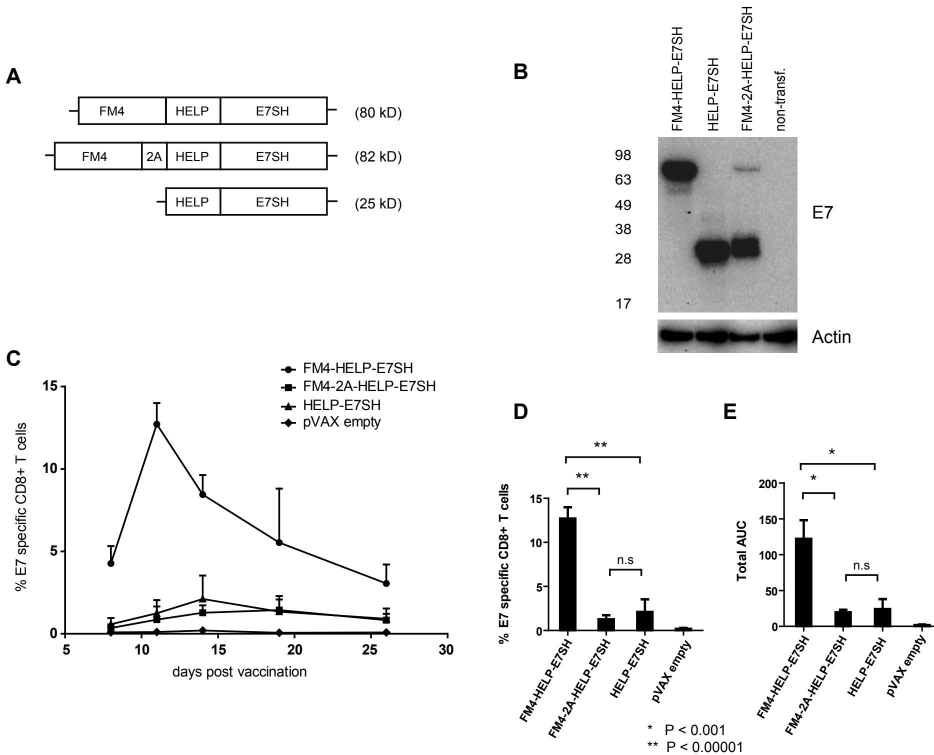
epitopes by modification of high affinity DR-blocking peptides. *Immunity* 1994;1:751-61.

36. Pancre V, Georges B, Angyalosi G, Castelli F, Delanoye A, Delacre M et al. Novel promiscuous HLA-DQ HIV Nef peptide that induces IFN-gamma-producing memory CD4+ T cells. *Clin Exp Immunol* 2002;129:429-37.
37. Rice J, Elliott T, Buchan S, Stevenson FK. DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines. *J Immunol* 2001;167:1558-65.
38. Livingston B, Crimi C, Newman M, Higashimoto Y, Appella E, Sidney J et al. A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. *J Immunol* 2002;168:5499-506.
39. Ohlschlager P, Pes M, Osen W, Durst M, Schneider A, Gissmann L et al. An improved rearranged Human Papillomavirus Type 16 E7 DNA vaccine candidate (HPV-16 E7SH) induces an E7 wildtype-specific T cell response. *Vaccine* 2006;24:2880-93.
40. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med* 2005;11:899-904.
41. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. *J Exp Med* 1997;185:2043-51.
42. Johnston SA, Talaat AM, McGuire MJ. Genetic immunization: what's in a name? *Arch Med Res* 2002;33:325-9.
43. Bevan MJ. Helping the CD8(+) T-cell response. *Nat Rev Immunol* 2004;4:595-602.
44. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol* 1999;17:51-88.
45. Wu A, Zeng Q, Kang TH, Peng S, Roosinovich E, Pai SI et al. Innovative DNA vaccine for human papillomavirus (HPV)-associated head and neck cancer. *Gene Ther* 2011;18:304-12.
46. Freigang S, Eschli B, Harris N, Geuking M, Quirin K, Schrempf S et al. A lymphocytic choriomeningitis virus glycoprotein variant that is retained in the endoplasmic reticulum efficiently cross-primes CD8(+) T cell responses. *Proc Natl Acad Sci U S A* 2007;104:13426-31.
47. Binder RJ, Srivastava PK. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat Immunol* 2005;6:593-9.
48. Golovina TN, Wherry EJ, Bullock TN, Eisenlohr LC. Efficient and qualitatively distinct MHC class I-restricted presentation of antigen targeted to the endoplasmic reticulum. *J Immunol* 2002;168:2667-75.
49. Martins I, Kepp O, Schlemmer F, Adjemian S, Tailler M, Shen S et al. Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. *Oncogene* 2011;30:1147-58.
50. Hung CF, Cheng WF, Hsu KF, Chai CY, He L, Ling M et al. Cancer immunotherapy using a DNA vaccine encoding the translocation domain of a bacterial toxin linked to a tumor antigen. *Cancer Res* 2001;61:3698-703.

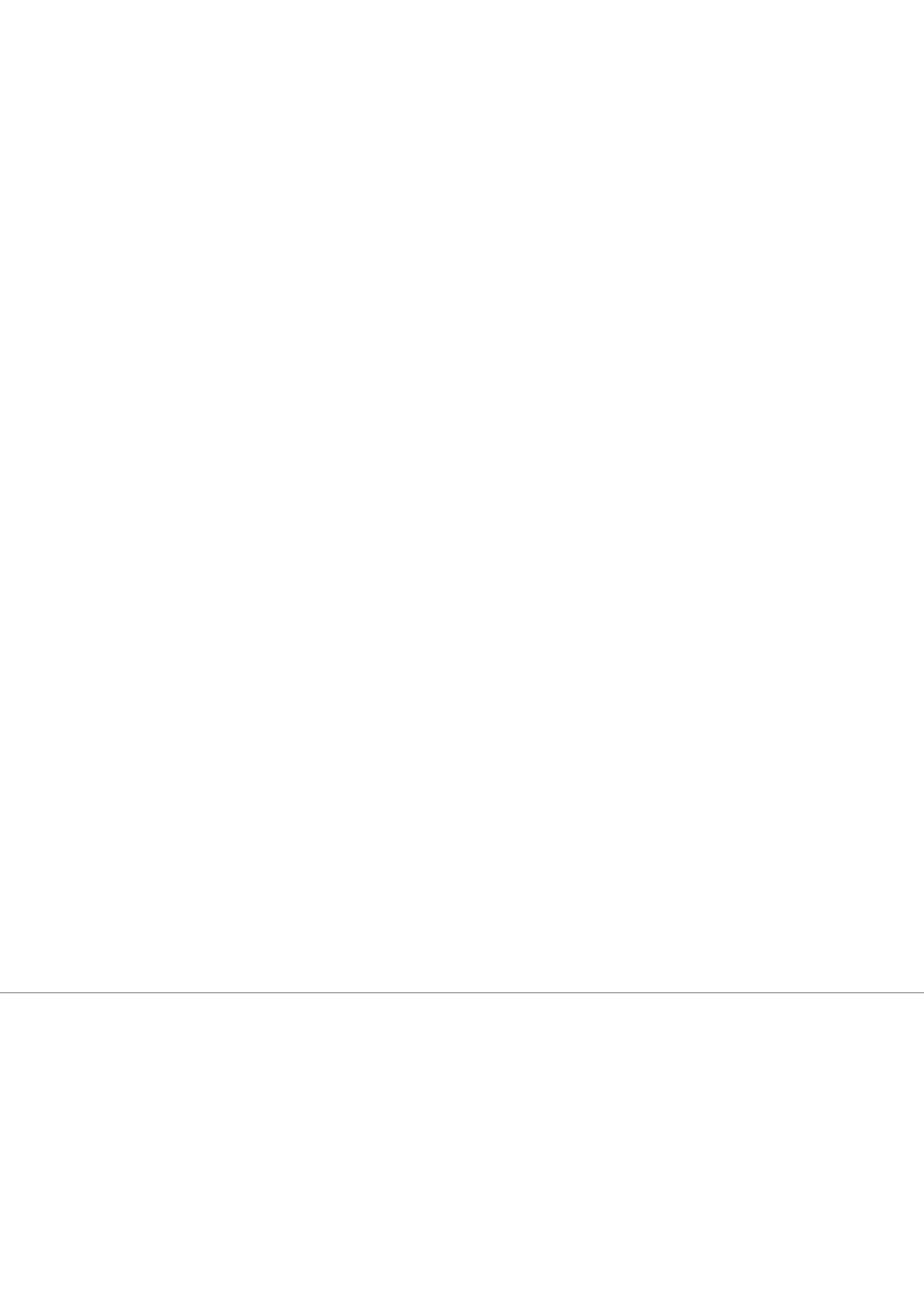
SUPPLEMENTARY FIGURES



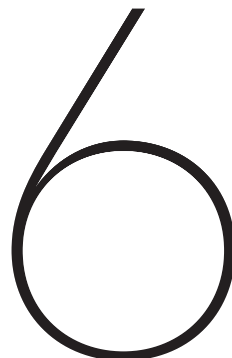
Supplementary figure 1. sig-HELP-E6SH-KDEL strongly outperforms TTFC-E6SH in HLA-A2 transgenic mice. HLA-A2 transgenic mouse class I knock-out (HHD) mice (n=3 per group) were immunized by DNA tattoo vaccination on day 0, 3 and 6, and peripheral blood was analyzed for antigen-specific $CD8^+$ T cells by MHC tetramer staining. **A)** Representative dot plots of A2-K^b $E6_{29-38}$ MHC tetramer stainings at the peak of the response for the different vaccines **B)** Plot depicting the mean percentage +/- S.D. of A2-K^b $E6_{29-38}$-specific $CD8^+$ T cells for the indicated groups over time. **C)** Bar diagram depicting the mean percentage +/- S.D. of A2-K^b $E6_{29-38}$-specific $CD8^+$ T cells for the indicated groups at the peak of the response. **D)** Bar diagram depicting the total area under the curve (AUC) +/- S.D. for each construct as measure for the total vaccine potency.



Supplementary figure 2. ER localization of the antigen and not the ER-stress provoked is causing the increased immunogenicity. A) Schematic representation and estimated molecular weights of the DNA vaccines used to test if ER-stress or ER localization is causing the increase in immunogenicity. **B)** Westernblot analysis of HEK 293 cells transfected with the vaccines shown in A. The FM4-2A-HELP-E7SH encoding constructs shows a dominant band of the size of HELP-E7SH and only a minor band of the size of FM4-HELP-E7SH demonstrating the functionality of the 2A linker. **C)** Curve depicting the mean percentage +/- S.D. of H-2D^b E7$_{49-57}$-specific CD8$^+$ T cells over time in C57BL/6J mice (n=5) tattoo vaccinated on day 0,3 and 6 with the indicated vaccines **D)** Bar diagram depicting the mean percentage +/- S.D. of H-2D^b E7$_{49-57}$-specific CD8$^+$ T cells for the indicated groups at the peak of the response. **E)** Bar diagram depicting the total area under the curve (AUC) +/- S.D. for each vaccine as measure for the total vaccine potency.



CHAPTER



SHIELDING THE CATIONIC CHARGE OF NANOPARTICLE-FORMULATED DERMAL DNA VACCINES IS ESSENTIAL FOR ANTIGEN EXPRESSION AND IMMUNOGENICITY

ABSTRACT

Nanoparticle-formulated DNA vaccines hold promise for the design of *in vivo* vaccination platforms that target defined cell types in human skin. A variety of DNA formulations, mainly based on cationic liposomes or polymers, has been investigated to improve transfection efficiency in *in vitro* assays.

Here we demonstrate that formulation of DNA into both liposomal and polymeric cationic nanoparticles completely blocks vaccination-induced antigen expression in mice and *ex vivo* human skin. Furthermore, this detrimental effect of cationic nanoparticle formulation is associated with an essentially complete block in vaccine immunogenicity. The blocking of DNA vaccine activity may be explained by immobilization of the nanoparticles in the extracellular matrix, caused by electrostatic interactions of the cationic nanoparticles with negatively charged extracellular matrix components. Shielding the surface charge of the nanoparticles by PEGylation improves *in vivo* antigen expression more than 55 fold. Furthermore, this shielding of cationic surface charge results in antigen-specific T cell responses that are similar as those induced by naked DNA for the two lipo- and polyplex DNA carrier systems. These observations suggest that charge shielding forms a generally applicable strategy for the development of dermally applied vaccine formulations. Furthermore, the nanoparticle formulations developed here form an attractive platform for the design of targeted nanoparticle formulations that can be utilized for *in vivo* transfection of defined cell types.

6

Joost H. van den Berg[a,b,c]*, Koen Oosterhuis[c], Wim E. Hennink[b], Gert Storm[b], Leonardus J. van der Aa[d], Johan F.J. Engbersen[d], John B.A.G. Haanen[c], Jos H. Beijnen[a,e], Ton N. Schumacher[c], Bastiaan Nuijen[a]

[a] Department of Pharmacy & Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC, Amsterdam, The Netherlands [b] Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands [c] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands [d] Department of Biomedical Chemistry , Faculty Science & Technology, University of Twente, 7500 AE, Enschede, The Netherlands [e] Department of Bioanalysis , Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands

J. Control. Release: 141(2), 234-40 (2010)

INTRODUCTION

Non-viral carrier systems are widely used as transfection reagents to deliver nucleic acids for both *in vitro* and *in vivo* applications. In these systems, negatively charged DNA is bound by electrostatic interaction to an excess of a positively charged carrier. In this complexation process, DNA is condensated into positively charged, nanosized particles and protected from nuclease degradation, resulting in substantially higher transfection efficiencies compared to naked nucleic acids in *in vitro* assays. The two most frequently used carriers to enhance transfection efficiency are cationic lipids and cationic polymers, and the resulting DNA nanoparticles are referred to as lipoplexes and polyplexes, respectively (1-4). In addition to the beneficial effect on *in vitro* transfection efficiency, formulation of DNA into cationic particles has also been shown to result in a higher transfection efficiency than naked DNA upon intramuscular injection (5,6).

While DNA vaccines were first described using intramuscular injection as an administration route (7), a growing interest has developed into intradermal DNA vaccine delivery. Specifically, because of its natural barrier function, the skin is perceived as a site that is well-equipped for the induction of adaptive immune responses and the high density of antigen-presenting cells in skin provides indirect support for this notion. Dermal DNA vaccines can be applied by various methods, including classical intradermal injection, gene gun and DNA tattoo (8). The latter strategy delivers naked plasmid DNA into the skin through thousands of punctures using an oscillating multiple needle tattoo device. DNA tattooing leads to the induction of strong and rapid antigen-specific cellular immune responses in mice (8). Furthermore, the immunogenicity of DNA tattoo is approximately 10-100 fold higher than that of classical intramuscular DNA vaccination in non-human primates (9). Importantly, in spite of the high immunogenicity of DNA tattoo, the *in vivo* transfection efficiency of naked DNA with this technique is extremely low, with approximately 1 out of $5x10^6$ to $5x10^9$ plasmid copies applied being taken up, transcribed and translated (10). Based on the strongly positive effect on transfection that is generally observed for DNA encapsulation in cationic lipo- and polyplexes in *in vitro* assays, we aimed to determine whether such formulations could also result in improved transfection and subsequent immune response for intradermal vaccines applied by DNA tattooing. Development of these nanoparticle-formulated DNA vaccines forms an essential first step towards the further development of targeted intradermal DNA vaccines.

MATERIALS AND METHODS

Materials

The pVAX:Luc-NP plasmid (8) encodes the influenza A $NP_{366\text{-}374}$ epitope as a genetic fusion with firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). pVAX:GFP was generated by inserting Green Fluorescence Protein (GFP) encoding DNA into the BamHI/NotI site of pVAX1. Plasmids were expressed and amplified in *E. Coli* DH5 and were purified by Endofree™ QIAGEN® Mega-kit (QIAGEN®, Hilden, Germany). 1,2-dioleoyl-oxypropyl-3-trimethyl-ammonium chloride (DOTAP) was obtained from Avanti Polar lipids (Alabster, AL, USA). Dioleoylphosphatidyl-ethanolamide (DOPE) and distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG) were a kind gift from Lipoïd GmbH (Ludwigshafen, Germany). NonPEGylated and PEGylated poly(amido amine) (PAA) copolymers possessing protonable amino groups and bioreducible disulfide linkages in

the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of *N,N'*-cystaminebisacrylamide with the appropriate amine according to the procedure described previously (11). The PEGylated analog was prepared using 11 mol% of MeO-PEG-NH_2 in the total amino monomer feed during the PAA synthesis. All other chemicals were of analytical grade.

Liposome preparation

NonPEGylated liposomes, composed of DOTAP-DOPE, were prepared in a 1:1 molar ratio. For PEGylated liposomes, DOPE was replaced by DSPE-PEG at different concentrations to keep the total molarity of lipids constant. Lipid mixtures were dissolved in chloroform/methanol (1:1 v/v) and mixed in a round-bottomed flask. Organic solvents were evaporated at 40 ºC using a vacuum evaporator and the obtained lipid films were purged with nitrogen for 30 min. Lipid films were rehydrated in 20 mM HEPES, pH 7.4, 10% sucrose, to give a final lipid concentration of 35 mM. The resuspended lipids were extruded 8 times through two stacked polycarbonate membranes (Poretics, Livermore, USA, 200 and 100 nm) to obtain small unilamellar vesicles of 100 nm.

Lipoplex and polyplex preparation and characterization

Lipo- and polyplexes were prepared by mixing an equal volume of plasmid and cationic liposomes or dissolved polymer. All formulations were prepared in 20 mM HEPES pH 7.4, 10% sucrose buffer with a high viscosity and a low ionic strength, conditions previously shown to be favourable for obtaining small and stable DNA complexes (12). Formulation characterizations were performed with the Luc-NP construct.

N/P ratios were defined as the charge ratio between cationic nitrogen residues in DOTAP or PAA and anionic phosphate groups in the DNA. Ratios were calculated assuming that 302 and 532 g/mol correspond with each (protonable) nitrogen containing-repeating unit of PAA and PEG-PAA, respectively. For DOTAP 699 g/mol is the mass bearing one cationic nitrogen. For plasmid DNA 330 g/mol corresponds with the average mass of a repeating unit bearing one negative phosphate group. For polyplexes, weight ratios polymer/DNA are also provided. All complexes were formulated with a final DNA concentration of 0.5 mg/mL. Control naked DNA was diluted to the same concentration in the same buffer. The size of obtained particles was measured in 20 mM HEPES pH 7.4, 10% sucrose, with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 20 mM HEPES pH 7.4, using a Zetasizer Nano Z (Malvern Instruments). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The presence of unbound DNA was visualized by electrophoresis at 85 V using a 1% agarose gel containing 0.5 μg/mL etidium bromide. Only particle formulations shown not to be aggregated and containing no free DNA were used in further experiments.

Transfection of epidermal cell suspensions

Healthy human abdominal skin from female patients (41-63 years) was obtained from the plastic surgery department of the institute according with the guidelines of the Antoni van Leeuwenhoek Hospital/ The Netherlands Cancer Institute. Subcutaneous fat was directly removed by blunt dissection. Skin was transported on ice and used within 2 hours after surgical removal.

To obtain an epidermal cell suspension, skin was incubated for 1 hour in 10 mg/mL dispase II (Sigma Aldrich, St. Louis, MO, USA) in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25 µg/mL amphotericin B (all Invitrogen) at 37 ºC, upon which the epidermis was mechanically peeled from skin samples. The obtained epidermal sheet was digested at 37 ºC in complete keratinocyte medium containing 0.05% trypsin. After 15 min, the epidermis was disrupted with a glass pipette and 10% FCS was added to the medium, after which the cells were filtered through 70 µm nylon gauze to remove debris.

Per well, 1 x 10^5 cells of a freshly prepared epidermal cell suspension were seed in 24-well tissue culture plates in complete keratinocyte medium. Cells were incubated with 50 µl naked pVAX:GFP or the indicated nanoparticle formulation (all at 0.04 mg/mL DNA to obtain a final concentration of 1 µg DNA/well) at 2% CO_2, 37 ºC. After 24 hours, cells were harvested and analyzed for GFP expression using a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) and data were analyzed using Flowjo software (Three Star, Ashland, USA). Live cells were selected based on propidium iodide exclusion.

DNA tattooing of *ex vivo* human skin

Formulations and naked DNA controls encoding firefly luciferase were administered to intact skin by DNA tattooing (8) to allow longitudinal luciferase expression measurements. Alternatively, formulations and naked DNA controls encoding GFP were used for flow cytometric analysis of transfected cell types. The skin model used in these experiments has been described previously for the optimization of tattooing of naked DNA in skin (10). In brief, 10µl of the indicated formulation at a final DNA concentration of 0.5 mg/ml was applied to the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8mm, surface 50 mm^2). The formulation was subsequently administered into the skin using a Permanent Make Up (PMU)® tattoo machine (kindly provided by MT Derm GmbH, Berlin, Germany). For all tattoos, 9-needle cartridges at an oscillating frequency of 100 Hz were used. The needle depth was adjusted to 1.5 mm and tattoo duration was 20 seconds.

After tattooing, skin samples were kept at 5% CO_2, 37 ºC in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25 µg/mL amphotericin B (all Invitrogen) to allow longitudinal expression measurements of luciferase. During this incubation, skin was cultured at the air-medium interface with the epidermis exposed to the air to mimic the natural situation.

Measurement of antigen expression using intravital imaging of *ex vivo* human skin

The expression of luciferase was measured in intact skin samples at the indicated time points after tattooing. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/mL. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 minutes after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS system 100 CCD camera (Xenogen, Hopkinton, USA).

Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. In all measurements, background luminescence was determined for non-treated skin to allow correction during data analysis. After each measurement, medium was refreshed to remove residual luciferin.

Flow cytometric analysis of DNA vaccine induced antigen expression

For flow cytometry experiments, tattooed areas of interest were removed from the intact skin with a 6 mm biopsy punch and transferred into 48-well plates. Directly upon tattooing, epidermal sheets were removed as described above and incubated overnight at 37 °C. After incubation, epidermal sheets were digested and stained with antibodies. The antibodies used were mouse anti-human CD1a allophycocyanin (APC) (Immunotech) and mouse anti human cytokeratin (equal mixture of clone LP34 and MNF116 (both Dako, Glostrup Denmark)), labelled with Alexa Fluor 647 (Invitrogen) according to manufacturer's protocol. Prior to cytokeratin staining, epidermal cell suspensions were permeabilized using the BD Cytofix/Cytoperm kit (BD Sciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. In case of anti CD1a staining, live cells were selected based on propidium iodide exclusion.

DNA immunization

C57BL/6J mice (6-8 weeks) were obtained from the experimental animal department of The Netherlands Cancer Institute. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the NKI-AVL Animal Research Committee.

To allow simultaneous measurement of antigen expression and T cell responses, mice were immunized by DNA tattooing with formulations containing the pVAX:Luc-NP construct. Before intradermal DNA vaccination, the hair at the administration sites was removed with depilatory cream (Veet sensitve, Reckitt Benckiser, Hull, UK). During immunization, 15 μl of lipo- or polyplex formulation or naked DNA solution at a final concentration of 0.5 mg/ml was applied to the skin of the hind leg and administered using a disposable 9-needle cartridge mounted on an PMU® tattoo machine. DNA vaccines were tattooed during 30 s at a needle depth of 1.0 mm, and the needle bar oscillated at 100 Hz. Using this needle depth setting, cells in both the epidermis and upper layer of the dermis are transfected (8). Mice were vaccinated on day 0, 3 and 6. All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment. At the indicated time points after immunization, approximately 50 μl of peripheral blood was collected by tail bleeding for the measurement of T cell responses.

Measurement of antigen expression using intravital imaging

Antigen expression upon DNA vaccination was measured by a light-sensitive camera to allow longitudinal *in vivo* expression of firefly luciferase. Mice were anesthetized with isoflurane. A solution of the substrate luciferin in PBS (150 mg/kg, Xenogen) was intraperitoneally injected and after 18 min, the luminescence produced by active luciferase was acquired during 30 s in an IVIS® system 100 CCD camera (Xenogen). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

Antigen-specific T cell assay

To measure antigen-specific T cell responses, peripheral blood lymphocytes were stained on different time points with H-2D^b/NP$_{366-374}$-tetramers and APC-conjugated CD8α antibody (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1× PBS, 0.5% BSA and 0.02% sodium azide) as described before (13). Cells were washed three times in FACS buffer and analyzed by flow cytometry. Live cells were selected based on propidium iodide exclusion.

Statistical Analysis

A two-tailed Mann-Whitney U-test was used for statistical analysis and a value of $p < 0.05$ was considered significant. A Bonferroni adjustment test was applied to correct the significant level when multiple groups were compared.

RESULTS

Cationic nanoparticles increase transfection efficiency in epidermal cell suspensions but decrease antigen expression in *ex vivo* human skin and in mice

In order to develop nanoparticle formulations for intradermal application of DNA vaccines, DNA was complexed with cationic DOTAP-DOPE liposomes or with cationic poly(amidoamine) (PAA) polymers, to form lipoplexes and polyplexes, respectively. DOTAP-DOPE was chosen since this is the most commonly used composition in liposomal based transfection experiments. PAA was chosen as a novel and biodegradable polymeric carrier system. As DNA vaccination is known to require high DNA concentrations (14-16), complexes were formulated with a final DNA concentration of 0.5 mg/mL, which is relatively high for these systems. The obtained lipoplexes and polyplexes were characterized for particles size and surface charge (as reflected by the zeta potential). Both types of DNA-nanoparticles had particle sizes below 240 nm, did not contain free DNA (as determined by gel electrophoresis) and exhibited a zeta potential above +40 mV.

To determine the effect of DNA formulation into nanoparticles on *in vitro* transfection efficiency, fresh suspensions of non-transformed human epidermal cells were used. These target cells were transfected *in vitro* with either lipoplex or polyplex nanoparticles that had been formulated with a GFP encoding construct, and transfection-induced GFP expression was analyzed 24 hrs after addition to the cells, by flow cytometry. For both types of nanoparticles, nanoparticle formulation resulted in marked increase in transfection efficiency when compared with naked DNA (by a factor of >26 and >900 for polyplexes and lipoplexes respectively, Figure 1A). These data demonstrate that the superior *in vitro* transfection properties of formulated cationic nanoparticles previously shown for human cell lines also apply to non-transformed human skin cells.

To study the performance of cationic DNA-nanoparticles in a clinically more relevant model, antigen expression was measured in intact *ex vivo* human skin upon tattooing of nanoparticles formulated with a luciferase encoding construct. Luciferase expression was measured with a light sensitive CCD camera. Surprisingly, antigen expression induced by application of both lipoplex and polyplex DNA nanoparticles was extremely low (see Figure 1B). In contrast, intradermal application of naked DNA resulted in robust levels of antigen expression, as observed previously (10). Consistent with the data obtained in human skin, application of lipo- or polyplexes to murine skin also yield very low levels of antigen expression (Figure 1C) and corresponding undetectable antigen-specific immune responses in vaccinated mice (see below).

These results demonstrate that *in vitro* transfection data of these positively charged DNA vaccine formulations bear little, if any, predictive value for *in vivo* expression in either murine or human skin. Furthermore, the data provide the more general indication that cationic nanoparticles are ill-suited for the intradermal application of DNA vaccines.

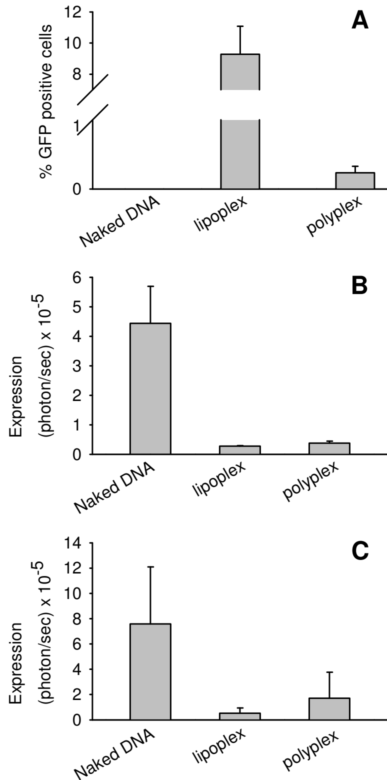


Figure 1. Discordant *in vitro* and *in vivo* performance of cationic nanoparticles. (**A**) Transfection of epidermal cell suspensions with naked DNA, DOTAP-DOPE/DNA complexes ('lipoplex'), and PAA/DNA complexes ('polyplex'). Bars represent the mean + SD of three independent measurements. (**B**) Luciferase activity upon application of naked DNA, lipoplex or polyplex formulation to intact *ex vivo* human skin by DNA tattoo. Data shown depict luciferase activity measured 5 hours after DNA application. The same poor performance of lipoplex and polyplex DNA formulations was observed after 21 hrs. Bars represent the mean + SD of 3-5 measurements. (**C**) Luciferase activity upon application of naked DNA, lipoplex or polyplex formulation to murine skin. Data shown depict the poor performance of lipoplex and polyplex DNA formulation when compared with naked DNA. Expression was measured 8 hours after tattooing. Bars represent the mean + SD of 5-8 mice. All formulations were prepared at a charge (N/P) ratio of 5 (lipoplexes) or 55 (polyplexes), to obtain particles with sizes below 240 nm and a zeta potential above +40 mV.

Shielding of the cationic surface charge restores transfection efficiency of nanoparticles in *ex vivo* human skin

To determine the underlying reason for the discordance between the effectiveness of DNA nanoparticles in *in vitro* and *in/ ex vivo* assays we focussed on potential differences between intact skin and skin cell cultures. First, the presence of the extracellular matrix (ECM) in skin tissue conceivably reduces free diffusion of particles in intact skin. As condensation of DNA into nanoparticles results in a reduced size compared to free DNA it is unlikely that a sieve function of the ECM is responsible for the reduction in effectiveness *in vivo*. However, several ECM components carry a net negative charge and are likely to interact with the positively charged nanoparticles, with the result that the nanoparticles become immobilized in the ECM. Thus, while in *in vitro* cultures the cationic charge of the nanoparticles is positively contributing to the transfection process by promoting binding to cell surfaces, this positive effect is most likely surpassed *in vivo* by electrostatic interactions with anionic ECM components and consequent immobilization of these nanoparticles in the ECM, preventing the particles to reach their target.

In order to evaluate whether the positive particle charge is responsible for the absence of vaccination-induced antigen expression in intact skin, we introduced poly (ethylene glycol) (PEG) moieties to both types of nanoparticles, a strategy that is known to reduce the surface charge of lipo-and polyplexes. The presence of increasing concentrations of PEG in both lipo- and polyplexes resulted in a reduction of the surface charge in a dose-dependent manner from

+40-60 to close to neutrality for both formulations, together with a modest (2-fold or less) reduction in particle size (see supplementary Figures 1-3).

Subsequently, PEGylated nanoparticles were applied to intact human skin by DNA tattooing and vaccination-induced antigen expression was analyzed. In agreement with our hypothesis that blocking of the intradermal expression as observed for the cationic nanoparticles is due to their positive surface charge, the PEGylated nanoparticles showed a very marked increase in antigen expression (~50-fold and ~20-fold for lipo- and polyplexes, respectively, see Figures 2 and 3).

For the PEGylated lipoplexes, antigen expression levels reached a plateau value at a DSPE-PEG content between 15 and 17.5% (Figure 2A) with an expression level that was 1.6 ± 0.3 fold (mean ± SD, measured in three independent pieces of skin) higher at the peak of expression than naked DNA. At a DSPE-PEG content of 10%, no difference in antigen expression was observed between N/P ratio 2 and 5 (data not shown).

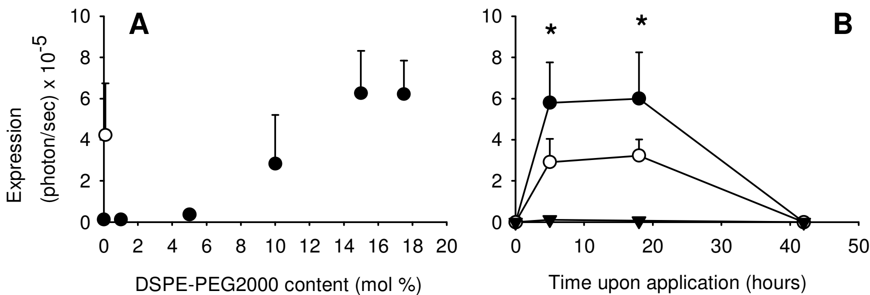


Figure 2. Antigen expression (luciferase) upon tattoo vaccination of PEGylated lipoplexes in *ex vivo* human skin. (**A**) Expression induced by administration of naked DNA (○), or by administration of DOTAP-DOPE/DNA complexes as a function of DSPE-PEG content at an N/P ratio of 5 (●). Expression was measured at 5 hrs post DNA application. (**B**) Longitudinal expression upon application of naked DNA (○) or application of DOTAP-DOPE/DNA lipoplexes (N/P of 5) with (●) or without (▼)17.5 mol % DSPE-PEG. Each point represents the mean + SD of 3-8 data points, randomized tattooed over one biopsy of skin. All experiments were performed in triplicate. * Values significantly different from naked DNA control.

The PEGylated polyplexes showed a plateau in antigen expression levels at a polymer/DNA w/w ratio between 25:1 and 50:1 (Figure 3B) that was 8.5 ± 4.4 fold (mean ± SD, measured in three independent pieces of skin) higher than naked DNA.

Finally, to determine whether the physical incorporation of PEG into nanoparticles is essential to restore antigen expression, a control experiment was performed in which unbound PEG 2000 was added to the nonPEGylated lipo- and polyplexes in the same concentrations as used in the PEGylated particles. Application of these formulations to human skin resulted in non-detectable levels of antigen expression levels (data not shown). This demonstrates that the observed effects are due to the PEG modification of the particles rather than to the presence of PEG itself in the formulation solutions.

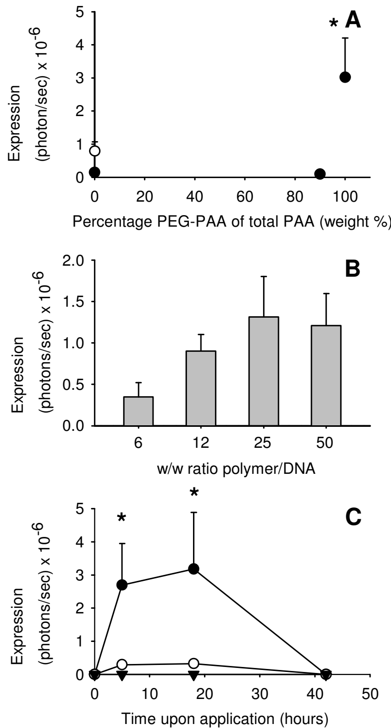


Figure 3. Antigen expression upon tattoo vaccination of PEGylated polyplexes in *ex vivo* human skin. (**A**) Expression upon application of naked DNA (○), or application of PAA/DNA polyplexes as a function of the percentage of PEGylated PAA at a w/w ratio polymer/DNA of 50 (●). (**B**) Expression upon application of PEGylated PAA /DNA polyplexes at different ratios polymer/DNA. Expression was measured at 5hrs post DNA application. (**C**) Longitudinal expression of luciferase in human skin upon application of naked DNA (○), PEGylated PAA/DNA polyplexes at a w/w ratio polymer/DNA of 50 (●) or nonPEGylated PAA/DNA complexes at a w/w ratio polymer/DNA of 50 (▼). Each point represents the mean + SD of 3-8 data points, randomized tattooed over one biopsy of skin. All experiments were performed in triplicate. * Values significantly different from naked DNA control.

PEGylated nanoparticles and naked DNA primarily transfect epidermal keratinocytes

It has been reported that vaccination by nanoparticles can result in preferential targeting of Antigen Presenting Cells (APCs) (17-19). Therefore, it is of interest to evaluate which type of cells are transfected upon DNA tattoo vaccination with PEGylated lipo-and polyplexes. To this purpose, a GFP encoding plasmid was applied by DNA tattooing to human skin biopsies, either as uncomplexed DNA, or encapsulated in PEGylated lipo- or polyplexes. After DNA application, the epidermis of the skin was removed and digested to a single cell suspension. Cells were subsequently stained with anti-cytokeratin and anti-CD1a antibodies to reveal transfection of cytokeratin positive epidermal keratinocytes and CD1a positive Langerhans Cells, respectively. Flow cytometric analysis of obtained cell populations demonstrated that, as is the case for uncomplexed DNA (10), intradermal application of DNA encapsulated into PEGylated lipoplexes or polyplexes resulted in the near-exclusive transfection of keratinocytes, with at most a sporadic GFP positive Langerhans Cell (LCs) (Figure 4). These data reveal that *ex vivo* nanoparticle administration by DNA tattooing, does not result in preferential expression in epidermal LCs.

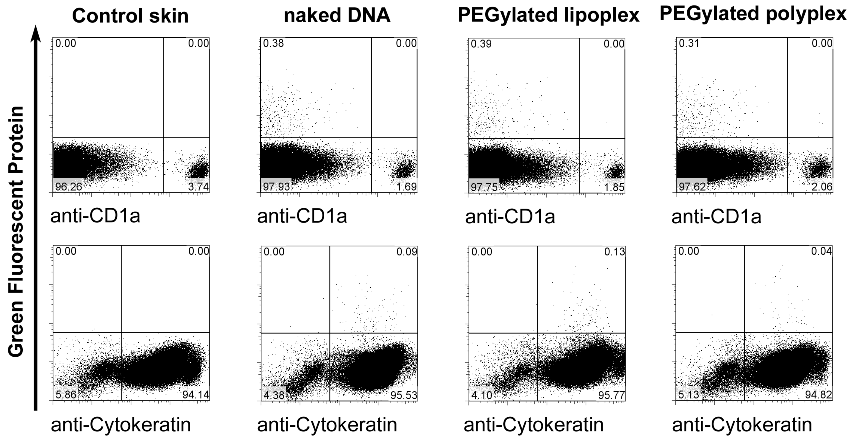


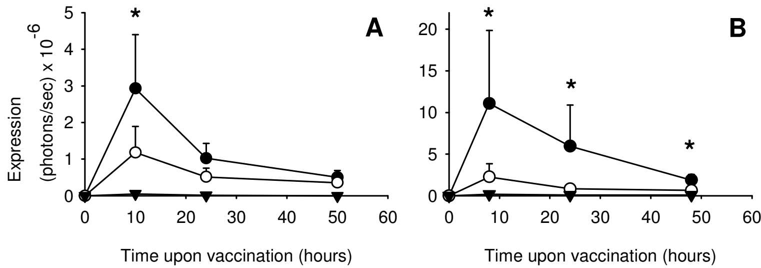
Figure 4. Flow cytometric analysis of epidermal cell suspensions of tattooed skin. Skin was tattooed with naked GFP-encoding DNA, DOTAP-DOPE/DNA lipoplexes with 17.5 mol % DSPE-PEG (N/P ratio 5), or PEG-PAA/DNA polyplexes (w/w ratio 50). Cell suspensions of tattooed or control skin were stained with anti CD1a (top) antibody or with anti-cytokeratin antibody (bottom).

Shielding of the cationic surface charge of nanoparticles results in an increased transfection efficiency and preserved immunogenicity of lipoplexes upon *in vivo* tattooing

The *in vivo* antigen expression and immunogenicity of the PEGylated nanoparticles was studied in C57/B6 mice. To this purpose, mice were vaccinated with naked DNA or lipo- or polyplex (both nonPEGylated and PEGylated) formulations using a standard tattoo vaccination protocol, with DNA administrations on day 0, 3 and 6 (8). To allow the simultaneous detection of vaccination-induced antigen expression and vaccination-induced antigen-specific T cell responses, a pVAX:Luc-NP model DNA vaccine was utilized. Use of this model DNA vaccine permits the monitoring of *in vivo* antigen expression by assessment of luciferase activity, while vaccine immunogenicity can be determined by monitoring of T cell responses against the vaccine-encoded influenza $NP_{366\text{-}374}$ epitope.

Consistent with the results from the *ex vivo* human skin model, PEGylation of nanoparticles was essential to obtain substantial antigen expression by either lipoplexes or polyplexes (with an increase in AUC of 73-fold and 55-fold by PEGylation for lipoplexes and polyplexes, respectively). Furthermore, PEGylated lipo- and polyplexes showed a significant increase in antigen expression as compared to the naked DNA control (Figure 5), where again the PEGylated polyplexes induced higher expression levels as compared to PEGylated lipoplexes. When compared to naked DNA, the AUC of antigen expression were 2.1 and 5.4 fold higher for lipo- and polyplexes, respectively.

To investigate whether shielding of the surface charge is sufficient to restore the immunogenicity of nanoparticle-formulated DNA vaccines, vaccine-induced, antigen-specific T cell responses were measured directly *ex vivo* in peripheral blood by staining with MHC tetramers (Figure 6). We focused exclusively on effector T cell immunity in this study since we are developing DNA tattooing as a method for therapeutic tumor immunization (20),



6

Figure 5. *In vivo* antigen expression in mice upon tattoo vaccination of: (**A**) Naked DNA (○) or DOTAP-DOPE/ DNA lipoplex with (●) or without (▼) 17.5 mol % DSPE-PEG (both lipoplexes at an N/P ratio of 5). (**B**) Naked DNA (○), PEGylated PAA/DNA polyplexes (●) or nonPEGylated PAA/DNA polyplex (▼) (both polyplexes at a w/w ratio polymer/DNA 50). Expression of the vaccine-encoded antigen (luciferase) was measured at the indicated time points upon tattooing with a light sensitive camera. * Values significantly different from naked DNA control. Each point represents the mean + SD of 8 mice.

which aims for high T cell titres (21). As expected, due to the absence of substantial levels of antigen expression, no significant T cell responses were detected in animals vaccinated with nonPEGylated nanoparticles. In contrast, vaccination with the matched PEGylated nanoparticles resulted in a strong T cell response that peaked between day 15 and 17, similar to that observed for the naked DNA control group. The magnitude of the antigen-specific T cell response induced by vaccination with naked DNA or with PEGylated lipoplexes or polyplexes was similar, with no significant difference (Figure 6). These data establish that the presence of the PEG moieties on the DNA nanoparticles is not only sufficient to restore vaccination-induced antigen expression in human and murine skin but also leads to a full restoration of vaccine immunogenicity.

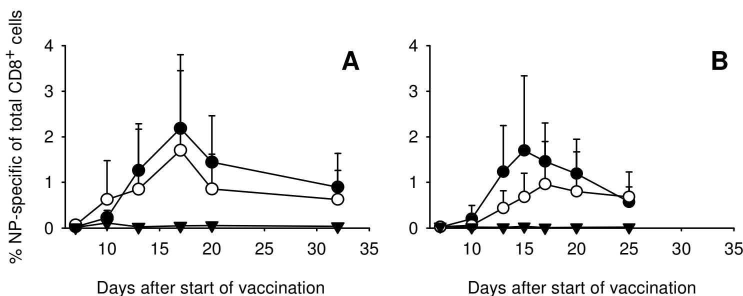


Figure 6. T cell responses upon tattoo vaccination of DNA nanoparticles. $NP_{366\text{-}374}$ specific T cell responses upon tattoo vaccination with the Luc-NP construct are shown for: (**A**) Mice vaccinated with naked DNA (○) or DOTAP-DOPE/ DNA lipoplex with (●) or without (▼)17.5 mol % DSPE-PEG (both lipoplexes at an N/P ratio of 5). (**B**) Mice vaccinated with naked DNA (○), PEGylated PAA/DNA polyplexes (●) or nonPEGylated PAA/DNA polyplexes (▼)(both polyplexes at a w/w ratio polymer/ DNA of 50). NP_{366}-specific T cell responses were measured by direct *ex vivo* MHC tetramer staining of peripheral blood lymphocytes. Each point represents the mean + SD of 8 mice.

DISCUSSION

Incorporation of DNA in nanoparticles may offer the possibility to enhance cellular uptake and may offer the opportunity to develop intradermal DNA vaccines that are amenable to target specific cell types. A first requirement in this research is the development of particles that can be active *in vivo*. This study shows that cationic lipo- and polyplexes that are highly active in *in vitro* assays (22) (Figure 1A) yield only marginal vaccination-induced antigen expression in either murine or human intact skin. We demonstrate that the poor performance of cationic nanoparticles in the latter cases can be significantly improved by shielding the positive surface charge of the nanoparticles by PEGylation to generate near-neutrally charged nanoparticles that yield robust vaccination-induced antigen expression in both murine and human skin.

A possible explanation for the marked discordance between the effectiveness of cationic nanoparticles in cell culture and intact skin is the presence of ECM in intact skin. It is known that major ECM components in the skin (like proteoglycans and hyaluronic acid) have a negative charge at physiological pH (23). These negatively charged components may be responsible for electrostatic binding and immobilisation of the positively charged nanoparticles in the matrix after intradermal administration. Indirect support for this hypothese is also provided by the reported observation that inclusion of ECM components in the transfection medium can inhibit cellular uptake of lipo-and polyplexes in *in vitro* assays (24-26). Although PEGylated particles induce low levels of *in vitro* transfection their performance upon *in vivo* tattooing was markedly improved compared to the unPEGylated particles and naked DNA. It is known that PEGylation of particles affects *in vitro* transfection both at the level of cellular uptake and intracellular trafficking. The transfection efficiency of PEGylated particles is generally 2-fold lower compared to nonPEGylated particles due to a decrease in cell binding and uptake (27,28). In contrast, the mobility of PEGylated particles through the cytosol upon *in vitro* microinjection is 2-fold faster than nonPEGylated particles (29). During intracellular trafficking, both PEGylated and nonPEGylated complexes are thought to be unpacked similar in the cytosol before the DNA can enter the nucleus (28). In our view, these *in vitro* data do not explain the marked difference in transfection properties observed between PEGylated and nonPEGylated particles upon *in vivo* tattooing but do indeed suggest that nonPEGylated particles do not reach the skin cells upon tattooing.

The current data demonstrate that PEGylation of the nanoparticles to a level that allows a near complete shielding of the surface charge suffices to restore and even enhance antigen expression in intact skin. These PEGylated nanoparticles give higher antigen expression than naked DNA controls in the *ex vivo* and *in vivo* experiments. It seems plausible that further optimization of nanoparticle properties and dosing may yield shielded formulations that give a further increase in *in vivo* antigen expression and immunogenicity.

In addition to the optimization of the properties of shielded nanoparticle formulations to enhance vaccination-induced antigen expression, a second key step is the introduction of defined ligands within these formulations (30-32). The current shielded nanoparticles form a highly suited platform for such introduction, as ligands can readily be attached to the terminal ends of the PEG chains, using standard protocols. Two specific goals may be achieved by introduction of such ligands. First, introduction of ligands for defined cell surface receptor may enhance cellular uptake or may be used to target defined epidermal cell types (for example epidermal LCs). Second, the introduction of Toll-like receptor ligands or ligands for other pathogen-associated molecular pattern receptors on the surface of the nanoparticles is an attractive option to further enhance the immunogenicity of shielded nanoparticle vaccines, by providing an intrinsic danger signal.

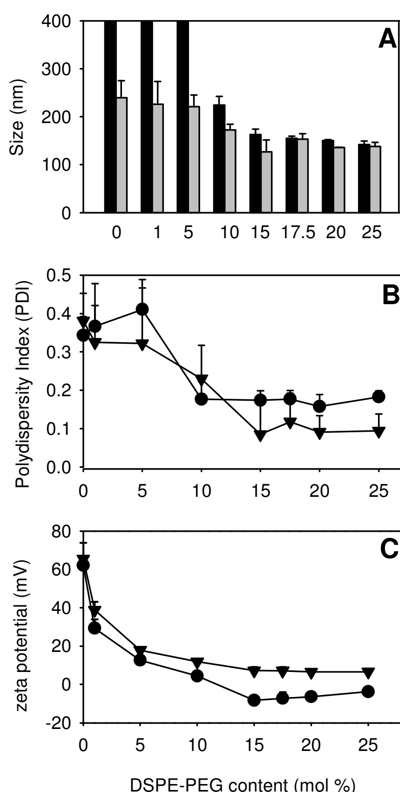
REFERENCE LIST

1. Zhang S, Xu Y, Wang B, Qiao W, Liu D, Li Z. Cationic compounds used in lipoplexes and polyplexes for gene delivery. *J.Control Release* 2004;100:165-80.
2. El Aneed A. An overview of current delivery systems in cancer gene therapy. *J.Control Release* 2004;94:1-14.
3. Luten J, van Nostrum CF, De Smedt SC, Hennink WE. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. *J.Control Release* 2008;126:97-110.
4. Mintzer MA, Simanek EE. Nonviral vectors for gene delivery. *Chem.Rev.* 2009;109:259-302.
5. Bos GW, Kanellos T, Crommelin DJ, Hennink WE, Howard CR. Cationic polymers that enhance the performance of HbsAg DNA in vivo. *Vaccine* 2004;23:460-9.
6. Singh M, Briones M, Ott G, O'hagan D. Cationic microparticles: A potent delivery system for DNA vaccines. *Proc.Natl.Acad.Sci.U.S.A* 2000;97:811-6.
7. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A et al. Direct gene transfer into mouse muscle in vivo. *Science* 1990;247:1465-8.
8. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat.Med.* 2005;11:899-904.
9. Verstrepen BE, Bins AD, Rollier CS, Mooij P, Koopman G, Sheppard NC et al. Improved HIV-1 specific T-cell responses by short-interval DNA tattooing as compared to intramuscular immunization in non-human primates. *Vaccine* 2008;26:3346-51.
10. van den Berg JH, Nujien B, Beijnen JH, Vincent A, Van Tinteren H, Kluge J et al. Optimization of intradermal vaccination by DNA tattooing in human skin. *Hum.Gene Ther.* 2009;20:181-9.
11. Lin C, Zhong Z, Lok MC, Jiang X, Hennink WE, Feijen J et al. Novel bioreducible poly(amido amine)s for highly efficient gene delivery. *Bioconjug.Chem.* 2007;18:138-45.
12. Cherng JY, Talsma H, Verrijk R, Crommelin DJ, Hennink WE. The effect of formulation parameters on the size of poly-((2-dimethylamino)ethyl methacrylate)-plasmid complexes. *Eur.J.Pharm.Biopharm.* 1999;47:215-24.
13. Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ et al. Design and use of conditional MHC class I ligands. *Nat.Med.* 2006;12:246-51.
14. Du X, Zheng G, Jin H, Kang Y, Wang J, Xiao C et al. The adjuvant effects of co-stimulatory molecules on cellular and memory responses to HBsAg DNA vaccination. *J.Gene Med.* 2007;9:136-46.
15. Heller LC, Jaroszeski MJ, Coppola D, McCray AN, Hickey J, Heller R. Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode. *Gene Ther.* 2007;14:275-80.
16. Pearton M, Allender C, Brain K, Anstey A, Gateley C, Wilke N et al. Gene delivery to the epidermal cells of human skin explants using microfabricated microneedles and hydrogel formulations. *Pharm.Res.* 2008;25:407-16.
17. Lu Y, Kawakami S, Yamashita F, Hashida M. Development of an antigen-presenting cell-targeted DNA vaccine against melanoma by mannosylated liposomes. *Biomaterials* 2007;28:3255-62.
18. Mahe B, Vogt A, Liard C, Duffy D, Abadie V, Bonduelle O et al. Nanoparticle-based targeting of vaccine compounds to skin antigen-presenting cells by hair follicles and their transport in mice. *J.Invest Dermatol.* 2009;129:1156-64.
19. Perrie Y, Frederik PM, Gregoriadis G. Liposome-mediated DNA vaccination: the effect of vesicle composition. *Vaccine* 2001;19:3301-10.
20. Quaak SG, van den Berg JH, Toebes M, Schumacher TN, Haanen JB, Beijnen JH et al. GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial. *Eur.J.Pharm.Biopharm.* 2008;70:429-38.
21. Spagnoli GC, Adamina M, Bolli M, Weber WP, Zajac P, Marti W et al. Active antigen-specific immunotherapy of melanoma: from basic science to clinical investigation. *World J.Surg.* 2005;29:692-9.
22. Paasonen L, Korhonen M, Yliperttula M, Urtti A. Epidermal cell culture model with tight stratum corneum as a tool for dermal gene delivery studies. *Int.J.Pharm.* 2006;307:188-93.
23. Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. *Annu.Rev.Biochem.* 1998;67:609-52.
24. Ruponen M, Ronkko S, Honkakoski P, Pelkonen J, Tammi M, Urtti A. Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. *J.Biol.Chem.* 2001;276:33875-80.
25. Ruponen M, Honkakoski P, Tammi M, Urtti A. Cell-surface glycosaminoglycans inhibit cation-mediated gene transfer. *J.Gene Med.* 2004;6:405-14.
26. van de WP, Schuurmans-Nieuwenbroek NM, Hennink WE, Storm G. Comparative transfection studies of human ovarian carcinoma cells in vitro, ex vivo and in vivo with poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes. *J.Gene Med.* 1999;1:156-65.
27. Luten J, Van Steenbergen MJ, Lok MC, de Graaff AM, van Nostrum CF, Talsma H et al.

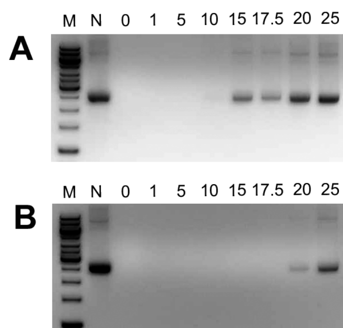
Degradable PEG-folate coated poly(DMAEA-co-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression. *Eur.J.Pharm. Sci*. 2008;33:241-51.

28. Mishra S, Webster P, Davis ME. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur.J.Cell Biol*. 2004;83:97-111.
29. Suh J, Choy KL, Lai SK, Suk JS, Tang BC, Prabhu S et al. PEGylation of nanoparticles improves their cytoplasmic transport. *Int.J.Nanomedicine*. 2007;2:735-41.
30. Kim KS, Lee YK, Kim JS, Koo KH, Hong HJ, Park YS. Targeted gene therapy of LS174 T human colon carcinoma by anti-TAG-72 immunoliposomes. *Cancer Gene Ther*. 2008;15:331-40.
31. Miller AM, Dean DA. Cell-specific nuclear import of plasmid DNA in smooth muscle requires tissue-specific transcription factors and DNA sequences. *Gene Ther*. 2008;15:1107-15.
32. Tang CK, Sheng KC, Apostolopoulos V, Pietersz GA. Protein/peptide and DNA vaccine delivery by targeting C-type lectin receptors. *Expert. Rev.Vaccines*. 2008;7:1005-18.

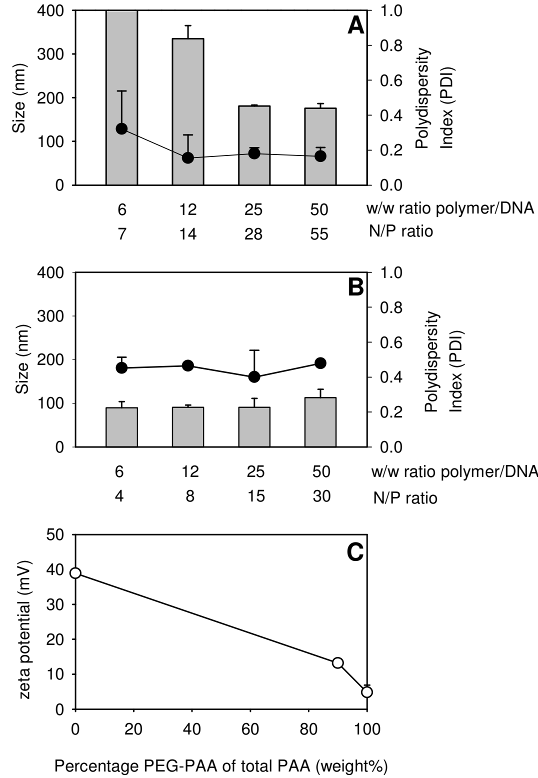
SUPPLEMENTARY FIGURES



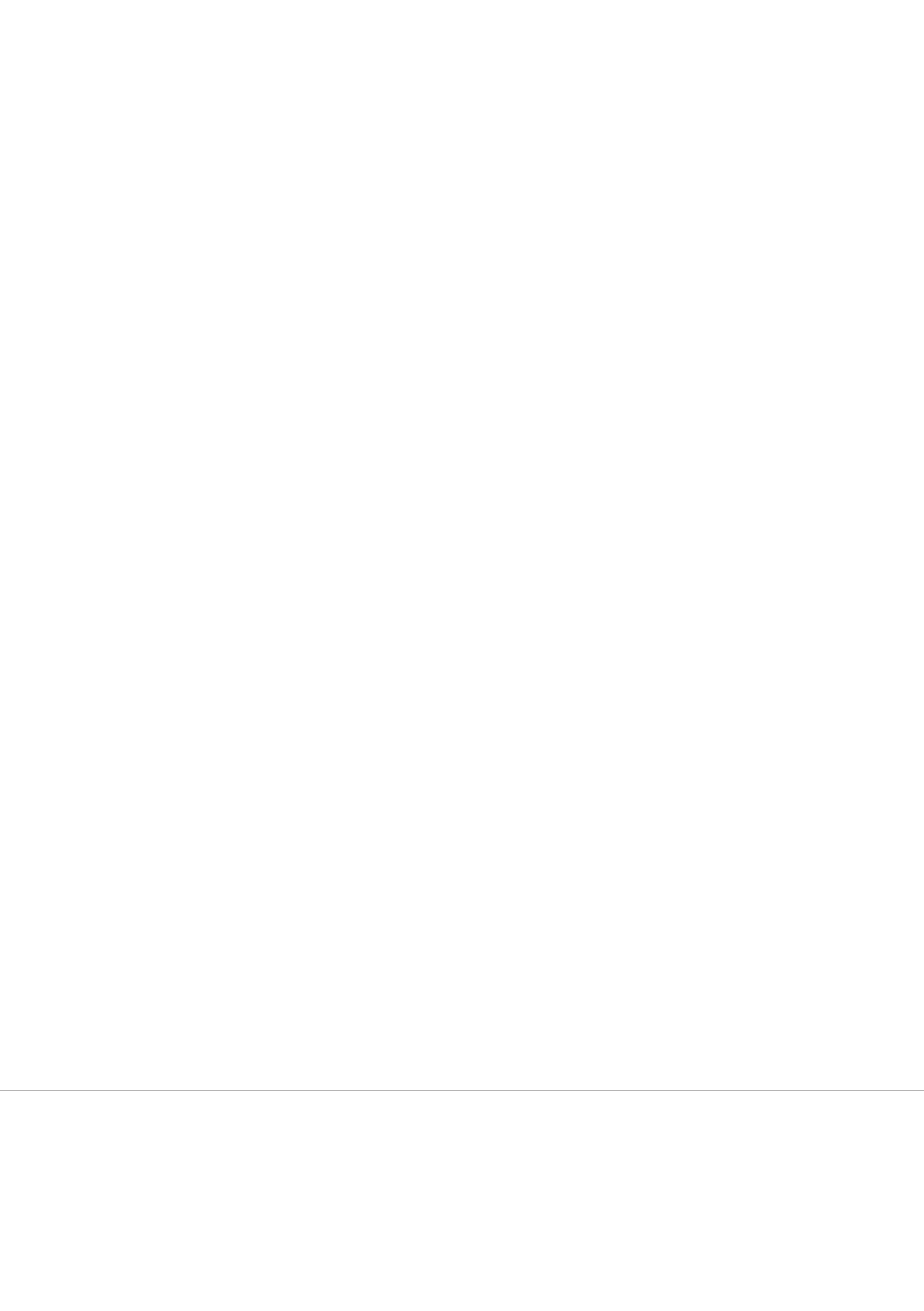
Supplementary Figure 1. Lipoplex characteristics: effect of increasing DSPE-PEG content on size (A), Polydispersity Index (PDI) **(B)** and ζ potential **(C)** of DOTAP-DOPE/DNA lipoplexes. Complexes were prepared at a charge (N/P) ratio of 2 (black bars or ●) and 5 (grey bars or ▼) at 0.5 mg/mL DNA in 20 mM HEPES pH 7.4, 10% sucrose. Bars or symbols represent the mean + SD of three independent measurements. Aggregated lipoplexes are indicated by bars that reach the upper axis.



Supplementary Figure 2. Condensation of plasmid DNA with DOTAP-DOPE liposomes containing increasing concentration of DSPE-PEG at a charge (N/P) ratio of 2 (A) or 5 **(B)**. Each lane was loaded with 2 µl formulation, corresponding to 1 µg DNA, and samples were analyzed by gel electrophoresis. Lane M; l/Hind III DNA molecular-weight markers; lane N, naked DNA control; lane 0 to 25, DOTAP-DOPE/ DNA lipoplexes prepared with an increasing mol percentage of DSPE-PEG. Data demonstrate that up to a DSPE-PEG percentage of 10% (for N/P 2) or 17.5% (for N/P 5) all DNA is complexed as no free DNA is detected.



Supplementary Figure 3: Polyplex characteristics: effect of PEGylation on particle size (bars) and Polydispersity Index (PDI) (lines) of polymer/DNA polyplexes. Polyplexes were prepared at the indicated polymer/DNA ratios, either with nonPEGylated PAA polymer **(A)** or with PEGylated PAA polymer **(B)**. **(C)** PEGylated and nonPEGylated PAA polymer was mixed at the indicated ratios (always in a 50/1 w/w ratio polymer/DNA) to obtain particles with a range of ζ potentials. Bars or symbols represent the mean + SD of three independent measurements. All complexes were prepared at 0.5 mg/mL DNA in 20 mM HEPES pH 7.4, 10% sucrose. Aggregated polyplexes are indicated by bars that reach the upper axis. Gel electrophoresis showed no free DNA in all polyplexes formulated at the tested ratios (gel not shown).



CHAPTER 7

SUMMARIZING DISCUSSION AND FUTURE OUTLOOK

SUMMARIZING DISCUSSION

As outlined in the introduction there is a strong rationale for the development of therapeutic HPV vaccines. In this thesis we focused on the preclinical development of DNA vaccine candidates targeting HPV16 E6 and E7. In short, we show that the immunogenicity of E6 and E7 encoding DNA vaccines can be strongly influenced by the design of the DNA vaccine. Beside this, we show that DNA vaccines encoding gene-shuffled versions of E6 and E7 no longer posses the transforming potential that is associated with the wild-type versions of these genes. Below we provide a summary of the chapters with a focus on open questions that remain.

Despite the high expectations in the 1990s, to date no DNA vaccines have been approved for human use. In **chapter 2** we provide a review that discusses the possibilities to improve the immunogenicity of DNA vaccines, with a focus on a delivery method called DNA tattooing that was developed in our lab (1). We think that DNA tattooing has great potential to overcome the so called 'simian-barrier': the observation that DNA vaccines although highly immunogenic in mice are only weakly immunogenic in non-human primates (2). This optimism is based on the 10- to 100-fold increase in the magnitude of vaccine specific T cell responses in peripheral blood from DNA tattooed rhesus macaques, as compared to T cell responses in animals immunized via intramuscular (IM) route (3). Advantages of DNA tattooing compared to other DNA delivery methods are: targeting of the skin being the ultimate organ for immune surveillance, provision danger signals by the thousands of inflictions made during tattooing, delivery without requirement of expensive instruments (such as the gene gun), and the relative ease to scale doses used in mice to equivalent doses in humans by increasing the surface of the tattooed area. The review also summarizes our knowledge on the mechanism of T cell priming upon DNA (tattoo) vaccination, providing clues for the improvement of DNA vaccines. We are optimistic that advances in the design and delivery of DNA vaccines will result in clinical application of DNA vaccines in the near future. The recent licensing of three different DNA vaccines in the field of veterinary medicine (4) and a recent clinical trial showing DNA vaccine mediated protection from influenza challenge (5) fuels this optimism.

In **chapter 3** we show that fusion with Tetanus Toxin Fragment C (TTFC) considerably increased the immunogenicity of both wild-type E7 as gene-shuffled versions of E6 and E7. These data form a nice illustration of the enormous impact of antigen design on DNA vaccine immunogenicity. TTFC was selected as carrier-molecule as it had been shown to improve the immunogenicity of C-terminally fused minimal T cell epitopes (6, 7). We thus extent this finding to full-length proteins. The function of TTFC is most likely to provide CD4+ T cell help and improve the stability/half-life of the antigen. Previous more fundamental studies in our lab already pointed towards an important role of these two factors in DNA vaccine immunogenicity (8, 9). We observed in this study that the effect for E7 is much more pronounced than for E6. This might be explained by distinct intrinsic properties of the antigens, such as the stability. On the other hand the lower impact of TTFC fusion on the immunogenicity of E6 can be explained by immunogenic competition (10). As TTFC is a large foreign protein it likely contains competing CD8+ T cell epitopes, possibly resulting in immunodominant immune responses against TTFC. This effect would be expected to be largely dependent on the HLA make up of the target species. Therefore, it is very well possible that the hierarchy that we observe in inbred mice is not predictive for the human situation. An experimental finding that supports this hypothesis is that in HLA-A2 transgenic mice we could show TTFC-E6SH responses after *in vitro* re-stimulation, but not against TTFC-E7SH (unpublished observation). However, it is also

possible that the inability to raise E7 directed responses in these mice reflect a short-coming of the HLA-A2 transgenic mice as a model (11). The use of detoxified versions of E6 and E7 is considered necessary to prevent the risk of cellular transformation at the vaccination side (see also chapter 4). Most often this is achieved by point mutations that affect the binding of E6 and E7 to their known cellular targets, respectively p53 and pRb. As gene-shuffling can be expected to result in much more drastic conformational changes it has the conceptual advantage of also preventing the binding to other cellular targets apart from p53 and pRb. This is of particular relevance as also the binding to such targets is believed to play a role in the transformation process (12-14). Therefore we think that TTFC-E7SH and TTFC-E6SH have a better safety profile than most other candidate DNA vaccines that rely on point mutations for detoxification.

Despite perceived good safety profile of TTFC-E6SH and TTFC-E7SH we wished to provide experimental proof for the absence of oncogenic potential, before moving to clinical application. **Chapter 4** reports on the detailed safety evaluation that we performed in order to demonstrate the absence of oncogenic potential of these candidate vaccines. To this end, two different cell-types were used namely murine NIH 3T3 cells and primary human foreskin keratinocytes (HFKs). In both assay systems we could show that TTFC-E6SH and TTFC-E7SH alone and in combination have lost the oncogenic potential that is associated with the wild-type proteins. Although HFKs are a common system to study the transforming potential of high-risk HPV types (15, 16), their use for the evaluation of the safety of E6 and E7 directed vaccines has not been reported before. We show that it is feasible to use these primary human cells for this purpose. We consider HFKs as a more relevant system compared to NIH 3T3 cells as HFKs are of human origin and because keratinocytes are the natural target cells of HPV infections and are also targeted by most DNA vaccination strategies. In this study TTFC-E6SH and TTFC-E7SH were compared with their wild-type counterparts. It would however be interesting to compare the various detoxification methods (i.e. gene-shuffling and the introduction of point-mutations) with each other, using HFKs, in a future study.

As we hypothesized that a large foreign carrier molecule carries the risk of inducing immunodominant immune responses directed against the carrier molecule, we aimed to further optimize our DNA vaccine design. In **chapter 5** we report on the development of rationally designed modular DNA vaccines encoding HPV16 E6SH and E7SH. We hypothesized that it would be possible to split the anticipated effect on antigen stability and the addition of CD4+ T cell help. To this end we used a self carrier protein and a so called helper-cassette consisting of 3 promiscuous minimal CD4 helper epitopes (PADRE, P30 and NEF). Using this modular design, we could show that addition of both elements was necessary for optimal DNA vaccine immunogenicity. When comparing a set of 5 self carrier molecules with different subcellular localization we found that only ER localized carriers improved the immunogenicity. Subsequently we demonstrated that the carrier effect could be entirely explained by ER targeting of the antigen. Thereby, suggesting an important role for ER localization for the improvement of DNA vaccine immunogenicity. Importantly, the resulting minimal ER-HELP design also resulted in optimal T cell responses against E6SH. Interestingly, many carrier-proteins used to improve the immunogenicity of HPV16 E7 encoding DNA vaccines are also ER localized (17-20). Based on our data we hypothesize that in those cases not so much the biological function of the carrier-protein explains the enhanced immunogenicity of the fusion product, but rather the effect on antigen localization. In light of this, it is interesting to note that the addition of ER localization and retention signals to an adenoviral vector encoded E7 improved its immunogenicity to the same extend as fusion with complete calreticulin (21). Moreover, these data suggest that the

mechanism that we defined in the context of DNA vaccination might also hold true for other vaccine vectors. It will therefore be interesting to test if our design rules apply in the context of for example adenovirus-based vaccines or Semliki Forest virus based vaccines. Experiments with the latter vector system are ongoing.

An interesting open question is whether the design rules defined in this chapter are also applicable to other antigens. To this end we selected 3 clinically relevant antigens namely Hepatitis B virus (HBV) core protein, human gp100 and Plasmodium Berghei circumsporozoite protein (Pb CSP) and compared the immunogenicity of the antigen alone (Ag), TTFC-Ag, HELP-Ag and sig-HELP-Ag-KDEL. Surprisingly, the design rules that resulted in highly immunogenic E6 and E7 directed DNA vaccines did not hold true for these 3 new antigens (unpublished observation). Only in the case of HBV core protein we could show improved immunogenicity after fusion with the helper-cassette albeit this effect was not significant (unpublished observation). In case of PbCSP there was no difference in immunogenicity between the antigen alone and the modified versions, and in case of human gp100 all modifications resulted in a loss of immunogenicity compared to the antigen alone. Possible explanation for this failure is that our modifications negatively impacted on other antigen properties that are important for DNA vaccine immunogenicity, for example antigen stability or the ability to form a particulate structure as has been reported for HBV core protein (22). An interesting experiment would be to gene shuffle these new antigens, to destroy any special property of the antigen, and see if our design rules would subsequently apply. Another possibility would be to select different (model) antigens that resemble E6SH and E7SH more closely, namely instable cytosolic antigens, and see if our design rules would apply to such a more specific category of antigens. Nonetheless these results make clear that optimization of antigens in the context of DNA vaccination requires detailed knowledge of the antigens and is not simply a generic process.

Besides optimization of the antigen design or the physical delivery method it is believed that DNA vaccine immunogenicity can be enhanced by improving the formulation for example by encapsulating DNA in so called nanoparticles. This assumption is based on the inefficient cellular uptake of naked/non formulated DNA upon vaccination, estimated to be extremely low in the order of 1 out of $1x10^6$ to $5x\ 10^9$ plasmids applied for DNA tattooing (23). These nanoparticles generally consist of a complex between the negatively charged DNA and with cationic polymers or lipids. It is thought that nanoparticle formulated DNA is better protected from degradation and by its condensed nature can more easily pass the cell membrane (24). In **Chapter 6** we show that, though efficient in vitro, these nanoparticles completely block DNA tattoo mediated gene expression and immunogenicity. Interestingly, the gene-expression could be completely restored by shielding positive charge of these nanoparticles by addition of polyethylene glycol (PEG) chains. Despite the fact that gene-expression in mice *in vivo* was up to 5 times higher than that of an equivalent dose of naked DNA, the immunogenicity was not significantly improved, suggesting that stronger improvement of the gene expression levels is necessary to have an impact on immunogenicity. On the other hand, other factors might play a role such as a reduced immune stimulatory capacity of the formulation compared to naked-DNA. Importantly, the current shielded nanoparticles form a highly suited platform for the introduction of targeting ligands (to improve cellular up-take or affect cell-type specificity) or the addition of immunostimulatory molecules such as TLR agonists. It will be very interesting to test if such modifications can further improve the immunogenicity of nanoparticle formulated DNA vaccines.

FUTURE OUTLOOK

Will the DNA vaccines candidates described in this thesis be the cure for HPV induced malignancies in the near future? To be able to provide a meaningful answer to this question, first of all, the outcome of clinical trials has to be awaited. Such clinical trials should show robust induction of E6 and E7 specific T cell immunity and ultimately objective and relevant clinical responses against HPV induced (pre-)malignancies. Important considerations regarding the planning of such clinical studies are discussed below.

Patient selection

The most important consideration will be the type of patient to select for clinical evaluation of the developed DNA vaccines. It is well documented that high-risk HPV types have evolved to escape the host immune response (25, 26). Mechanisms that are thought to play a role are: the ability of the virus to maintain a very low profile (e.g. low expression levels of the viral proteins, absence of a blood-borne phase etc.), modulation of antigen presentation (e.g. by loss of MHC class I expression (27)) and local immune suppression (e.g. by suppression of IFN transcription (28, 29)). Thus, even in case vaccination would yield powerful systemic E6 and E7 specific T cell responses, such mechanisms can result in immune escape of the HPV induced lesions. Treatment of early stage patients, that are generally thought to be less immune suppressed, is likely to result in a more favorable outcome than treatment of late stage patients (30). This is clearly demonstrated by the high response rates (47% complete regression) in VIN 3 patients with a vaccine that consist of multiple E6 and E7 derived overlapping synthetic long-peptides (SLP) (31). Notably the spontaneous regression rate for these types of lesions is below 1.5% (32). The same vaccine did induce vaccine specific immune responses and lesion regression in end-stage cervical cancer patients, but only 1 out of 35 patients (not tested for HPV16 positivity) experienced complete regression (33). Thus in order to demonstrate clinical efficacy it seems highly important to treat early-stage patients. Nevertheless, for ethical reasons safety will have to be established first in end-stage patients.

Combining vaccination with (local) immune modulation

Another important consideration, related to the ability of high-risk HPV to escape the host immune response, will be to combine systemic vaccination with local or systemic immune modulation. This is even more crucial in the treatment of patients with late-stage lesions as they are oftentimes severely immunosuppressed, as mentioned above.

As systemic immune modulation can induce considerable side effects (34-36), local immune modulation would be my first choice (37). A compound that has the ability to non-specifically activate the immune system is Toll-like receptor 7/8 agonist imiquimod (38). Imiquimod can be administered locally as a 5% cream (Aldara®) and is registered for the treatment of genital warts. Interestingly, imiquimod has also been shown to result in complete histological regression in 35% of treated VIN stage 2/3 patients after 16 weeks of treatment (39). The clinical benefit of this treatment is associated with normalization of immune cell counts at the site of the lesion, suggesting that HPV specific adaptive immunity played a (direct or indirect) role in the success of the treatment (40). The promise of combining local immune modulation with systemic vaccination is demonstrated by a recent clinical study. In this study a protein-based vaccine TA-CIN (HPV16 L2/E6/E7 fusion protein) was applied to VIN 2/3 patients after 8 weeks of pre-treatment with imiquimod. This combination elicited durable clinical responses in 63%

of the patients and a significant increase in both systemic and local vaccine specific cellular responses was observed in the clinical responders (41). The same vaccine was shown to result in detectable E6 and E7 specific T cell immunity when combined with a viral boost (see below) in a previous study, albeit without clinically relevant responses (42). Other toll-like receptors agonists like MPL-A (TLR-4) and CpG (TLR-9) should in principle be able to exert similar effects. Although these molecules are available in the clinic as vaccine adjuvants, their usefulness for the local treatment of HPV induced malignancies has not been explored so far (43). Interestingly also more conventional treatments such as chemotherapy and irradiation are also believed to impact on the local tumor environment by inducing (immunogenic) tumor cell death (44, 45). And indeed, the combination of radiation or chemotherapy (Cisplatin as well as DMXAA) and HPV specific vaccination, improved the anti tumor effect compared to vaccination alone in the TC-1 model in mice (46-48). Importantly clinical evaluation of such conventional therapies in combination with vaccination seems rather straightforward.

Nowadays a wide array of therapeutics is available to systemically impact on T cell regulation. Most of these therapeutics are antibodies that impact on co-inhibitory or co-stimulatory signaling, thereby lowering the threshold for T cell activation. Well known examples of antibodies that inhibit co-inhibitory signaling are CTLA4 blocking antibodies (49, 50) anti-PD-1 antibodies (51) and anti-GITR antibodies (49). Examples of antibodies that aim to induce co-stimulatory signaling are agonistic anti-CD40 antibodies (36) and agonistic anti-4-1BB ligand antibodies (52). Monotherapy with these type of agents has been shown to induce tumor regression in both preclinical models as well as in clinical trials (53, 54). Interestingly, for both for anti-4-1BB antibodies and anti-GITR antibodies the combination of E7 specific vaccination has been shown to be much more effective in the eradication of established TC-1 tumors in mice than vaccination alone (52, 55). However the latter examples still need validation in human subjects and, as mentioned above, the fact that this class of therapeutics has considerable side effects may limit their application as 'vaccine adjuvants'.

Prime-boost regimens

If DNA vaccines might turn out to be not powerful enough as a stand alone method for T cell induction in humans, they could still be highly useful as part of heterologous prime-boost regimens (56). For this purpose viral vaccines are attractive as they are generally considered more powerful than DNA vaccines for the induction of T cell immunity, but have the drawback that they cannot be administered repeatedly. This is explained by the induction of vector specific immunity, most often neutralizing antibodies against viral (capsid) components (57, 58). As DNA vaccination only results in expression of the transgene and no other foreign proteins are present, DNA vaccines do not induce vector specific immune responses and can thus be administered repeatedly (59). Many reports, including clinical studies, have shown that DNA priming followed by viral boosting can induce highest responses when compared with homologous prime-boost regimens (60). Therefore we consider it worthwhile to evaluate our vaccine candidates in the context of prime-boost regimens. For this purpose Modified Vaccinia Ankara (MVA) based vectors, poxvirus vectors or replication deficient adenoviral vectors are the most obvious candidates, as clinical trials or non-human primate studies have already shown the beneficial effect of DNA priming followed boosting with these vaccine platforms (60-63). Nevertheless it would also be valuable to validate the effect of prime boosting for other vaccine systems such as the Semliki Forest virus (SFV). HPV16 E6 and E7 encoding SFV based vaccines have shown promising anti tumor effects in pre-clinical models (64). Before

testing such prime boost regimens it would be interesting to evaluate if our E6 and E7 specific antigen designs, as developed in the context of DNA vaccines, are also superior in the context of the above-mentioned viral vectors. Evidence that this could be the case comes from the finding that optimizations that have been shown to improve the immunogenicity of antigens in the form of naked DNA also improved their immunogenicity in the context of an adenoviral vectors (65-67). Another argument to test our designs in other vectors is that we found it to be essential to boost with the very same antigen for optimal induction of memory T cell responses in the context of DNA vaccination (non published observation). As mentioned before, validation of our DNA vaccine design rules in the context of SFV is currently ongoing. Finally, also the combination of DNA vaccination with non viral vaccine modalities such as peptide or protein based vaccines holds promise. Many preclinical studies have for example shown improved (Th1 type) immunogenicity and efficacy of DNA prime protein boost strategies compared to homologous prime boosting with either vectors (68-70). An obvious vaccine candidate to test in combination with our DNA vaccines would be the E6 and E7 derived overlapping SLP vaccine that has proven clinical efficacy ((31) and see above).

Further optimization of the current candidate vaccines

The results obtained in this thesis show the enormous potential of modifications of the antigen as such. To the best of our knowledge the E6 and E7 specific DNA vaccines tested so far in clinical trials were only moderately immunogenic in mice compared to our optimal DNA vaccine candidates: sig-HELP-E6SH-KDEL and sig-HELP-E7SH-KDEL. Therefore we are optimistic that our vaccines will result in stronger E6 and E7 specific T cell responses in human subjects. It is however not unlikely that further improvements of the antigen design are possible, for example inclusion of more diverse set of CD4+ helper epitopes to further improve CD4+ T cell help. An obvious set of epitopes to test are those included in the so called N19 polytope, a string of 19 universal CD4+ T cell epitopes that has extensively been tested as a carrier protein in conjugate vaccines (71, 72). Also other possibilities exist to further improve the immunogenicity our candidate vaccines such as the inclusion (via bisistronic expression) or co-delivery of molecular adjuvants such as GM-CSF (73), IL-12 (74), IL-15 (75) and HGMB1 (76). Such strategies have shown promise in pre clinical models in conjunction with a wide variety of antigens (see for more examples (77), and can easily be tested in combination with our vaccine candidates in the available pre-clinical models. Finally optimization of DNA delivery is a very active field (see also chapter 2). The recent improvements particularly in the field of electroporation mediated DNA delivery in preclinical models (78, 79) and the recent demonstration of significant increase in humoral immunity by combining intramuscular delivery and electroporation in human subjects reveal the great potential of this delivery method (80). It is however difficult to judge whether results of such preclinical studies should be awaited before initiating clinical trials with the current candidate vaccines.

CONCLUSION

Taken together we have developed highly effective and safe DNA vaccines targeting HPV16 E6 and E7 in preclinical models, warranting their clinical evaluation. Might these vaccines turn out to be not powerful enough as stand alone treatment, many adjuvant strategies are available to improve clinical outcome. Of the adjuvant strategies discussed, the combination with local imiquimod and prime-boost strategies in conjunction with the clinically available overlapping

SLP vaccines are the most obvious options. The promising clinical responses in recent human vaccination trials fuel the optimism that the treatment of HPV induced (pre-)malignancies via induction of E6 and E7 specific T cell responses is a realistic scenario.

REFERENCE LIST

1. Bins AD, Jorritsma A, Wolkers MC, Hung CF, Wu TC, Schumacher TN et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med* 2005;11:899-904.
2. Johnston SA, Talaat AM, McGuire MJ. Genetic immunization: what's in a name? *Arch Med Res* 2002;33:325-9.
3. Verstrepen BE, Bins AD, Rollier CS, Mooij P, Koopman G, Sheppard NC et al. Improved HIV-1 specific T-cell responses by short-interval DNA tattooing as compared to intramuscular immunization in non-human primates. *Vaccine* 2008;26:3346-51.
4. Redding L, Weiner DB. DNA vaccines in veterinary use. *Expert Rev Vaccines* 2009;8:1251-76.
5. Jones S, Evans K, McElwaine-Johnn H, Sharpe M, Oxford J, Lambkin-Williams R et al. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. *Vaccine* 2009;27:2506-12.
6. Rice J, Elliott T, Buchan S, Stevenson FK. DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines. *J Immunol* 2001;167:1558-65.
7. Stevenson FK, Rice J, Ottensmeier CH, Thirdborough SM, Zhu D. DNA fusion gene vaccines against cancer: from the laboratory to the clinic. *Immunol Rev* 2004;199:156-80.
8. Bins AD, Wolkers MC, van den Boom MD, Haanen JB, Schumacher TN. In vivo antigen stability affects DNA vaccine immunogenicity. *J Immunol* 2007;179:2126-33.
9. Wolkers MC, Toebes M, Okabe M, Haanen JB, Schumacher TN. Optimizing the efficacy of epitope-directed DNA vaccination. *J Immunol* 2002;168:4998-5004.
10. Yewdell JW, Bennink JR. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol* 1999;17:51-88.
11. Street MD, Doan T, Herd KA, Tindle RW. Limitations of HLA-transgenic mice in presentation of HLA-restricted cytotoxic T-cell epitopes from endogenously processed human papillomavirus type 16 E7 protein. *Immunology* 2002;106:526-36.
12. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. *Virology* 2009;384:324-34.
13. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. *Virology* 2009;384:335-44.
14. Yugawa T, Kiyono T. Molecular mechanisms of cervical carcinogenesis by high-risk human papillomaviruses: novel functions of E6 and E7 oncoproteins. *Rev Med Virol* 2009;19:97-113.
15. Snijders PJ, Steenbergen RD, Heideman DA, Meijer CJ. HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* 2006;208:152-64.
16. Steenbergen RD, de Wilde J, Wilting SM, Brink AA, Snijders PJ, Meijer CJ. HPV-mediated transformation of the anogenital tract. *J Clin Virol* 2005;32 Suppl 1:S25-S33.
17. Cheng WF, Hung CF, Chai CY, Hsu KF, He L, Ling M et al. Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J Clin Invest* 2001;108:669-78.
18. Hung CF, Hsu KF, Cheng WF, Chai CY, He L, Ling M et al. Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. *Cancer Res* 2001;61:1080-8.
19. Peng S, Ji H, Trimble C, He L, Tsai YC, Yeatermeyer J et al. Development of a DNA vaccine targeting human papillomavirus type 16 oncoprotein E6. *J Virol* 2004;78:8468-76.
20. Smahel M, Polakova I, Pokorna D, Ludvikova V, Duskova M, Vlasak J. Enhancement of T cell-mediated and humoral immunity of beta-glucuronidase-based DNA vaccines against HPV16 E7 oncoprotein. *Int J Oncol* 2008;33:93-101.
21. Loera-Arias MJ, Martinez-Perez AG, Barrera-Hernandez A, Ibarra-Obregon ER, Gonzalez-Saldivar G, Martinez-Ortega JI et al. Targeting and retention of HPV16 E7 to the endoplasmic reticulum enhances immune tumour protection. *J Cell Mol Med* 2010;14:890-4.
22. Kuhober A, Pudollek HP, Reifenberg K, Chisari FV, Schlicht HJ, Reimann J et al. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2b mice. *J Immunol* 1996;156:3687-95.
23. van den Berg JH, Nuijen B, Beijnen JH, Vincent A, van Tinteren H, Kluge J et al. Optimization

of intradermal vaccination by DNA tattooing in human skin. *Hum Gene Ther* 2008.

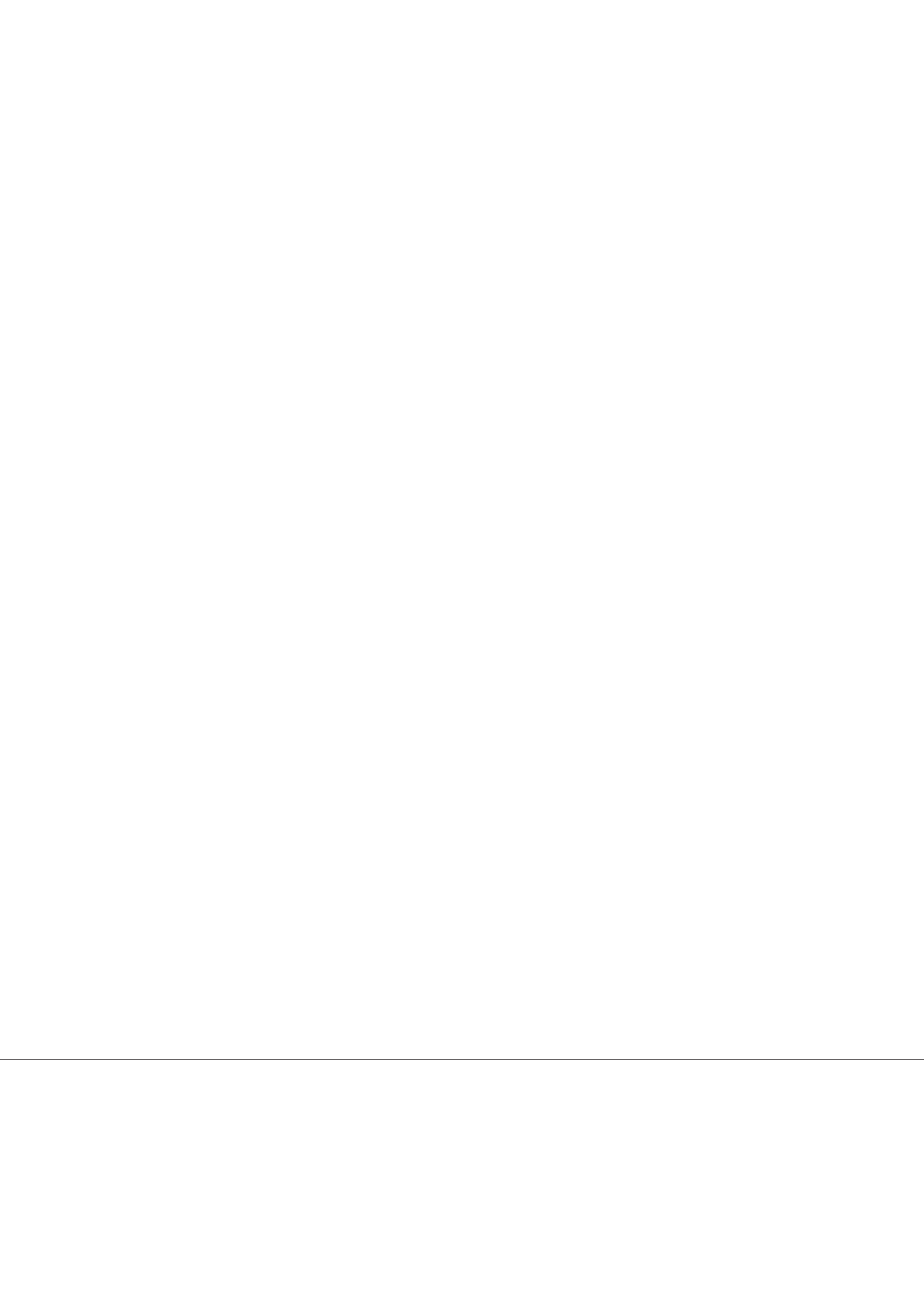
24. Gao X, Kim KS, Liu D. Nonviral gene delivery: what we know and what is next. *AAPS J* 2007;9:E92-104.
25. Kanodia S, Fahey LM, Kast WM. Mechanisms used by human papillomaviruses to escape the host immune response. *Curr Cancer Drug Targets* 2007;7:79-89.
26. Tindle RW. Immune evasion in human papillomavirus-associated cervical cancer. *Nat Rev Cancer* 2002;2:59-65.
27. Bontkes HJ, Walboomers JM, Meijer CJ, Helmerhorst TJ, Stern PL. Specific HLA class I down-regulation is an early event in cervical dysplasia associated with clinical progression. *Lancet* 1998;351:187-8.
28. Park JS, Kim EJ, Kwon HJ, Hwang ES, Namkoong SE, Um SJ. Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. *J Biol Chem* 2000;275:6764-9.
29. Ronco LV, Karpova AY, Vidal M, Howley PM. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev* 1998;12:2061-72.
30. Brinkman JA, Hughes SH, Stone P, Caffrey AS, Muderspach LI, Roman LD et al. Therapeutic vaccination for HPV induced cervical cancers. *Dis Markers* 2007;23:337-52.
31. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* 2009;361:1838-47.
32. van Seters M, van Beurden M, de Craen AJ. Is the assumed natural history of vulvar intraepithelial neoplasia III based on enough evidence? A systematic review of 3322 published patients. *Gynecol Oncol* 2005;97:645-51.
33. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP et al. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. *Clin Cancer Res* 2008;14:169-77.
34. Bakacs T, Mehrishi JN, Moss RW. Ipilimumab (Yervoy) and the TGN1412 catastrophe. *Immunobiology* 2011.
35. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD et al. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* 2006;355:1018-28.
36. Vonderheide RH, Flaherty KT, Khalil M, Stumacher MS, Bajor DL, Hutnick NA et al. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol* 2007;25:876-83.
37. Fransen MF, Sluijter M, Morreau H, Arens R, Melief CJ. Local activation of CD8 T cells and systemic tumor eradication without toxicity via slow release and local delivery of agonistic CD40 antibody. *Clin Cancer Res* 2011;17:2270-80.
38. Schiller M, Metze D, Luger TA, Grabbe S, Gunzer M. Immune response modifiers--mode of action. *Exp Dermatol* 2006;15:331-41.
39. van Seters M, van Beurden M, ten Kate FJ, Beckmann I, Ewing PC, Eijkemans MJ et al. Treatment of vulvar intraepithelial neoplasia with topical imiquimod. *N Engl J Med* 2008;358:1465-73.
40. Terlou A, van Seters M, Kleinjan A, Heijmans-Antonissen C, Santegoets LA, Beckmann I et al. Imiquimod induced clearance of HPV is associated with normalization of immune cell counts in usual type vulvar intraepithelial neoplasia. *Int J Cancer* 2010.
41. Daayana S, Elkord E, Winters U, Pawlita M, Roden R, Stern PL et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. *Br J Cancer* 2010;102:1129-36.
42. Smyth LJ, van Poelgeest MI, Davidson EJ, Kwappenberg KM, Burt D, Sehr P et al. Immunological responses in women with human papillomavirus type 16 (HPV-16)-associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV-16 oncogene vaccination. *Clin Cancer Res* 2004;10:2954-61.
43. Coban C, Ishii KJ, Akira S. Immune interventions of human diseases through toll-like receptors. *Adv Exp Med Biol* 2009;655:63-80.
44. Ferguson TA, Choi J, Green DR. Armed response: how dying cells influence T-cell functions. *Immunol Rev* 2011;241:77-88.
45. Ma Y, Kepp O, Ghiringhelli F, Apetoh L, Aymeric L, Locher C et al. Chemotherapy and radiotherapy: cryptic anticancer vaccines. *Semin Immunol* 2010;22:113-24.
46. Tseng CW, Hung CF, Alvarez RD, Trimble C, Huh WK, Kim D et al. Pretreatment with cisplatin enhances E7-specific CD8+ T-Cell-mediated antitumor immunity induced by DNA vaccination. *Clin Cancer Res* 2008;14:3185-92.
47. Tseng CW, Trimble C, Zeng Q, Monie A, Alvarez RD, Huh WK et al. Low-dose radiation enhances therapeutic HPV DNA vaccination in tumor-bearing hosts. *Cancer Immunol Immunother* 2009;58:737-48.
48. Zeng Q, Peng S, Monie A, Yang M, Pang X, Hung CF et al. Control of Cervicovaginal HPV-16 E7-Expressing Tumors by the Combination of Therapeutic HPV Vaccination and

Vascular Disrupting Agents. *Hum Gene Ther* 2011;22:809-19.

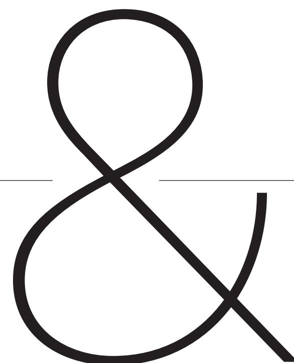
49. Avogadri F, Yuan J, Yang A, Schaer D, Wolchok JD. Modulation of CTLA-4 and GITR for cancer immunotherapy. *Curr Top Microbiol Immunol* 2011;344:211-44.
50. Callahan MK, Wolchok JD, Allison JP. Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. *Semin Oncol* 2010;37:473-84.
51. Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol* 2007;19:309-14.
52. Sharma RK, Schabowsky RH, Srivastava AK, Elpek KG, Madireddi S, Zhao H et al. 4-1BB ligand as an effective multifunctional immunomodulator and antigen delivery vehicle for the development of therapeutic cancer vaccines. *Cancer Res* 2010;70:3945-54.
53. Blank CU, Hooijkaas AI, Haanen JB, Schumacher TN. Combination of targeted therapy and immunotherapy in melanoma. *Cancer Immunol Immunother* 2011.
54. Wolchok JD, Yang AS, Weber JS. Immune regulatory antibodies: are they the next advance? *Cancer J* 2010;16:311-7.
55. Hoffmann C, Stanke J, Kaufmann AM, Loddenkemper C, Schneider A, Cichon G. Combining T-cell vaccination and application of agonistic anti-GITR mAb (DTA-1) induces complete eradication of HPV oncogene expressing tumors in mice. *J Immunother* 2010;33:136-45.
56. Lu S, Wang S, Grimes-Serrano JM. Current progress of DNA vaccine studies in humans. *Expert Rev Vaccines* 2008;7:175-91.
57. Lasaro MO, Ertl HC. New insights on adenovirus as vaccine vectors. *Mol Ther* 2009;17:1333-9.
58. de Mare A, Lambeck AJ, Regts J, van Dam GM, Nijman HW, Snippe H et al. Viral vector-based prime-boost immunization regimens: a possible involvement of T-cell competition. *Gene Ther* 2008;15:393-403.
59. Ingolotti M, Kawalekar O, Shedlock DJ, Muthumani K, Weiner DB. DNA vaccines for targeting bacterial infections. *Expert Rev Vaccines* 2010;9:747-63.
60. Lu S. Heterologous prime-boost vaccination. *Curr Opin Immunol* 2009;21:346-51.
61. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med* 2003;9:729-35.
62. Harari A, Bart PA, Stohr W, Tapia G, Garcia M, Medjitna-Rais E et al. An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J Exp Med* 2008;205:63-77.
63. Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002;415:331-5.
64. Daemen T, Pries F, Bungener L, Kraak M, Regts J, Wilschut J. Genetic immunization against cervical carcinoma: induction of cytotoxic T lymphocyte activity with a recombinant alphavirus vector expressing human papillomavirus type 16 E6 and E7. *Gene Ther* 2000;7:1859-66.
65. Gomez-Gutierrez JG, Elpek KG, Montes de Oca-Luna R, Shirwan H, Sam Zhou H, McMasters KM. Vaccination with an adenoviral vector expressing calreticulin-human papillomavirus 16 E7 fusion protein eradicates E7 expressing established tumors in mice. *Cancer Immunol Immunother* 2007;56:997-1007.
66. Holst PJ, Sorensen MR, Mandrup Jensen CM, Orskov C, Thomsen AR, Christensen JP. MHC class II-associated invariant chain linkage of antigen dramatically improves cell-mediated immunity induced by adenovirus vaccines. *J Immunol* 2008;180:3339-46.
67. Holst PJ, Bassi MR, Thomsen AR, Christensen JP. DNA fusion gene vaccines. *Curr Opin Mol Ther* 2010;12:47-54.
68. Tanghe A, D'Souza S, Rosseels V, Denis O, Ottenhoff TH, Dalemans W et al. Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. *Infect Immun* 2001;69:3041-7.
69. Sin JI, Bagarazzi M, Pachuk C, Weiner DB. DNA priming-protein boosting enhances both antigen-specific antibody and Th1-type cellular immune responses in a murine herpes simplex virus-2 gD vaccine model. *DNA Cell Biol* 1999;18:771-9.
70. Song MK, Lee SW, Suh YS, Lee KJ, Sung YC. Enhancement of immunoglobulin G2a and cytotoxic T-lymphocyte responses by a booster immunization with recombinant hepatitis C virus E2 protein in E2 DNA-primed mice. *J Virol* 2000;74:2920-5.
71. Baraldo K, Mori E, Bartoloni A, Petracca R, Giannozzi A, Norelli F et al. N19 polyepitope as a carrier for enhanced immunogenicity and protective efficacy of meningococcal conjugate vaccines. *Infect Immun* 2004;72:4884-7.
72. Baraldo K, Mori E, Bartoloni A, Norelli F, Grandi G, Rappuoli R et al. Combined conjugate vaccines: enhanced immunogenicity with the N19 polyepitope as a carrier protein. *Infect Immun* 2005;73:5835-41.
73. Loudon PT, Yager EJ, Lynch DT, Narendran A, Stagnar C, Franchini AM et al. GM-CSF increases mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into

the epidermis of non-human primates. *PLoS One* 2010;5:e11021.

74. Katae M, Miyahira Y, Takeda K, Matsuda H, Yagita H, Okumura K et al. Coadministration of an interleukin-12 gene and a Trypanosoma cruzi gene improves vaccine efficacy. *Infect Immun* 2002;70:4833-40.
75. Eickhoff CS, Vasconcelos JR, Sullivan NL, Blazevic A, Bruna-Romero O, Rodrigues MM et al. Co-administration of a plasmid DNA encoding IL-15 improves long-term protection of a genetic vaccine against Trypanosoma cruzi. *PLoS Negl.Trop Dis* 2011;5:e983.
76. Fagone P, Shedlock DJ, Bao H, Kawalekar OU, Yan J, Gupta D et al. Molecular adjuvant HMGB1 enhances anti-influenza immunity during DNA vaccination. *Gene Ther* 2011.
77. Abdulhaqq SA, Weiner DB. DNA vaccines: developing new strategies to enhance immune responses. *Immunol Res* 2008;42:219-32.
78. Bodles-Brakhop AM, Heller R, Draghia-Akli R. Electroporation for the delivery of DNA-based vaccines and immunotherapeutics: current clinical developments. *Mol Ther* 2009;17:585-92.
79. Luxembourg A, Evans CF, Hannaman D. Electroporation-based DNA immunisation: translation to the clinic. *Expert Opin Biol Ther* 2007;7:1647-64.
80. Low L, Mander A, McCann K, Dearnaley D, Tjelle T, Mathiesen I et al. DNA vaccination with electroporation induces increased antibody responses in patients with prostate cancer. *Hum Gene Ther* 2009;20:1269-78.



ANNEX



NEDERLANDSE SAMENVATTING
CURICULUM VITAE
LIST OF PUBLICATIONS

NEDERLANDSE SAMENVATTING

HPV en kanker

Dit proefschrift beschrijft de ontwikkeling van DNA vaccins voor de behandeling van humaan papillomavirus (HPV) geïnduceerde vormen van kanker. HPV virussen zijn wijdverspreid en veroorzaken onder andere (genitale) wratten. Voor bepaalde seksueel overdraagbare hoog-risico subtypes, voornamelijk subtype 16 en 18, is overduidelijk aangetoond dat ze kanker kunnen veroorzaken. Dit betreft voornamelijk baarmoederhalskanker (met ongeveer 300.000 doden per jaar wereldwijd), maar ook andere anogenitale vormen van kanker zoals vulvakanker, anuskanker en peniskanker en ook bepaalde vormen van kanker in de mond- en keelholte. De huidige behandeling van baarmoederhalskanker is gericht op vroegtijdig opsporen van afwijkingen (door middel van het zogenaamde uitstrijkje) en chirurgische verwijdering van het verdachte weefsel. Deze behandeling is ingrijpend en het is niet altijd mogelijk al het geïnfecteerde weefsel weg te halen. Omdat HPV geïnduceerde tumoren een duidelijke virale oorsprong hebben, betekent dit dat er in de tumor lichaamsvreemde antigenen tot expressie komen. In theorie kunnen tegen deze lichaamsvreemde eiwitten krachtige immuunresponsen opgewekt worden. Hierdoor zou het mogelijk moeten zijn heel gericht de tumor cellen op te ruimen met behulp van het immuunsysteem, zonder gezond weefsel aan te tasten. Dit is zo ongeveer de heilige graal op het gebied van de oncologie. Voor tumoren waarin een virale component ontbreekt is dat veel moeilijker omdat het immuunsysteem is gebaseerd op herkenning van “niet-eigen” eiwitten.

&

Profylactische vaccins

Tegenwoordig zijn er twee vaccins beschikbaar (Gardasil® en Cervarix ®) die infectie met een aantal subtypes van het HPV verhinderen. Deze vaccins bestaan uit de structurele eiwitten (L1) die de mantel van het virus vormen. Deze eiwitten vormen op het virus lijkende deeltjes waarop het immuunsysteem reageert met een krachtige B-cel respons. Een B-cel respons leidt tot de productie van antistoffen die het virus kunnen herkennen en vervolgens onschadelijk maken. Deze antistoffen hebben dus een profylactische werking, in andere woorden ze beschermen tegen een infectie met HPV. Uit grootschalig onderzoek in de mens is gebleken dat dit type vaccin bijna 100% bescherming biedt tegen de ontwikkeling van door deze HPV subtypes geïnduceerde tumoren in gevaccineerde individuen. Daarbij is het belangrijk dat het vaccin wordt toegediend voordat de HPV infectie optreedt. De praktische consequentie is dat dit vaccin al op jonge leeftijd moeten worden toegediend, namelijk voordat het individu sexueel actief wordt. Het is echter nog niet bekend hoe lang de bescherming aanhoudt. Het belangrijkste nadeel van dit type vaccin is, dat voor een optimale bescherming, de hele bevolking gevaccineerd dient te worden terwijl de incidentie van bijvoorbeeld baarmoederhalskanker in de westerse wereld maar 7 per 100.000 inwoners bedraagt. Dit betekent dat er veel individuen in feite onnodig zullen worden gevaccineerd, wat ongunstig is voor de kosteneffectiviteit. Gebaseerd op de incidentie en de kosten per vaccinatie (360 dollar) kost het voorkomen van 1 geval van baarmoederhalskanker maar liefst 5 miljoen dollar! Hierbij moet wel opgemerkt worden dat de profylactische vaccins ook de voorstadia van kanker kunnen vóórkomen waarvan de incidentie aanmerkelijk hoger ligt. Voor het behandelen van dergelijke laesies is veelal chirurgie nodig met mogelijk ernstige complicaties.

Noodzaak voor ontwikkeling van therapeutische vaccins

Een waarschijnlijk veel goedkoper alternatief is het ontwikkelen van een therapeutisch vaccin. Met andere woorden, een vaccin dat kan worden gegeven op het moment er een HPV geïnduceerde tumor, of een voorstadium daarvan, wordt geconstateerd. Hierdoor is het niet nodig de gehele bevolking op voorhand te vaccineren, maar alleen diegenen met een voorstadium van een HPV geïnduceerde tumor. Toepassing van een dergelijk vaccin is daarom waarschijnlijk ook haalbaar in ontwikkelingslanden. De profylactische vaccins zijn hiervoor momenteel nog te duur, terwijl daar notabene 80% van de HPV gerelateerde slachtoffers vallen. Een dergelijk therapeutisch vaccin moet tegen andere antigenen van het virus gericht worden omdat de structurele eiwitten in dit stadium niet meer tot expressie komen. De virale eiwitten die hiervoor bij uitstek in aanmerking komen zijn E6 en E7. Deze eiwitten spelen een grote rol bij het ontstaan van kanker als gevolg van HPV infectie. E6 en E7 zijn namelijk in staat te binden aan bepaalde tumorsurpressoreiwitten (respectievelijk p53 en pRb) en deze uit te schakelen, waardoor de controle over de celdeling wegvalt. Expressie van E6 en E7 is noodzakelijk voor de HPV geïnfecteerde cel om zich te ontwikkelen tot tumorcel, bovendien kunnen de eenmaal gevormde tumorcellen niet overleven zonder de expressie van E6 en E7. Ook is een ander type immuunrespons nodig, namelijk een T-cel respons in plaats van een B-cel/antilichaam respons. T-cellen kunnen virus geïnfecteerde cellen van buitenaf herkennen via presentatie van kleine eiwitfragmenten afkomstig van het virus op het oppervlakte van de cel. Na herkenning kan een T-cel een geïnfecteerde cel doden zonder verdere schade aan te richten. Er zijn vele mogelijke manieren om E6 en E7 specifieke T-cel immuniteit op te wekken, zoals eiwit/peptide vaccinatie, vaccinatie met behulp van virale vectoren en DNA vaccinatie. DNA vaccinatie heeft een aantal voordelen ten opzichte van de andere methoden. Zo wordt DNA vaccinatie als veiliger beschouwd dan virale methoden en effectiever voor het opwekken van T-cel immuniteit dan de andere non-virale methoden zoals peptide en eiwit vaccinatie. Ook is de productie van DNA vaccins relatief eenvoudig. Hoewel enkele studies in mensen al hebben laten zien dat het opwekken van E6 en E7 specifieke T-cel responsen gunstige effecten heeft in patiënten met een voorstadium van baarmoederhalskanker, zijn er met DNA vaccins tot nu toe nog geen duidelijke successen geboekt. In dit proefschrift hebben we geprobeerd nieuwe DNA vaccin kandidaten te ontwikkelen die veel krachtigere T-cel responsen opwekken dan de bestaande vaccin kandidaten. Hierbij hebben we bestaande inzichten gecombineerd en zijn we ook tot nieuwe inzichten gekomen over hoe DNA vaccins te optimaliseren.

Inhoud van de verschillende hoofdstukken

In **hoofdstuk 1** wordt het verband tussen HPV infectie en baarmoederhalskanker en de keuze voor DNA vaccinatie, zoals hierboven uitgelegd, besproken.

Hoofdstuk 2 is een overzichtsartikel over DNA-tatoeage vaccinatie. Deze techniek is in het NKI-AVL ontwikkeld om de immunogeniciteit van DNA vaccins te verbeteren. De methode bestaat uit het aanbrengen van een DNA oplossing op de huid, die vervolgens met behulp van een tatoeage-apparaat in de opperhuid/epidermis wordt geïnjecteerd. De gedachte is dat de huid het ideale orgaan is om een immuunrespons in op te wekken omdat de huid de natuurlijke barrière met de buitenwereld vormt en erg rijk is aan immuuncellen. Ook de huidschade die ontstaat als het gevolg van het tatoeëren is waarschijnlijk van groot belang omdat het immuunsysteem hierdoor wordt gealarmeerd. Aan de orde komen onder andere: de voordelen van DNA vaccinatie ten opzichte van meer conventionele vaccinatie methoden zoals immunisatie met subunitvaccins of met geïnactiveerde pathogenen, de mogelijke

mechanismen waarop DNA tatoeage leidt tot T-cel immuniteit, de voordelen van de DNA-tatoeage ten opzichte van andere toedieningsmethoden zoals gene-gun (techniek waarbij het DNA gekoppeld aan goudbolletjes in de huid wordt geschoten) en electroporatie (een techniek die gebruikt maakt van stroomstootjes om het DNA de cel in te krijgen).

In **hoofdstuk 3** wordt de ontwikkeling van twee kandidaatvaccins voor de behandeling van HPV geïnduceerde vormen van kanker. Omdat E6 en E7 kankerverwekkende/transformerende eigenschappen hebben, hebben we gekozen voor het gebruik van 'door elkaar gehusselde' (in het Engels: 'shuffled') en daardoor niet functionele versies van E6 en E7, E6SH en E7SH genoemd. Dit om het risico uit te sluiten dat er kanker kan ontstaan op de plaats van de vaccinatie. Het bleek echter dat deze 'shuffled' versies veel lagere T-cel responsen induceerden dan de ongemodificeerde/wild-type varianten. Om dit verlies van immunogeniciteit te herstellen hebben we genetische fusies gemaakt van E6SH en E7SH met een deel van tetanus toxine fragment C (TTFC). De resulterende fusie vaccins TTFC-E7SH en TTFC-E6SH hebben een sterk verbeterde immunogeniciteit ten opzichte van E7SH en E6SH. In een muismodel voor HPV geïnduceerde kanker resulteert dit ook in zeer sterk verbeterde antitumorreacties na vaccinatie met TTFC-E7SH in vergelijking met E7SH. Een belangrijke verklaring voor de verbeterde immunogeniciteit is dat TTFC universele 'helper T-celepitopen' bevat. De gedachte is dat hierdoor een zogenaamde 'helper T-cel' reactie wordt opgewekt waardoor de cytotoxische/celdodende T-cel respons, die nodig is om geïnfecteerde cellen te doden, verbetert. Een andere verklaring is dat de stabiliteit van het antigeen toeneemt en daardoor ook de hoeveelheid antigeen die door het immunsysteem kan worden opgemerkt.

In **hoofdstuk 4** worden de veiligheidsstudies beschreven die we hebben uitgevoerd om aan te tonen dat TTFC-E6SH en TTFC-E7SH inderdaad geen kankerverwekkende/transformerende eigenschappen meer hebben. Hiervoor hebben we 2 verschillende uitleessystemen gebruikt namelijk een uit muizen afkomstige cellijn (NIH 3T3 cellen) en primaire humane huidcellen afkomstig uit de voorhuid van donoren. Het inbrengen van onze kandidaatvaccins in beide typen cellen leidt niet tot transformatie, terwijl het inbrengen van de wild-type varianten wél tot transformatie leidt. Ook laten we in de primaire humane cellen zien dat de geshufflede varianten niet meer kunnen binden aan hun belangrijkste targets, de tumorsuppressoreiwitten p53 en pRb. Op grond van deze data beschouwen wij het gebruik van onze kandidaatvaccins in de mens veilig.

In **hoofdstuk 5** wordt de ontwikkeling van nog een tweetal E6 en E7 coderende kandidaatvaccins beschreven. Deze nieuwe vaccins bevatten in plaats van het complete drager-eiwit Tetanus Toxine Fragment C (TTFC), een set van 3 gedefinieerde universele helper T-celepitopen. De gedachte is dat hierdoor het risico op eventuele ongewenste cytotoxische T-cel responsen tegen TTFC vrijwel is uitgesloten. Het vaccin wordt hierdoor als het ware doelgerichter. Daarnaast zijn deze vaccins naar het endoplasmatisch reticulum (ER, een afgegrensd organel binnen de cel) gestuurd met behulp van uitsluitend (korte) signaalsequenties. Veel E6 en E7 gerichte DNA vaccins die in de literatuur beschreven staan, bestaan uit een fusie met een compleet in het ER gelokaliseerd drager-eiwit. Het voordeel van het gebruik van alleen signaalsequenties is het verminderde risico op het induceren van auto-immuniteit. Belangrijk voordeel van de nieuwe vaccins, sigHELPE6SHKDEL en sigHELPE7SHKDEL genoemd, is dat deze vele malen immunogener zijn dan de in hoofdstuk 3 ontwikkelde vaccin kandidaten TTFC-E6 en TTFC-E7. Het is dan ook de bedoeling om deze vaccins te gaan testen bij patiënten met baarmoederhalskanker.

In **hoofdstuk 6** is een methode ontwikkeld om de opname van naakt-DNA (DNA zonder toevoegingen zoals gebruikt in hoofdstuk 3 en 5) vaccins te verbeteren. Het is algemeen bekend dat de opname van naakt DNA door cellen een uiterst inefficiënt proces is. Wij hebben berekend dat na DNA tatoeage slechts 1 op de miljoen tot slechts 1 op de 5 miljard DNA moleculen wordt opgenomen. Dit wordt waarschijnlijk veroorzaakt door snelle afbraak van het DNA in de huid en het feit dat het DNA de celmembraan moeilijk kan passeren. Een mogelijke oplossing is het DNA te verpakken in zogenaamde nanobolletjes. Deze nanobolletjes bestaan uit een complex van het negatief geladen DNA en een positief geladen polymeer. Hierdoor wordt het DNA beschermd tegen afbraak en zou het de celwand makkelijker kunnen passeren doordat deze bolletjes compacter zijn dan naakt DNA. Onze studie laat zien dat voor twee gangbare typen nanobolletjes de opname van het DNA sterk wordt verbeterd in gekweekte cellen (*in vitro*), maar dat de opname in intacte huid (*ex vivo*) of in muizen (*in vivo*) compleet wordt geremd. Dit duidt op verschillen in het opname mechanisme in *in vitro* condities versus *in vivo* condities. Door de lading van de nanobolletjes af te schermen kon de opname van het DNA sterk worden verbeterd met als gevolg een 5x hogere expressie van een model vaccin in vergelijking met naakt DNA. De verhoogde expressie leidde echter niet tot een significante toename in immunogeniciteit. Dit suggereert dat er een sterkere verbetering nodig is of dat andere factoren dan alleen de hoogte van de expressie een rol spelen bij de immunogeniciteit van in nanobolletjes verpakte DNA vaccins.

Hoofdstuk 7 bevat een samenvattende discussie van het proefschrift en bespreekt een aantal factoren die het succes van DNA vaccinatie tegen HPV geïnduceerde tumoren kunnen verbeteren. Een belangrijke factor is de selectie van patiënten. Uit verschillende onderzoeken blijkt dat het behandelen van patiënten in een vroeg stadium de kans op succes verbeterd. Daarnaast kan de uitkomst van de behandeling verbeterd worden door vaccinatie te combineren met locale immuunactivatie op de plaats van de tumor. Een hiervoor bij uitstek geschikt middel is het geregistreerde Imiquimod (Aldara®). Deze stof wordt al toegepast bij de behandeling van genitale wratten en voorstadia van vulvakanker en ook is er al een studie gepubliceerd die een gunstig effect laat zien van de combinatie van vaccinatie en Imiquimod. Ook kan het gunstig zijn verschillende vormen van vaccinatie (zoals virale vaccinatie en DNA vaccinatie) met elkaar te combineren in een zogenaamd heteroloog prime-boost protocol. Hierdoor worden de sterke punten van de verschillende systemen als het ware gecombineerd. Verschillende studies hebben al laten zien dat dergelijke prime-boost protocollen de efficiënte van vaccinatie sterk kunnen verbeteren. Tenslotte is het niet onwaarschijnlijk dat onze kandidaat DNA vaccins nog verder kunnen worden verbeterd, bijvoorbeeld door het toevoegen van bepaalde immuun-activerende genen zoals GM-CSF, IL-12 en IL-15. Onze verwachting is dat de behandeling van HPV geïnduceerde tumoren door middel van immunotherapie een realistisch scenario is voor de toekomst.

CURRICULUM VITAE

Koen Oosterhuis was born on the 22nd of April 1980 in Breukelen, The Netherlands. After finishing secondary education at the Gomarus College in Groningen in 1998 he started his Pharmacy studies in the same year at the University of Groningen. During his studies he performed a research project at the department of Therapeutic Gene Modulation, with the aim to redirect adenoviral vectors expressing therapeutic genes specifically to tumor endothelial cells and graduated Cum Laude in 2003. After passing the qualifying examination in pharmacy in 2006, he temporarily worked as a project-pharmacist in the University Medical Center Groningen (UMCG) on a project that aimed to identify the number of Hospital Admissions Related to Medication (HARM study). Subsequently he started his PhD project at the Immunology Department of Netherlands Cancer Institute in the group of John Haanen and Ton Schumacher. The results of the research performed during his PhD project are described in this thesis. The author is currently working at Crucell as scientist Vaccine Generation.

LIST OF PUBLICATIONS

Preclinical safety evaluation of DNA vaccines encoding modified HPV16 E6 and E7. Henken FE, Oosterhuis K, Öhlschläger P, Bosch L, Hooijberg E, Haanen JB, Steenbergen RD. *Vaccine, accepted for publication*.

Preclinical development of highly effective and safe DNA vaccines directed against HPV 16 E6 and E7. Oosterhuis K, Ohlschläger P, van den Berg JH, Toebes M, Gomez R, Schumacher TN, Haanen JB. *Int J Cancer. 2011 Jul 15;129(2):397-406*

DNA Vaccines and Intradermal Vaccination by DNA Tattooing. Oosterhuis K, van den Berg JH, Schumacher TN, Haanen JB. *Curr Top Microbiol Immunol. 2012;351:221-50*

DNA vaccination in oncology: current status, opportunities and perspectives. van den Berg JH, Oosterhuis K, Beijnen JH, Nuijen B, Haanen JB. *Curr Clin Pharmacol. 2010 Aug;5(3):218-25*.

Shielding the cationic charge of nanoparticle-formulated dermal DNA vaccines is essential for antigen expression and immunogenicity. van den Berg JH, Oosterhuis K, Hennink WE, Storm G, van der Aa LJ, Engbersen JF, Haanen JB, Beijnen JH, Schumacher TN, Nuijen B. *J Control Release. 2010 Jan 25;141(2):234-40*.

DNA tattoo vaccination: effect on plasmid purity and transfection efficiency of different topoisoforms. Quaak SG, van den Berg JH, Oosterhuis K, Beijnen JH, Haanen JB, Nuijen B. *J Control Release. 2009 Oct 15;139(2):153-9*.

Frequency of and risk factors for preventable medication-related hospital admissions in the Netherlands. Leendertse AJ, Egberts AC, Stoker LJ, van den Bemt PM; HARM Study Group. *Arch Intern Med. 2008 Sep 22;168(17):1890-6*.

Functional inhibition of NF-kappaB signal transduction in alphavbeta3 integrin expressing endothelial cells by using RGD-PEG-modified adenovirus with a mutant IkappaB gene. Ogawara K, Kułdo JM, Oosterhuis K, Kroesen BJ, Rots MG, Trautwein C, Kimura T, Haisma HJ, Molema G. *Arthritis Res Ther. 2006;8(1):R32*.

Differential effects of NF-{kappa}B and p38 MAPK inhibitors and combinations thereof on TNF-{alpha}- and IL-1{beta}-induced proinflammatory status of endothelial cells in vitro. Kuldo JM, Westra J, Asgeirsdóttir SA, Kok RJ, Oosterhuis K, Rots MG, Schouten JP, Limburg PC, Molema G. *Am J Physiol Cell Physiol. 2005 Nov;289(5):C1229-39*.