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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 possess 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
DISCUSSION AND FUTURE OUTLOOK

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 adenviral vector encoded E7 improved its immunogenicity to the same extent 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 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.

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