Formulation of peptide-based cancer vaccines

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CHAPTER 9

Summary, discussion & conclusion

SUMMARY

This thesis describes the development and immunological evaluation of two different cancer vaccination strategies for peptide-based personalized cancer vaccines. Both strategies, liposomal encapsulation and adjuvanting by direct conjugation to a TLR-ligand, are aimed to be readily combined with neoantigen-containing synthetic peptide (SP) sequences. In order to formulate such personalized cancer vaccines a flexible platform is required that can harbor a wide range of physiochemically different SPs, because multiple neoepitopes are uniquely expressed per patient (1-5). Upon formulation, the vaccination platform should be able to induce effective tumor-specific immune responses, which was evaluated in this thesis in *in vitro* and *in vivo* preclinical models.

In **chapter 2** the literature on cationic nanoparticle based cancer vaccines is reviewed and discussed. Because (neo)antigen only is not an effective (personalized) cancer vaccine, it needs to be formulated in order to effectively induce tumor-controlling immune responses. To be a successful cancer vaccine, cancer vaccine formulations must ensure antigen delivery to dendritic cells (DCs) and induce the priming of antigen specific T-cells (1, 2, 5-9). Cationic nanoparticles have been shown to improve vaccine efficacy for a variety of tumors by efficient antigen delivery to and subsequent activation of DCs (6, 10). The DCs subsequently efficiently induce antigen-specific cellular immune responses, which play a major role in cancer immunity (6). The nanoparticles can be combined with synthetically produced antigens (synthetic peptides, mRNA and DNA) and allow production of multi-epitope vaccines under current Good Manufacturing Practice (cGMP) conditions. The intradermal administration of such vaccines is of special interest, because relatively large amounts of DCs are present in the skin and are well accessible for drug delivery.

For personalized cancer vaccination it is envisioned that a high number of patient-specific peptides (~20) with a large variety of physicochemical properties need to be formulated in a single personalized vaccine. Therefore, in **chapter 3** cationic liposomes three methods based on a dehydration-rehydration have been developed to encapsulate SPs with a wide range of psychochemical properties while containing a reporter $CD8^+$ T-cell epitope for immunological readout. The physicochemical characteristics (hydrodynamic diameter, polydispersity index and zeta-potential) of all liposomal formulations were comparable. All the formulations efficiently delivered the SP to DCs that subsequently activated specific $CD8^+$ T-cells *in vitro*. Indicating the improved immunological activity of the SPs upon encapsulation. Furthermore, modelling indicated that the physicochemical range of SPs, selected in this study, covered the majority of SPs (n=5546) that can theoretically be derived from 10 representative proteins. Combined these results indicated that cationic liposomes offer a promising formulation strategy for multi-epitope personalized cancer vaccines (11).

Intradermal vaccination has shown great potential for the administration of cancer vaccines, however, the classical ”Mantoux” method requiring hypodermic needles and syringes and injecting relatively large volumes has multiple drawbacks. Therefore, **in chapter 4,** the administration of cationic liposomes by hollow-microneedle mediated micro-injections was studied. The microneedle system was able to accurately dispense volumes in between 1 – 10 μL in a repeatable manner. In *ex vivo* human skin the microneedle system was able to deliver similar drug doses compared to classical hypodermic needle-mediated injections, but at much lower volumes. This is especially of interest for personalized cancer vaccines in which multiple neoepitopes will be included and only limited quantities of vaccine will be produced. Cationic liposomes, loaded with HPV-E7 derived SPs, efficiently induced functional $CD8^+$ as well as $CD4^+$ T-cells in mice upon vaccination by the hollow microneedle system. While administering similar doses via classical injection needles and hollow microneedles, the latter made use of a 6-fold lower volume and resulted in improved immunogenicity. Additionally, the injection depth was fully controlled by the microneedle system resulting in depth- and volume-controlled and minimally-invasive administration of the vaccine.

In order clinically translate SP-loaded cationic liposomes, analytical methods should be in place to quantify both the lipid and peptide content in the (personalized) liposomal cancer vaccine formulations. Therefore, in **chapter 5** a reversed-phase ultra-performance liquid chromatography (RP-UPLC) method was developed that separates and quantifies both lipids (DOTAP, DOPC) and two physicochemically different SPs. Upon separation, peptides and lipids were quantified with acceptable accuracy and precision as described in the ICH guideline validation of analytical procedures (12). The lipids and peptides did not mutually influence their quantification and therefore eliminated the need for lipid extraction in sample preprocessing. This procedure is especially important for peptide-based personalized cancer vaccines, since a large number of peptides (~20) with a wide variety of physicochemical properties (see chapter 3) are envisioned in a single personalized vaccine, making extraction optimization during sample analysis very laborious and a source of error.

In **chapter 6** a multi-epitope vaccine is described that is composed of seven different SPs comprising neoepitopes, both MHC class I and MHC class II, individually encapsulated in cationic liposomes. The neoepitopes used in this studied originated form the mouse colorectal cancer model (MC-38) and liposomes were prepared and analyzed as described in chapters 3 and 5. All SPs were individually encapsulated and resulting formulations had comparable size distributions and were positively charged. Recovery of both DOTAP and DOPC in the final formulation was comparable and SP recovery was on average 25 %, indicating efficient loading of the SPs. The liposomally formulated MHC class I neoepitopes efficiently activated neoepitope-specific $CD8^+$ T-cells *in vitro*. Combined vaccination with four MHC class I neoepitopes, individually loaded in cationic liposomes efficiently induced neoepitope-specific $CD8^+$ T-cells This indicates that the

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liposomal neoepitopes can be administered as a single cocktail injection. Vaccination with a cocktail of two MHC-I neoepitopes and three MHC-II neoepitopes significantly improved neoepitope-specific $CD8^+$ T-cell induction. Furthermore, the liposomal combination vaccine of MHC-I and MHC-II neoepitopes prophylactically protected mice against outgrowth of MC-38 tumors. Re-challenged with a lethal tumor cell dose, revealed long-term tumor immunity. The study shows that cationic liposomes are a powerful delivery system for multiple synthetic peptide-based neoepitope vaccines.

An explorative study on the mechanism of action of cationic liposomes in peptide based cancer vaccines is described in **chapter 7.** Liposomal biodistribution upon intradermal vaccination was studied by making use of near-infrared labeled lipids and (lipo)peptides. The influence of liposomal charge on *in vivo* biodistribution, T-cell priming and functionality was studied by making use of lipopeptide loaded cationic, neutral and anionic liposomes. Cationic liposomes loaded with lipopeptide were detectable at the injection site up to 2 weeks post vaccination, followed by neutral and anionic liposomes, 6 and 2 days respectively. Only the formulations that contained cationic liposomes and antigen, either encapsulated or admixed, were able to induce specific $CD8^+$ T-cells capable of inhibiting tumor outgrowth. Vaccination with encapsulated antigen outperformed the admixed antigen by inducing 10-fold higher antigen specific $CD8^+$ T-cells. The vaccine containing peptide encapsulated in cationic liposomes prevented tumor outgrowth in 100% of the mice, while peptide admixed with cationic liposomes only protected 25% of the mice. Cationic liposomes enhanced uptake of peptide and protein by dendritic cells *in vitro.* The antigen was detectable up to 72 hours post incubation and the dendritic cells were still able to activate antigen-specific $CD8^+$ T-cells *in vitro*. The results of this explorative study indicate that cationic liposomes mediate prolonged antigen exposure and facilitate sustained antigen cross-presentation capacity by dendritic cells.

Chapter 8 describes chemical conjugation of the novel TLR-2 ligand mini-UPam to two different human neoepitopes, a MHC class I and MHC class II epitope, derived from a melanoma patient. This direct conjugation resulted in a two-in-one system: one molecule that contains both antigen and adjuvant (13). Since the mini-UPam contains only one palmitoyl chain, instead of three as in the classical TLR-2 ligand Pam_3CysSk_4, the ligand is better applicable for production of self-adjuvanting peptide based cancer vaccines by avoiding solubility issues of more hydrophobic SPs (13, 14). Covalently attached mini-UPam to both neoepitope containing SPs, containing human melanoma derived neoepitopes, could properly activate human TLR-2. Human antigen presenting cells loaded with the mini-UPam-SP conjugates were able to efficiently activate patient-derived neoepitope specific $CD8^+$ and $CD4^+$ T-cells. In conclusion, the mini-UPam offer an immunogenic modifier for peptide-based personalized cancer vaccines.

GENERAL DISCUSSION

The capacity of the immune system to attack tumor cells throughout the body and, upon clearance, form long-lasting immunological memory form a powerful weapon against cancer. Cancer vaccines have therefore been of interest for several decades, however, with only limited clinical successes so far (1, 15, 16). During the past decade the major role of neoantigen-specific T-cells in tumor immunity became clear and offered new, highly-specific tumor targets for cancer vaccines (1, 2, 4, 6, 17). Design and production of neoantigen-based vaccines became possible through advancements as next-generation sequencing, novel bioinformatic tools and full synthetic antigen formats (e.g., synthetic peptides and mRNA) (1, 17-20). These neoepitope-based cancer vaccines have shown to amplify preexisting and induce new subsets of tumor specific T-cells, both $CD8^+$ as wells as $CD4^+$ T-cells, in recent preclinical and clinical trials (2, 5, 7, 17, 18, 20-23). In order to unleash the full potential of personalized cancer vaccines, adequate delivery to and subsequent activation of the cellular immune system is required (1, 5-7). In this thesis the formulation of peptide-based personalized cancer vaccines via two different strategies have been studied.

9.1 Liposomal based cancer vaccines

9.1.1 Pharmaceutical perspective

Nanoparticles have been extensively studied for vaccine delivery purposes in both prophylactic and therapeutic vaccines (1, 6, 10, 24). With the approval of two lipid nanoparticle-based COVID-19 vaccines (Moderna's mRNA-1273 and Pfizer-BionTech's Comirnaty) the world has seen, for the first time, large scale administration of synthetically produced nanoparticle based vaccines (25-27). Despite the fact that both vaccines aim to induce a prophylactic immune response, they have established clinical application of nanoparticles in vaccine delivery. Cationic nanoparticles have shown to efficiently induce cellular immune responses and are therefore of interest for the formulation of cancer vaccines. The cellular immune response plays a major role in tumor cell recognition and clearance (chapter 2) (6, 10, 28-32). The use of the liposomal dehydration-rehydration preparation method allowed preclinical development and evaluation of DOTAP:DOPC liposomes as a delivery system for personalized cancer vaccines. However, due to the use of chloroform based rotary-evaporation it will be cumbersome to perform this formulation method under cGMP conditions. Dissolution of both lipids in an organic solvent that can be removed by freeze-drying (e.g., DMSO) could be a strategy to circumvent rotary evaporation. The SP can then either be added in the organic solvent, to incorporate the SP in the lipid bilayer, or post freeze-drying in an aqueous solvent such as 0.04% NH_4OH to load the SP in the aqueous core of the liposomes (chapter 3). Currently, the final formulation is an aqueous liposomal dispersion, not ideal for long-term storage and transport for multiple reasons (e.g., chemical and physical stability). Incorporation of a lyoprotectant (e.g., sucrose, trehalose) and a buffer that is suitable for freeze-drying

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(e.g., histidine) allows lyophilization of the final product, yielding a dry product that can be reconstituted prior to injection (33, 34). This dry end product would facilitate prime-boost regimes over longer periods of time without the need of manufacturing new vaccine batches.

9.1.2. Immunological perspective

Cationic liposomes increase vaccine immunogenicity via various mechanisms: enhancement of antigen uptake, dendritic cell activation and formation of a depot at the SOI (side of injection) leading to prolongation of antigen exposure. For the SP-based neoantigen vaccine described in chapter 6 the *in vivo* priming of 4 different neoepitope-specific $CD8^+$ T-cells was most efficient when the SP was encapsulated, admixing of the SP with empty cationic liposomes did only moderately improve T-cell induction in compared to SP-loaded liposomes. Encapsulation resulted in prolonged retention of the SP at the SOI and thereby increased antigen exposure at the SOI (chapter 7). Additionally, by encapsulation the likelihood is increased that both antigen and immunostimulant are internalized by the same DCs, which is important to efficiently generate signal 1, 2 & 3 (figure 2, chapter 2), a requirement for tumor-specific T-cell priming (6, 8, 9, 35, 36). To further unravel the mechanism of action, further studies will show which subsets of DCs internalize cationic liposomes at the SOI. Distinct subsets of DCs, $CD8^+α$, have shown to be able to cross-present antigen up to 72 hours post uptake and hereby facilitate prolonged T-cell priming (37). This prolonged antigen presentation is facilitated by antigen storage in lysosome-like compartments that allow prolonged supply to MHC-class I molecules (38). The explorative *in vitro* data in chapter 7 suggests a similar routing for cationic liposomes, since prolonged antigen presence as well as prolonged $CD8^+$ T-cell presentation were observed for SP cationic liposomes. The infrared dye labelled SPs and lipids, developed in chapter 7, offer possibilities to determine *in vivo* which subsets of DCs and APCs (e.g., macrophages) engulf SP loaded cationic liposomes at the SOI and in the draining lymph nodes. Additionally, these compounds allow experiments to study intracellular trafficking of SP loaded cationic liposomes. In order to efficiently induce anti-tumor immunity, both $CD8^+$ and $CD4^+$ T-cells neoepitopes should be included in personalized cancer vaccines (5, 8, 21, 39-42). During T-cell priming several subsets of DCs are involved in priming of $CD8^+$ and $CD4^+$ T-cells and separate delivery of MHC class I and MHC class II neoepitopes to distinct DC subsets could improve tumor vaccine efficacy (8, 43, 44). $CD4^+$ T-cells have shown to play a fundamental role in T-cell mediated tumor immunity and specific formulation strategies for MHC class II neoepitopes could therefore be of great benefit for personalized cancer vaccines(4, 5, 8, 21, 23, 39-41). Mechanistic understanding of the *in vivo* behavior of SP loaded cationic liposomes and interaction with the cellular immune system allow further optimization of liposomal cancer vaccines.

Conjugate based vaccines

Pharmaceutical perspective

The mini-UPam conjugated to human melanoma $CD8^+$ and a $CD4^+$ T-cell neoepitopes effectively activated patient derived neoepitope-specific T-cells (14). Combined with the promising clinical results of the UPam-based HPV conjugates the mini-UPam offers a well-defined two in one vaccination system. In comparison to UPam the described TLR-2 ligand mini-UPam is less hydrophobic, only one palmitoyl chain instead of three, and therefore there is a lower risk of solubility issues when conjugated to more hydrophobic SPs (**chapter 8**) (13, 14, 45). Nevertheless, the varying physicochemical characteristics of the neoepitope-containing mini-UPam SP conjugates should be taken into account during further conjugate and formulation development. Varying solubilities of the SPs can limit application and influence biodistribution upon administration. Hydrophilic-based SPs are likely to rapidly leave the site of injection (SOI), while hydrophobic-based SPs potentially form supramolecular structures (e.g., micelles) and promote deposition at the SOI (46). As described in various reports and chapter 7, biodistribution profiles could influence induction of tumor-specific T-cells. Incorporation of SP conjugates in cationic liposomes could be a strategy to circumvent this problem, after all when successfully encapsulated the liposomes will have similar physicochemical characteristics (chapters 3 & 6). A recent study reported self-assembling nanoparticles, composed of lipopeptides, for the delivery of cancer vaccines. These lipopeptides contained a tumor epitope, TLR ligand and a charge modifying groups, ionizable lipids and specific amino acid sequences, to ensure nanoparticle formation upon addition of an aqueous solvent (47). These self-assembling nanoparticles were able to induce functional, tumor-specific T-cells in multiple tumor bearing mice models (47, 48). This work shows the potential of a nanoparticulate-based vaccine containing SP-adjuvant conjugates. Cationic liposomes loaded with mini-UPam based SP conjugates is a promising future perspective for an optimal peptide-based vaccine platform.

Immunological perspective

Delivery of both antigen and adjuvant in one molecule has shown efficient induction of tumor specific T-cell responses in both mice and man for various tumor antigen types (model antigens, viral oncoproteins and neoantigens) (chapter 8) (13, 14, 49-51). Recently, in a phase I/II clinical trial, the HPV-based UPam conjugates were found to be well tolerated and induced a 100-fold higher immune response compared to unconjugated SPs (51). These results clearly illustrate the clinical potential of the direct conjugation of antigenic SPs to TLR ligands. In this thesis the mini-UPam, conjugates have been evaluated with monocyte derived DCs, these DCs are derived from peripheral blood monocytes by cell culture based differentiation. The moDCs they most likely do not have full T-cell priming capabilities compared to primary DCs. Nevertheless, moDCs loaded with the UPam based conjugates improved priming of neoepitope specific $CD8^+$ T-cells compared to an equimolar mixture of UPam and the free SP. The $CD4^+$ T-cell

neoepitope based mini-UPam conjugate did not interfere with induction of neoepitope specific $CD4^+$ T-cells, however, the conjugate did not induce higher levels of *ex vivo* activation (chapter 8) (14). Previous studies with UPam based conjugates made similar observations during *in vitro* studies but when administered *in vivo* the conjugated $CD4^+$ T-cell epitopes improved tumor-specific T-cell responses, stressing their importance (49). Evaluation of mini-UPam-based conjugates in primary like DCs can provide further insight in the induction of $CD4^+$ T-cells by these conjugates (52). Little is known yet on the biodistribution of the studied conjugates upon intradermal injection. Addition of relatively large fluorescent labels is not a suitable option since these will most likely alter the biodistribution profile of the conjugate molecule. The use of biorthogonal- or radio labeling would provide a strategy to circumvent this problem since it makes use of small groups and has shown not to interact with peptide processing (53).

Cancer vaccines in combination with immunomodulation

The combination of cancer vaccines and checkpoint inhibiting therapies has shown promising results, both in animal models as well as in humans (5, 7, 17, 20, 47, 48, 54-57). The addition of checkpoint blockade ensures optimal T-cell functioning and can prevent T-cell exhaustion. Additionally, checkpoint inhibiting-molecules (e.g., PD-1/PD-L1) play a role during T-cell priming in the lymph nodes and can thus amplify T-cell induction by personalized cancer vaccines (9, 58, 59). The increased understanding of the interactions in-between tumor and immune cells have shown that limited successes of the first cancer vaccines could be attributed to dynamic tumor-antigen expression, immune escape and immune suppression by the tumor (5, 6, 15, 16). In order to fully benefit from personalized cancer vaccines a balanced combination of the vaccine and immune modulating molecules has to be selected according to the type of tumor. After all, the newly induced tumor-specific T-cells have to be able to reach the tumor, recognize the tumor cells and should not be switched off by the tumor micro-environment (5, 8, 9, 58). The rapidly advancing understanding of the tumor microenvironment and the effect on tumor specific T-cells allows further possibilities to develop and incorporate personalized cancer vaccines in clinical practice. For example, biomarkers determination of susceptibility of a patient to checkpoint inhibiting molecules and high resolution immune monitoring allow rational development of the concomitant administration of cancer vaccines with classical and novel therapeutic options for cancer patients.

CONCLUSION

The encapsulation of SPs in cationic liposomes or the direct conjugation to mini-UPam have shown to be potent vaccination strategies for peptide-based personalized cancer-specific vaccines. Manufacturing of liposomal formulations and subsequent characterization, both chemically and physicochemically, have been optimized in such a way that a wide range of SPs neoantigens can be encapsulated into cationic liposomes. The SP loaded cationic liposomes were able induce functional tumor-specific immune

response when used as a formulation strategy for multiple $CD8^+$ as well as $CD4^+$ T-cell neoepitopes *in vivo*. The TLR-2 ligand mini-UPam conjugated to two different SPs that contained human melanoma neoepitopes induced efficient activation of neoepitope specific $CD8^+$ or $CD4^+$ patient T-cells. The research described in this thesis has shown the potential of cationic liposomes and TLR ligand conjugates in neoepitope based peptide vaccines. This thesis offers a starting point for the further immunological evaluation of these molecularly defined cancer vaccines, which hold great potential for specific immunotherapy of cancer.

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