Liposome-based synthetic long peptide vaccines for cancer immunotherapy

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LIPOSOME-BASED SYNTHETIC LONG PEPTIDE VACCINES FOR CANCER IMMUNOTHERAPY

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"It's the time you have spent on your rose that makes your rose so important"

Antoine de Saint-Exupéry*, The Little Prince*

To my parents

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General Introduction

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General Introduction

Cancer Immunotherapy

Immunotherapy has gained interest as a promising strategy to treat cancer, a leading cause of death worldwide, since the role of the immune system in tumours is rapidly becoming more clear [1]. In contrast to conventional treatments including surgery, chemotherapy and radiotherapy, immunotherapy can specifically target cancer cells without significantly attacking healthy cells. The aim of immunotherapy against cancer is to use the patients' own immune system to specifically kill tumour cells [2,3] and many different approaches towards this direction are currently being developed.

It was in 1909 that Paul Ehrlich suggested the concept of immune surveillance, according to which our immune system is constantly fighting against eventually occurring tumour cells. About 50 years later Thomas and Burnt suggested the existence of the tumour associated antigens (TAA) [4]. TAA are proteins that are uniquely or differentially expressed by tumour cells [5,6]. Today, the existence of the TAA is not only generally accepted, but also underlies the basic principles of cancer immunotherapy: raising adequate immune reactions against cancer requires the induction of a cell-mediated immune response towards these antigens. Cell types that need to be induced for such a response include antigen presenting cells (APCs) from the innate immune system, (mainly) dendritic cells (DCs), and the participation of the adaptive immune system including $CD4^+$ T helper (T_H) and $CD8^+$ cytotoxic T cells (CTLs) [7,8]. $CD8^+$ CTLs have a crucial role in immune reactions against cancer and thus approaches for the development of immunotherapy have focused on inducing cellular $CD8^+$ T cell-mediated responses [9].

Immunotherapeutic approaches involve, among others, initiation and development of an immune response against TAA upon vaccination or adoptive transfer of TAA-pulsed DCs. Furthermore, administration of immunostimulatory cytokines can enhance the induced immunity and direct immune cells to the tumour site. During the last years, monoclonal antibody therapies towards target molecules, such as the checkpoint-blocking inhibitors CTLA-4 and PD-1 were also introduced [3,10,11], functioning as T cell activation enhancers that can be used in patients with infections or autoimmunity disorders where any beneficial T cell activation is in a suppressed state. Recently, the FDA approval of such regimens, including the ipilumumab and nivolumab as a standard treatment for melanoma and non-small-cell lung carcinoma (NSCLC), respectively, showed their potential and the importance of specific T cell activation. However,

although these immunotherapeutic approaches were initially expected to be more specific and leading to less side effects compared to conventional therapies, they can result in toxicixity effects against healthy tissues [3]. This treatment-related toxicity can vary from minor problems to severe conditions against vital organs, and nowadays it has become clear that modern molecularly well characterised vaccines are needed using defined antigens, adjuvants and delivery systems.

Therapeutic vaccination against cancer

So far, most attempts at therapeutic vaccination against tumours have been disappointing [7,12]. Possible explanations for the lack of clinical efficacy may be found in the poor delivery of antigens, the tumour microenvironment and its heterogeneity and also the kinetics and phenotype of the induced T cell responses [13]. Furthermore, the evasion of the immune response by tumour cells, for instance, through loss of MHC class I molecule expression by the tumour cells [14], can limit the efficacy of therapeutic vaccines. Also important in the induction of tolerance is the tumour microenvironment which contains tolerance-signalling tumour-associated macrophages and regulatory T cells (Treg) [14-17]. Nevertheless, a recent development based on T cell stimulation against tumour-specific antigens raised the hopes in the field [sipuleucel-T (APC8015) with the brand name Provenge] and is already approved for use in prostate cancer patients [15,18]. In this approach, autologous dendritic cells are loaded with prostate antigens aimed to induce T cell responses targeted at the patient's cancer. Other areas of intensive research for therapeutic vaccination include melanoma, lung cancers as well as cervical cancer [18-21].

HPV-induced tumours: infection and therapeutic vaccination

Genital infections with HPV are common and can normally be cleared within a year [20, 22]. However, chronic infections with high-risk human papillomaviruses (HPV), such as HPV16, can lead to the development of tumours and even cervical cancer [23]. Oncogenic viruses infect cells and interfere with their normal mechanisms to regulate cell division and, as a result, cells start proliferating and producing viral proteins. A recent success in reducing the mortality associated with HPV-induced cervical cancer has been the development of preventive vaccines for HPV infections [24]. Nevertheless, there is no evidence for efficacy of these vaccines against established genital lesions.

HPV's viral DNA is associated with proteins similar to histones, and wrapped in capsomeres consisting of structural proteins L1 and L2. The viral genome is divided in genes encoding early (E) and late (L) proteins, and contains a local control region (LCR).

The E region contains genes involved in replication of the virus, including genes for transformation (E5, E6 and E7) and immortalization (E6, E7). The E6 and E7 proteins can stimulate proliferation of the infected cells by suppressing the p53 and pRb function, whereas the genes of the L region encode the capsid proteins [25-27]. Since the early proteins mediate transformation of the cells, and can eventually lead to tumour development and cervical cancer, E6 and E7 are the most commonly targeted proteins in therapeutic vaccination against HPV-induced cervical cancer [28,29]. The late proteins, including L1, have mainly been used to develop prophylactic approaches.

Clearance of the virus and induction of an immune response upon therapeutic vaccination can occur through pathways involving innate and adaptive immunity. An innate immune response is active at the site of infection, while the adaptive immune response operates from secondary lymphoid tissues, comprising both cellular and humoral immune responses. Humoral immune responses are generally important in preventive vaccination, whereas cellular responses are the driving force in anti-tumour immunity and antiviral immunity [27,30]. At the site of an HPV infection or vaccination with HPV-derived antigens, such as E6 or E7 proteins, professional APCs can take up antigens and transport them to the secondary lymphoid tissues. Concerning therapeutic vaccination against cervical cancer, DCs are superior in presenting antigens when compared to macrophages, since some subsets are migratory and can present antigens at secondary lymphoid tissues [30].

In order to optimally deliver antigens to fight HPV-induced carcinomas, it is important to understand how DCs induce, maintain and regulate T cell immunity [31, 32]. Two major classes of DCs exist: classical DCs – including Langerhans cells – and plasmacytoid DCs, each having different functions. The eventual immune reaction initiated in response to the viral antigen greatly depends on maturation signals and as a result, different signals can induce different cellular responses [31]. Vaccines aimed at the treatment of HPV-induced carcinomas will require long-lived antigen presentation of antigenic protein fragments by appropriately activated DCs [7] and subsequent activation of naive T cells that will recognize the processed antigens in the context of their major histocompatibility complex (MHC).

MHC class I molecules are present on almost all cell types and DCs can use them to present antigens synthesized inside the cell – “direct presentation” – or antigens originating from outside the cell – “cross- presentation”. Upon antigen uptake and processing by the proteasome, the fragmented peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP).

In the ER, the peptide fragments are loaded onto MHC class I molecules and the MHC class I/peptide complex is transported to the cell membrane, where they are presented to naïve $CD8^+$ T cells. In the context of the appropriate co-stimulatory signals, $CD8^+$ T cells differentiate into CTLs. MHC class II molecules, located only on a few cell types, present extracellular antigens to naive $CD4^+$ T cells which subsequently differentiate into $CD4^+$ T helper (T_H) cells. T_H1 $CD4^+$ T cell responses appeared to be of major importance in anti-tumour immunity for cellular memory function and enhancement of the inflammation through cytokines secretion [33]. Cross-presentation, performed by highly specialised DCs, is considered to be of vital importance when designing vaccination approaches for cancer and infectious diseases, where the success of the intervention is dependent on activation of both $CD4^+$ and $CD8^+$ T cells.

In order to raise a therapeutic response upon vaccination, four components are essential: a) the quality of the activated CTLs, b) the quality of the elicited T_H1 response, c) the elimination of Treg cells and d) the breakdown of the immunosuppressive tumour microenvironment [9,15,31]. In addition, the duration of antigen presentation is an important parameter for the efficacy of a therapeutic cancer vaccine [34]. For therapeutic vaccination against cancer, including HPV-induced tumours, different vaccine modalities are under development, including recombinant live vector vaccines, nucleic acid vaccines, protein- and peptide-based vaccines, virus-like particle vaccines (VLP), whole cell vaccines and combination vaccines [7,35].

Peptide-based vaccines and the synthetic long-peptide concept

Peptide-based vaccines offer great promise for the treatment of cancer, and vaccination with minimal/short MHC-binding peptides has been so far the most popular approach [7]. These minimal peptides, deriving from TAA sequences, bind directly to MHC molecules, so their applicability is limited to cancer patients with the appropriate MHC haplotype. Therefore, efforts have been focused on peptides presented by the most common HLA alleles [1]. Some advantages of short peptides are the relatively easy production and formulation, the low production cost and the fact that they are generally well tolerated. Unfortunately, up until now short-peptide vaccinations have shown only limited successes in the induction of clinical responses [36]. This has been ascribed not only to their restriction to a specific MHC class I phenotype, but also their presentation by non-professional APCs, such as B cells [34]. Another possible explanation can be found in the rapid degradation of short peptides since they lack a tertiary structure, which renders them an optimal target for peptidases [36]. Furthermore, the poor immunogenicity of such antigens requires their administration together with

immunomodulatory agents, such as TLR agonists, cytokines and other co-stimulatory molecules, while it was also found that short peptides could only transiently induce CTL activity, possibly limiting their usefulness in cancer vaccination [34,37]. In addition, it has been reported that the length of short peptides determines the magnitude of the immune response, and minimal peptides elongated with a $CD4^+$ helper epitope can be more potent [7]. Hence, elongation of minimal peptides could strengthen the anti-tumour response.

Using vaccines based on full proteins can circumvent the problems arising from the use of short peptides, since they contain a wide range of $CD4^+$ and $CD8^+$ epitopes. Additionally, they are well-characterized molecules with a good safety profile in general [38]. Yet, processed protein antigens are also poorly immunogenic and it has been suggested that they bias the antigen-specific immune response towards humoral immunity, rather than T cell immunity, thereby possibly also limiting their applicability for therapeutic vaccination against cancer [38,39].

Synthetic long peptide (SLP) vaccines have originated as a solution to the problems posed by short peptide-based and protein-based vaccines. SLPs, in contrast to short peptides, are unable to bind directly to MHC class I molecules, but have to be taken up and processed by the APCs [7]. Furthermore, whereas short peptides can be presented by both professional and non-professional APCs, SLPs are dependent on cross-presentation and will be mainly presented by DCs in the draining lymph nodes [34]. In addition, the superiority of SLPs over short peptides is for a large part based on the increased duration of antigen presentation by DCs [34]. In contrast to full-protein and short peptide based vaccines, SLP are known to induce $CD8^+$ T cell responses to the injected antigens, while administration of a T helper-peptide can enhance the induced immune response [34,37,40].

So far, great progress has been made with SLPs derived from HPV16 for therapeutic vaccination against cervical cancer. In trials, clinical responses have been observed in women with HPV16-positive grade 3 vulvar intraepithelial neoplasia after vaccination with a SLP-based vaccine against E6 and E7 oncoproteins in Montanide oil-in-water emulsion, which is a clinically approved incomplete Freund's adjuvant (IFA) variant [21]. In fact, 83% of all subjects developed a T cell response to the vaccine, and 5 out of 20 subjects showed a complete clinical response, that is, a complete regression of lesions. Based on the immune responses induced by SLPs and the amount of clinical data in pre-malignant conditions already present, SLP-based vaccination is considered to be a very

promising approach that could, however, greatly benefit from proper immunostimulatory adjuvants and delivery methods.

Immunostimulators and delivery systems

Adjuvants can be broadly divided into immunostimulatory adjuvants and delivery systems [41]. An important function mediated by immunostimulatory adjuvants is inducing the maturation of APCs through stimulation of innate receptors, in order to induce a cellular immune response and prevent tolerance. Ideally, an adjuvant should be safe, stable, biodegradable, cheap and able to induce the desired cellular immune response [42]. Currently, only very few adjuvants are available for use in humans and they are mainly used in a prophylactic setting (induction of humoral immunity) and are therefore considered less suitable for therapeutic vaccination against (HPV-induced) cancer. These adjuvants include aluminium salts, AS04, influenza virosomes, VLPs and the oil-in-water emulsions MF59, AS03 and AF03 [43-45,46].

Toll-like receptor (TLR) ligands

A very potent category of immunostimulatory compounds for therapeutic vaccine formulations is the Toll-like receptor (TLR) ligands, which are able to optimally co-stimulate and mature DCs [9]. TLRs as well as Nod-like (NLR) and RIG-I-like receptors (RLR) can recognize pathogen-associated molecular patterns (PAMP) and lead to the activation of APCs. Up to date, ten human and 15 mammal TLRs are known [47]. Most of them are located at the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are located in the endosomal compartment. TLRs can induce distinct responses in different DCs and other APCs [47], and in general they are involved in all major phases of the immune response, starting with the expression of pro-inflammatory cytokines and chemokines, directing DCs migration towards the lymph nodes and, finally, affecting adaptive immunity [48]. Furthermore, TLRs represent an attractive target for improving immunotherapy by inducing cytokine release such as IL-12, triggering a T_H1-type immune reaction [49,50]. TLR activation results in phenotypic and functional DCs' maturation, but the effects upon targeting the same TLR in different DCs subsets can be distinct. Additionally, inducing the desired maturation by targeting specific TLRs *in vivo* remains challenging, since TLRs are also expressed on non-APCs [51, 52].

TLR2 is expressed on immune cells and some TLR2-positive tumours and, after heterodimerization with TLR1 or TLR6, TLR2 recognises peptidoglycan, lipoprotein and zymosan [51]. TLR3 is expressed by classical human DCs [47,53] and can be stimulated by *poly(I:C)*, which represents a mix of single-stranded (ss-) and double-stranded

(ds)RNA (ranging from 1.5-8 kb) [54], resulting in IFN-gamma production. Importantly, TLR3 is known as an enhancer for cross-priming CTLs [55], but it was reported to be rapidly degraded and to have a high toxicity in clinical trials [56]. TLR4 ligands are currently the most developed adjuvants, since TLR4 can utilize both the MyD88 and TRIF signalling pathways. Most bacterial endotoxins, including monophosphoryl lipid A (MPL), act through TLR4 and DCs stimulated with endotoxin secrete IL-12, which is important in anti-tumour immunity [57]. Currently there are no TLR7/8 agonists approved for use as adjuvants, but they are available as stand-alone entities: imiquimod and resiquimod. Imiquimod is used for the topical treatment of basal cell carcinoma and both of them are able to induce a CTL response [58, 59]. TLR9 agonists are available in the form of CpG motif-containing oligodeoxynucleotides (ODN), which mimic bacterial DNA in their immunostimulatory activity [60]. The effects of CpG ODN may be of great use for cancer immunotherapy [60] although their need to enter the endosome in order to stimulate their cognate receptor supports the requirement of a delivery system for the induction of a strong response [61].

Emulsion-based adjuvants

Classical water-in-oil emulsion adjuvants, such as IFA, have been used in therapeutic peptide-based vaccines [20,43,44]. The CimaVax EGF vaccine against non-small-cell lung cancer that is currently licenced in Latin America is also adjuvanted with IFA [43,44,62]. In addition to water-in-oil emulsions, oil-in-water emulsions can be used as adjuvants: MF59, AF03 and AS03 are approved for human use. MF59, however, is used in the seasonal influenza vaccine *Fluad*™, and it primarily induces a humoral immune response [63,64]. Emulsion adjuvants may cause a depot effect and can thereby prolong antigen presentation; however, their main drawback lies with clinical safety: IFA and its clinical form of Montanide are known to cause severe local skin reactions, sterile abscesses and persistent painful granulomas at the injection site. Despite the activation of CTLs achieved by some formulations based on these adjuvants, there is an urgent need to develop safer delivery systems for antigens [61].

Polymeric nanoparticles

Polymer-based particles are generally made from polylactide-co-glycolide (PLGA) or polylactide (PLA). These delivery systems do not only provide antigen delivery, but they also serve as an immunopotentiating adjuvant, promoting humoral as well as cellular immune responses [65]. Furthermore, they rescue DCs from tumour-induced immunosuppression by causing DC activation and induction of a new pro-inflammatory environment [41]. PLGA microspheres have been found to target DCs and macrophages

[61] and it is believed that particles in the nanometre range will mostly induce T_H1 responses, whereas micrometre-sized particles will most likely induce humoral responses. This allows for precise modulation of the immune response by altering the particle size [65, 66]. A major advantage of PLGA-based particles is that PLGA is already used in products approved for use in humans (for surgical structures and controlled-release drugs) and they can be used to accommodate peptides, proteins, RNA or DNA-based vaccines [41,61]. Additionally, OVA-loaded PLGA microspheres have been shown to be able to induce a CTL response but not a Treg response, showing the possible applicability of these particles for therapeutic vaccination against cancer [61]. Finally, physicochemical properties of PLGA-based particles can easily be manipulated to alter the degradation time, which is an important parameter for providing a depot effect [41].

Liposomes

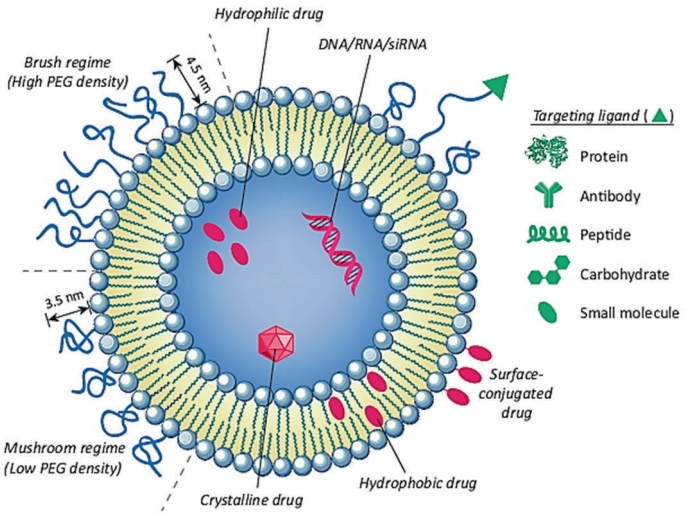
Liposomes are small vesicles consisting of one or more lipid bilayer [67,68]. They can be as small as 100 nm, but can also be engineered to become several micrometres in diameter [69]. Liposomes have been traditionally used for the delivery of anti-cancer drugs like doxorubicin or anthracycline [70,71], but they can also be used for the delivery of antigens to APCs [44,72]. Liposomes can accommodate hydrophilic compounds, such as nucleic acids, in the aqueous compartment as well as lipophilic compounds incorporated in the liposomal membrane [71]. They are known to be able to protect the loaded drugs/antigens against enzymatic degradation and thereby can improve the compounds' stability and bioavailability. Advantages also include the excellent biocompatibility, biodegradability, and capacity for size and surface manipulations [71]. From an immunological point of view, they can be very useful since they can be taken up by DCs therefore enhancing cross-presentation [67].

Liposomes can have a positive, negative or no net charge on the outer membrane by the incorporation of anionic, cationic or zwitterionic lipids. The charge can also determine the affinity for specific APCs and, while scavenger receptors present on macrophages and Kupffer cells have a high affinity for anionic particles [73], anionic liposomes barely interact with human DCs [74]. The high affinity of cationic liposomes can be attributed to the electrostatic interactions between lipids and the anionic cell surface of DCs, leading to advantages such as the formation of an antigen depot and the improved activation of DCs [75].

Apart from lipid composition and charge, also other properties of liposomes can be manipulated. The size of liposomes, that is partly determined by its composition and dehydration method, but also controlled by sizing methods, can greatly affect the

preferential target and elicited immune response; Larger particles (>300 nm) are more readily taken up by macrophages, whereas smaller particles (<300 nm) are preferentially processed by DCs [65,76,77]. The fluidity of the lipid membrane, mainly dependent on the lipid composition, has been correlated with the induced immune response as well: in principle, an ordered membrane organisation strongly contains the encapsulated molecules, while a more liquid organisation can easier allow content leakage [78]. Finally, the tuneable properties of liposomes include the incorporation and attachment of molecules or antibodies for targeting of specific tissues or cell types [79]; Poly(ethylene glycol) (PEG) significantly enhances the circulation time of liposomes by evading accumulation in the liver resulting in "stealth liposomes", which can be useful to increase the exposure of tumours to cytostatic drugs [79]. In addition, the latest developments have been the creation of liposomes that are responsive to external or environmental stimuli, including pH, enzymes and temperature [71].

So far, a number of FDA approved liposomal formulations are available on the market, such as the Doxil (Johnson&Johnson) and AmBisome (Gilead Sciences), while several other are currently being tested in clinical trials; some examples are the AS01a (GSK) for malaria, the Vaxisome (NasVax) and JVRS-100 (Juvaris BioTherapeutics) against influenza, the Stimuvax (Merck) for lung cancer treatment and the CAF01 (Statens Serum Institut) for tuberculosis [80,81] and human immunodeficiency virus (HIV) vaccines [82,83].



Picture 1: Structural and design considerations for liposomal compounds delivery. Melis Çağdaş 2014.

Additionally, as it is showed in this thesis, liposomal nanoparticles can be used for safe and efficient delivery of tumour-associated peptide vaccines when combined with defined immunostimulators, resulting potentially in a successful immunotherapy for cancer.

Aim and outline of this thesis

The main objectives of the research presented in this thesis were:

- Design of an SLP vaccine formulation concept for cancer immunotherapy based on particulate delivery systems
- Investigation of cationic liposomes as a flexible platform for defined therapeutic vaccines
- Improvement of the immunogenicity of synthetic long peptide-based vaccines
- Replacement of the Montanide ISA-51 emulsion by safe, biodegradable nanoparticles for clinical use

The research presented in *chapter 2* focuses on the investigation of a cationic, DOTAP-based liposomal formulation loaded with a model SLP harbouring the CTL epitope of ovalbumin (OVA) and adjuvanted with poly(I:C) – a TLR-3 ligand agonist. The major aim of this study was the feasibility of such a system: whether it is possible to generate cationic liposomes loaded with the model SLP in a reproducible way. The successfully formulated liposomes were physicochemically characterised and they were assessed *in vitro* and *in vivo* for their potency to induce a functional $CD8^+$ T cell cytotoxic immune response that is required for cancer immunotherapy.

Chapter 3 shows the improvement of the efficacy of the successfully developed liposomal formulation by incorporating a second SLP covering a T_H epitope of OVA. The latter was introduced in the CTL epitope-containing SLP-loaded formulation co-encapsulated in the same particle or loaded in separate liposomes, in order to analyse for the formulations potential and whether co-delivery is needed. The efficacy of the liposomes (either combined or not with TLR ligands) on the induction of antigen-specific T cells was investigated. Finally, the most potent SLP liposome formulations were tested *in vivo* as a therapeutic vaccine in an aggressive melanoma tumour model.

Chapter 4 provides a comparative study of cationic liposomes and PLGA nanoparticles, regarding their effectiveness as SLP-based vaccine candidates for the induction of cell--mediated immunity. For this reason, PLGA NPs and liposomes loaded with the model SLPs were adjuvanted with different TLR ligands and directly compared *in vitro* and *in vivo* with clinically used formulations (Montanide ISA-51 and a squalene-based oil-in-water emulsion) regarding their efficiency in inducing antigen-specific and functional T cells after subcutaneous immunisation in mice.

The research described in *chapter 5* provides the proof of concept that cationic DOPC:DOTAP liposomal formulation loaded with an SLP of the oncoprotein E7 of human papillomavirus HPV type 16 can be an efficient therapeutic vaccine. The goal of this study was to attain a better insight in whether this formulation can be considered as an alternative to Montanide ISA-51 for the treatment of HPV-induced lesions. The induced E7-specific T cell immune responses were characterised and the liposomes' capacity as a therapeutic vaccine was assessed *in vivo*, in a direct comparison with the curently used Montanide ISA-51 SLP vaccine in a tumour-bearing mouse model.

In *chapter 6* the full potential of the developed DOPC:DOTAP cationic liposomal formulation is tested for its use as a flexible platform for the delivery of multiple SLP antigens. The standardized validated protocol for the preparation of the cationic liposomes is applied for the loading of 11 SLPs in separate particles. All the different SLPs harbour the SIINFEKL CTL model epitope, but cover different physicochemical properties, such as amino acid sequence, charge and hydropathicity. The immunogenicity of the produced liposomes was analysed in an *in vitro* read out that assesses the efficiency of the formulations to activate DCs and subsequently present the processed SLP to SIINFEKL-specific T cells.

Finally, in *chapter 7*, the results and conclusions of this thesis are summarised and discussed. Moreover, prospects of SLP-based vaccination with particulate delivery systems for cancer immunotherapy are described and suggestions are given for further research.

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Chapter 2

Cationic liposomes loaded with a synthetic long peptide and poly(I:C): a defined adjuvanted vaccine for induction of antigen specific T cell cytotoxicity

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Abstract

For effective cancer immunotherapy by vaccination, co-delivery of tumour antigens and adjuvants to dendritic cells and subsequent activation of antigen-specific cytotoxic T cells (CTLs) is crucial. In this study, a synthetic long peptide (SLP) harbouring the model CTL epitope SIINFEKL was encapsulated with the TLR3 ligand poly(I:C) in cationic liposomes consisting of DOTAP and DOPC. The obtained particles were down-sized to around 140 nm, (measured by dynamic light scattering) and had a positive zeta-potential of approximately 26 mV (according to laser Doppler Electrophoresis). SLP loading efficiency was around 40% as determined by HPLC. Poly(I:C) loading efficiency was about 50%, as assessed from the fluorescence intensity of fluorescently labelled poly(I:C). Immunogenicity of the liposomal SLP vaccine was evaluated *in vitro* by its capacity to activate DCs and present the processed SLP to SIINFEKL-specific T cells. The effectiveness of the vaccine to activate $CD8^+$ T cells was analysed *in vivo* after intradermal and subcutaneous immunisation in mice, by measuring antigen-specific T cells in blood and spleens, and assessing their functionality by cytokine production and *in vivo* cytotoxicity. The liposomal formulation efficiently delivered the SLP to DCs *in vitro* and induced a functional $CD8^+$ T cell immune response *in vivo* to the CTL epitope present in the SLP. The SLP-specific $CD8^+$ T cells frequency induced by the poly(I:C)-adjuvanted liposomal SLP formulation showed an at least 25-fold increase over the T cell frequency induced by the poly(I:C)-adjuvanted soluble SLP. In conclusion, cationic liposomes loaded with SLP and poly(I:C) have potential as a powerful therapeutic cancer vaccine formulation.

Keywords: Peptide antigen, Cationic liposomes, Cancer immunotherapy, CTL epitope, Poly(I:C)

Introduction

Therapeutic vaccination has recently gained interest as a potential strategy to treat cancer and chronic infectious diseases, but it is not yet established as a standard treatment modality [1]. It is obvious that treatment of an ongoing disease by vaccination to (re)activate the immune system is more demanding as compared to classical prophylactic vaccines and requires optimal formulation of the specific vaccines. Therefore, in contrast to preventive vaccination, for which the induction of neutralising antibodies can be adequate in most cases, therapeutic vaccination relies significantly on the induction of cell-mediated immunity. Cell types essential for a cellular response include $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper (T_H) cells, and both cell types' activation requires antigen presentation by specialised antigen-presenting cells (APCs) [1,2]. Of these, dendritic cells (DCs) located in the lymphoid tissues (such as $CD8\alpha^+$ DCs subset) or in the mucosa ($CD103^+$) are the most efficient APCs, highly specialised at taking up exogenous antigens and presenting them to $CD8^+$ T cells, as processed peptides in the groove of MHC class I molecules [3,4]. Optimal vaccine formulations should therefore be targeted to DCs to be processed and presented to antigen-specific T cells. As $CD8^+$ CTLs possess a central role in the response to viral infections and cancer, research strategies for the development of therapeutic vaccines are focusing on ways to induce a strong CTL response [5-8].

Therapeutic vaccination with overlapping synthetic long peptides (SLPs), including all potential MHC class I and II restricted epitopes of a tumour associated antigen (TAA), has been proven superior to vaccination with protein antigen [9] or minimal synthetic peptide epitopes [10] for the induction of a long-term effector $CD8^+$ T cell immune response in mouse models. Impressive clinical responses have been observed also in women with HPV16 positive grade 3 vulvar intraepithelial neoplasia (VIN3 pre-malignant lesions) after therapeutic vaccination with a SLP based vaccine against E6 and E7 oncoproteins in Montanide, an incomplete Freund's adjuvant (IFA)-like mineral oil emulsion [11]. However, end stage $HPV16^+$ cervical cancer patients did not benefit from this type of vaccine, despite induction of significant levels of HPV-specific T cells [11]. These studies showed that SLP vaccines have functional potency when applied to pre-malignant stage patients, but need to be further improved for use as a therapeutic vaccination against cancer. So far, SLPs have been administered in Montanide ISA-51 as the most common vehicle used for peptide vaccinations in clinical trials; however, the use of such water-in-oil formulations have some important draw-backs, such as their non-biodegradability, low long-term stability and induction of local side effects [12]. Therefore, there is an urgent need for replacement of Montanide emulsion-based formulations by more potent ones that, in addition, do not provoke serious side effects.

In this work we propose cationic liposomes, as a suitable alternative to Montanide, as adjuvant for SLP antigens. Liposomes have traditionally been used for the delivery of anti-cancer drugs like doxorubicin [13], but especially cationic liposomes can also efficiently target APCs, resulting in improvement of the induced humoral and cellular immune response, in particular when formulated with other adjuvants, such as Toll-like receptor (TLR) ligands [14-16]. Advantages of liposomes as vaccine delivery system include their excellent biocompatibility, their superior to Montanide biodegradability and capacity for size and surface manipulations [17,18]. The adjuvanticity of liposomes highly depends on their physicochemical properties such as lipid composition, size and surface charge [19]. Size is a key factor for the adjuvanticity of liposomes. Particles with a size below 300 nm are efficiently taken up DCs, whereas larger particles will most probably be taken up preferentially by macrophages, before they are scavenged by DCs [20-22].

In this study, we investigated the potential of a poly (inosinic-polycytidylic acid) (poly (I: C), a TLR3 ligand)-adjuvanted cationic liposomal formulation, as a delivery system for SLPs for cancer immunotherapy, upon vaccination. As a model peptide, a 24-amino acid long SLP (referred to as OVA24) was used, covering the immunodominant SIINFEKL CTL epitope of ovalbumin (OVA). OVA24-loaded liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), with or without poly(I:C), were characterised for particle size, zeta-potential and for peptide and TLR loading efficiencies. The obtained liposomal formulations were assessed *in vitro* and *in vivo* for their potency to induce a functional $CD8^+$ T cell immune response. The observed T cell immune responses induced by our cationic adjuvanted SLP-loaded liposomal formulation were clearly superior to the one observed by the soluble SLP mixed with poly (I: C).

Materials and methods

Materials

The ovalbumin (OVA) derived 24-mer SLP OVA24 [DEVSGLEQLESIINFEKLAAAAAK], including the immunodominant cytotoxic T-lymphocyte (CTL) epitope [SIINFEKL], and the CTL $OVA_{257-264}$ SIINFEKL (OVA8), were produced and purified at the GMP facility of the Clinical Pharmacy and Toxicology Department of Leiden University Medical Center [23]. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and poly(I:C) and its rhodamine-labelled analogue were obtained from InvivoGen (Toulouse, France). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoehanol (Sigma-Aldrich, Zwijndrecht, Netherlands) and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionised water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ water). Phosphate buffer, was composed of 7.7 mM $Na_2HPO_4 \cdot 2\ H_2O$ and 2.3 mM $NaH_2PO_4 \cdot 2\ H_2O$, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, which was used for all the *in vitro* and *in vivo* assays (PBS: 140 mM NaCl, 8.7 mM $Na_2HPO_4 \cdot 12\ H_2O$, 1.8 mM $NaH_2PO_4 \cdot 2\ H_2O$, pH 7.4), was purchased from B. Braun (Meslungen, Germany).

Mice

Female C57BL/6 (H-2^b) mice were purchased from Charles River (L'Arbresle, France) and congenic CD45.1 (Ly5.1) mice were bred at the Leiden University Medical Center animal facility and used at 8-14 week of age according to the Dutch Experiments on Animal Act, which serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe.

Liposome preparation

Cationic liposomes loaded with OVA24 were prepared by using the thin film dehydration-rehydration method [24], followed by extrusion. In detail, DOTAP and DOPC in chloroform, in a 1:1 molar ratio were mixed in a round-bottomed flask to reach

a concentration of 10 mg lipid per ml of final liposome dispersion. A lipid film was formed by chloroform evaporation in a rotary evaporator for 1 hour at 37°C. The film was then rehydrated either with filtered MQ water (2 ml, pH 5.5 – 5.8) for non-loaded (empty) liposomes, or with 2 ml of a solution of 1 mg/ml OVA24 in ACN/H_2O 1:1 (v/v) (OVA24-loaded liposomes); the dispersion was mixed briefly in the presence of glass beads and equilibrated for 1 h at room temperature. For poly(I:C) loaded liposomes, the ligand including 0.5% fluorescently-labelled equivalent was added dropwise to the rehydrated lipid film until a concentration of 200 µg poly(I:C) per ml of liposome dispersion was reached. Next, the liposome dispersion was snap-frozen in liquid nitrogen, followed by freeze-drying in a Christ alpha 1-2 freeze-dryer (Osterode, Germany) overnight. Dehydration-rehydration liposomes were generated by gradually hydrating the freeze-dried lipid cake in 10 mM PB, pH 7.4. To prepare 2 ml liposome dispersion, two volumes of 400 µl and one volume of 1200 µl PB were successively added, with intervals of 20 minutes between each addition. The mixture was vortexed well during the rehydration steps and the resulting dispersion was kept at room temperature for at least 1 h.

Sizing of the obtained multilamellar vesicles was performed by high-pressure extrusion at room temperature, by using a Lipex extruder (Northern Lipids Inc., Canada). To obtain monodisperse liposomes, the liposome mixture was passed four times through a 400-nm pore size membrane and four times through a 200-nm pore size membrane (polycarbonate Track Etch membranes, Nucleopore Millipore, Kent, UK).

Purification (removal of free OVA24 peptide) and concentration of OVA24-loaded liposomes was performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa): 1300 µl of the liposome dispersion were loaded in the concentrator, which was then centrifuged at 2000 rpm and 20°C for 6-7 hours, till the suspension was concentrated fivefold. The filtrate, containing the free OVA24, was collected and the concentrated liposome dispersion was diluted by adding 1000 µl PB, followed again by a fivefold concentration-wash to remove most of the free soluble peptide. Samples of the liposome fraction and the free fractions were taken after each step for OVA24 analysis (see below). The concentrated-purified liposomes were physicochemically stable for at least six weeks in the refrigerator, as assessed by dynamic light scattering (DLS) and zeta-potential measurements.

Liposome characterisation

Dynamic light scattering and zeta-potential

Average diameter (Z_{ave}) and polydispersity index (PDI) of the liposomes were determined by dynamic DLS using a Zetasizer (NanoZS, Malvern Ltd. UK). The zeta-potential was determined by laser Doppler electrophoresis using the same device. For these measurements liposome samples were diluted 100-fold in PB.

Peptide and poly(I:C) loading efficiency

Peptide loading efficiency was determined by extracting OVA24 from the loaded liposomes and measuring the extracted fraction by reversed-phase HPLC. For that purpose, a modified Bligh-Dyer method was used: 250 µl of methanol (including 62.5 µg/ml of insulin as internal standard) and 125 µl of chloroform were added to 100 µl of aqueous liposomal dispersion. The mixture was vortexed well, followed by addition of 250 µl of 0.1 M HCl and another 125 µl of chloroform. After vortexing and centrifuging for 5 min at 1000 x g, the upper water-methanol phase containing the peptide OVA24 (as well as insulin) was carefully collected and analysed by HPLC (see below). In addition to the extracted samples from liposomes, the PB filtrates from the purification/concentration of the liposomes (before wash and after wash) containing the free OVA24 were also analysed by HPLC. The loading efficiency was expressed as a percentage (%LE) of the initial peptide amount that was loaded into the VivaSpin 2 centrifugation concentrator.

For the OVA24 peptide quantification the HPLC method described before [25] was used. In detail, 50 µl of the water-methanol extract (see above) was injected onto a reversed-phase HPLC column C18-AQ 3 µm (150x 4 mm) (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) by using an HPLC system from Waters (Saint-QuentinEn Yvelines Cedex, France). The mobile phases 5% ACN in MQ water with 0.1% TFA (Solvent A) and 95% ACN in MQ water with 0.1% TFA (Solvent B) were applied in a linear gradient from 0-78 % solvent B over 20 min at a flow rate of 1 ml/min. Detection of the peptide was achieved by measuring the absorbance at 220 nm through the ultraviolet system detector (Waters 486) and the peptide concentration for each sample was determined based on a calibration curve with known OVA24 concentrations (0.5 – 500 µg/ml).

The amount of poly(I:C) in the liposomes was determined by measuring the fluorescence [excitation (ex.) 546 and emission (em.) 576 nm] of its rhodamine labelled analogue in collected non-solubilised (i.e., intact) liposomal samples and the PB filtrates from the

purification/concentration process (before wash and after wash) containing the free poly(I:C) (assuming that the 0.5 % of the used labelled compound behaved the same as the non-labelled equivalent). A calibration curve of the fluorescently labelled poly(I:C) was made, ranging from 7.81 ng/ml to 1000 ng/ml and all samples were measured using a fluorescence micro plate reader (Tecan, Salzburg, Austria).

DC maturation evaluated by IL-12 ELISA and flow-cytometric analysis of CD80 DC marker

Immature D1 cells ($5x10^4$ cells/well) (i.e., a long-term growth factor-dependent dendritic cell line derived from spleens of C57BL/6 mice that was cultured as described before [26]) were incubated in 96-well round-bottomed plates at 37°C in supplemented IMDM with OVA24-loaded liposomes or free OVA2 at different concentrations. After overnight incubation at 37°C the plates were centrifuged and the supernatant was collected for IL-12p40 measurement by ELISA (BD Biosciences) according to the manufacturer's protocol. In addition, the cells were stained in staining buffer (PBS + 0.1% (w/v) bovine serum albumin (BSA) + 0.05% (w/v) sodium azide) for 30 min. with fluorescently labelled FITC antibody specific for the mouse CD80 DC surface marker (eBiosciences). 7-Aminoactinomycin D (7AAD) (Life Technologies) was used for the exclusion of dead cells and data acquisition of samples was done with a BD LSR II flow cytometer. The data were analysed by using the FlowJo software (Tree Star).

In vitro MHC class I antigen presentation

The immunogenicity of the OVA24 formulations was initially tested in an *in vitro* readout, with regard to their efficiency to activate immature DCs and present SIINFEKL to $CD8^+$ antigen specific T cells, leading to their activation. Immature D1 cells were incubated in 96-well flat-bottomed plates at 37°C in supplemented IMDM with OVA24-loaded liposome formulation or plain OVA24 in PBS solution at different concentrations. The stability of the liposomes did not change when diluted in PBS, as assessed by DLS analysis (unpublished data). After 2.5 hours the plates were washed three times with supplemented IMDM culture medium, in order to remove excess antigen. Subsequently, T cell hybridoma B3Z cells ($50x10^5$/well) were added followed by overnight incubation at 37°C [27]. Chlorophenol red-β-galactopyranoside (CPRG) was used as lacZ substrate in cell lysates and the colour conversion was measured by detecting absorbance at 590 nm.

Immunisation of mice

Mice were immunised with OVA24-loaded liposomes or free OVA24 (with or without poly(I:C)) by intradermal injection in the abdominal skin area [28]. All formulations were prepared on the day of injection. Vaccination dose was 5 nmol (12.5 μg) of peptide in a total volume of 30 μl, and immunisations were performed on day 0 (prime immunisation) and repeated after two weeks on day 14 (boost injection). Vaccinations with adjuvanted liposomes included 4 μg of poly(I:C)/mouse. Besides intradermal injection, which was chosen based on preliminary *in vivo* experiments, subcutaneous immunisation (in the flank) of the formulations in a total volume of 200 μl PBS, served as a control (data not shown in all experiments). During the *in vivo* studies, blood samples were obtained from the tail vein at different time points. After the mice were sacrificed on day 28/29, spleens were removed and processed for splenocytes' isolation.

Analysis of antigen-specific $CD8^+$ T cell responses by flow cytometry

Staining of the cell surface was performed on freshly isolated PBMCs after red blood lysis. Cells were stained in staining buffer for 30 min with allophycocyanin (APC)-labelled tetramer-$OVA_{257-264}$ (TM-SIINFEKL) and fluorescently labelled antibodies specific for mouse CD3 (BD Biosciences), CD4, CD8 and the killer cell-lectin-like receptor G1 (KLRG1) (eBiosciences). 7-Aminoactinomycin D (7AAD) (Life Technologies) was used for the exclusion of dead cells.

Overnight intracellular cytokine analysis of PBMCs was performed after incubating the cells with 2 μM of minimal CTL $OVA_{257-264}$ SIINFEKL (OVA8) in presence of brefeldin A (7.5 μg/ml) (BD Biosciences, Breda, the Netherlands). The next day after cells' surface staining with fluorescently labelled antibodies to mouse CD3 and CD8, cells were fixed with Cytofix/Cytoperm solution (BD Biosciences, Breda, the Netherlands) and permeabilised with Perm/Wash solution. Subsequently, cells were stained with fluorescently labelled antibodies against IFN-γ and TNF-α at 4°C for 30 min. Finally, data acquisition of samples was done with a BD LSR II flow cytometer and data analysed by using the FlowJo software (Tree Star).

In vivo cytotoxicity assay

Splenocytes from naive congenic $CD45.1^+$ mice were counted and split into two equal parts, after lysis of erythrocytes. Cells were labelled with CFSE for 1 h at 37°C, with either 5 μM (target population) or 0.5 μM (control) final concentration. The CFSE staining reaction was blocked with 10% (v/v) foetal calf serum and the cells of the target

population were pulsed for 1 h with OVA8 in complete culture medium at 37°C. Next, the cells were washed four times with PBS before the two fractions (target population-control) were mixed in a 1:1 number ratio and adoptively transferred intravenously in recipient previously immunised C57BL/6 mice in a volume of 200 µl in PBS. Two days after the adoptive transfer of the target cells (day 24 of vaccination schedule), mice were sacrificed, spleens were isolated and single cell suspensions were analysed by flow cytometry for specific killing following the equation below:

$$\mathrm{SK} = \left\{ 1 - \frac{\left[\frac{\text{CFSE high}}{\text{CFSE low}}\ \text{vaccinated mice}\right]}{\left[\frac{\text{CFSE high}}{\text{CFSE low}}\ \text{naive mice}\right]} \right\} \text{x } 100\ \%$$

Statistical analysis

The significance of differences in the *in vitro* and *in vivo* assays testing the properties of the induced $CD8^+$ T cells was evaluated by GraphPad Prism 5 (GraphPad) software, using an analysis of variance (ANOVA) at a 0.05 significance level, followed by Tukey's and non-parametric Mann Whitney post-test.

Results

Peptide and adjuvant successfully loaded in cationic liposomes

Following the film dehydration-rehydration method cationic DOTAP-based liposomal formulation were formed [29] loaded with the ovalbumin-derived SLP (OVA24), containing the well-defined CTL epitope SIINFEKL. The obtained OVA24- liposomes had an average size of about 140 nm, a low PDI (<0.2) and a positive zeta-potential. Measurement of the extracted SLP from concentrated liposomes revealed a final peptide loading efficiency of about 40% (table I).

Table I: Physicochemical properties of liposomal formulations. Data are averages ± SD of at least 5 independent batches.

	Z_{ave} diameter (nm)	PDI	ZP (mV)	OVA24 LE (%)	Poly(I:C) LE (%)
Empty liposomes	142 ± 5	0.15 ± 0.02	34 ± 4	NA	NA
OVA24 liposomes	130 ± 5*	0.12 ± 0.02	25 ± 3 *	40 ± 8	NA
OVA24/poly(I:C) liposomes	148 ± 8	0.16 ± 0.05	28 ± 3	38 ± 5	50 ± 4

Z_{ave}: average particle diameter in nm, PDI: polydispersity index, ZP: zeta-potential, LE: loading efficiency, NA: not applicable. Size and ZP significantly different from empty liposomes (evaluated by 1-way ANOVA w/Tukey's) are indicated by * $p < 0.05$

The co-encapsulation of poly(I:C) did not substantially influence the liposome characteristics. The zeta-potential of the adjuvanted liposomes was comparable to that of the non-adjuvanted ones, while the average size was only slightly larger (ca. 150 nm; PDI <0.2). This could be expected, because the initial molar ratio of negatively charged poly(I:C) subunits (MW = 335.5 g/mol) and DOTAP (MW =698.5 g/mol) was only about 0.06 and most of the poly(I:C) was likely localised inside the liposomes. Furthermore, the loading efficiency of OVA24 was very similar in the poly(I:C)-loaded liposomes and that of poly(I:C) was about 50% (table I), yielding a final formulation containing about 500 µg/ml OVA24 peptide and 100 µg/ml poly(I:C). Our data show that both poly(I:C)-adjuvanted and non-adjuvanted OVA24-liposomes can be prepared in a reproducible fashion, despite potential competition between these two negatively charged compounds regarding their association with the cationic liposomes.

In vitro antigen cross-presentation

The efficiency of the OVA24-loaded liposomes to activate DCs and induce the uptake and processing of the peptide for MHC class I cross-presentation and subsequent activation of $CD8^+$ antigen-specific T cells, was assessed in *in vitro* assays. DCs were incubated with the liposomal formulations and the controls, followed by washing steps to remove the excess of the formulations. Subsequently, DCs were co-cultured with the OVA-specific B3Z hybridoma to sensitively detect MHC class I presented SIINFEKL epitope. B3Z is a $CD8^+$ T cell line specific for the H-2 K^b-restricted SIINFEKL CTL epitope of ovalbumin; it contains the lacZ reporter gene under the regulation of NF-AT element of the IL-2 promotor [27]. Therefore, after ligation of the T cell receptor (TCR), the β-galactosidase protein is expressed, driven by the IL-2 promotor, allowing quantitative measurement of T cell activation by this reporter enzyme in a colorimetric assay.

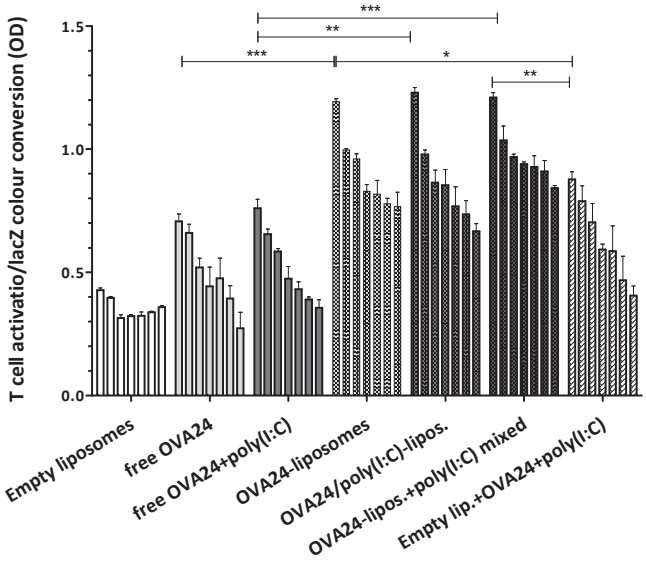

Figure 1: Activation of SIINFEKL-specific B3Z CD8+ T cells after culturing overnight with DCs pulsed for 2.5 h with titrated amounts (4.0 – 0.063 μM) of OVA24 formulations (empty liposomes were diluted to lipid concentrations corresponding to those of OVA24- liposomes). Graphs depict T cell activation based on the OD 590 nm value measured upon colour conversion of cell lysates after addition of CPRG (lacZ substrate). Data analysed with 1-way ANOVA w/Tukey's multiple comparison test, and *p* value calculated with non-parametric Mann Whitney test significant data with $p < 0.0005$ presented as ***, $p < 0.005$ as ** and $p < 0.05$ as * (results represented as mean ± SD; n = 3).

Incubation of DCs with OVA24- liposomes led to an improved activation of OVA-specific $CD8^+$ T cells *in vitro,* in comparison with free OVA24, indicating a more efficient antigen presentation by the DCs (figure 1). All SLP-loaded liposome formulations showed a comparable efficacy regardless the inclusion of the poly(I:C) in the formulation. The latter was expected, since antigen presentation to the B3Z reporter T cell hybridoma is independent of B7-ligation signalling.

DC maturation

Dendritic cells (DCs) are the most potent APCs and express several sensing receptors including TLR3, the receptor for the immunostimulant poly(I:C). Signalling through the TLRs leads to the activation and maturation of DCs, as can be measured by enhanced expression of the co-stimulatory marker CD80 and the production of the effector cytokine IL-12. These are essential signals for an effective DC–T cell interaction and the initiation of an adaptive immune response. Figure 2A shows that all poly(I:C)-adjuvanted formulations induced an enhanced expression of the DC surface marker CD80 *in vitro*,

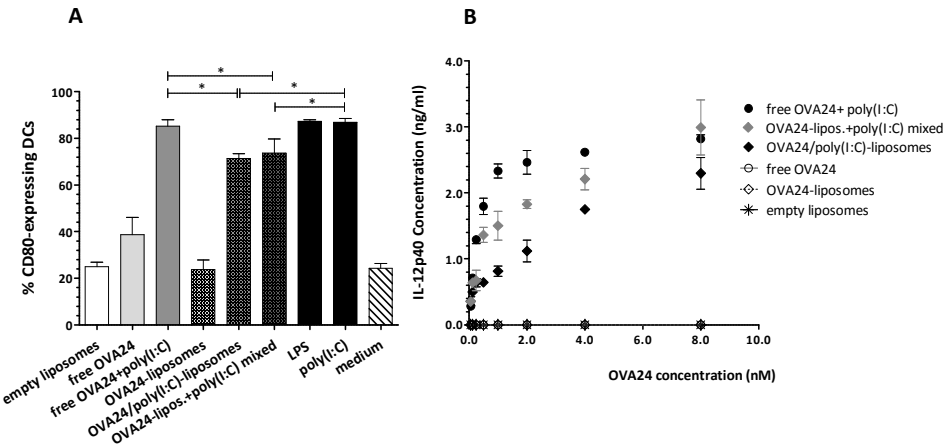

Figure 2: DC activation *in vitro* as measured by the expression of CD80 activation marker on the DC surface after overnight incubation of DCs at 37°C with titrated amounts of OVA24 formulations. The cells were stained with fluorescently-labelled anti-CD80 antibody and analysed by flow cytometry. Graphs depict the percentages of activated DC cells according to the expression of CD80 protein (A) and IL-12 cytokine concentration in culture medium after overnight incubation of DCs at 37°C with titrated amounts of OVA24 formulations. Cells incubated with LPS (control) produced 3.0 ng/ml IL-12, while poly(I:C) in solution followed the same concentration trend as free OVA24 + poly(I:C) sample (B). Data analysed with 1-way ANOVA w/ Tukey's multiple comparison test, and *p* value calculated with one-tailed Mann Whitney test. Significant data with $p \leq 0.05$ presented as *.

comparable to stimulation with LPS, a well-known TLR4 and a commonly studied TLR ligand that induces robust DC maturation (positive control). Additionally, the elevated

levels of IL-12 that were detected in the culture medium of the activated cells (figure 2B), indicate that poly(I:C) maintains its bioactivity after encapsulation or co-delivery (mixed) with the liposomes, albeit slightly reduced compared to free poly(I:C) (figure 2).

OVA24- specific CD8⁺ T cell responses in vivo

To investigate the efficacy of OVA24-loaded liposomal vaccines to induce T cell -mediated immunity *in vivo*, a series of vaccination studies was performed. Naive wild type BL6 mice were injected with different SLP formulations; the strength of OVA-specific CD8⁺ T cell responses was assessed by detecting antigen-specific CD8⁺ T cell frequencies in blood and spleens with MHC K^b-tetramer-$OVA_{257-264}$ (TM-SIINFEKL) APC--labelled conjugates.

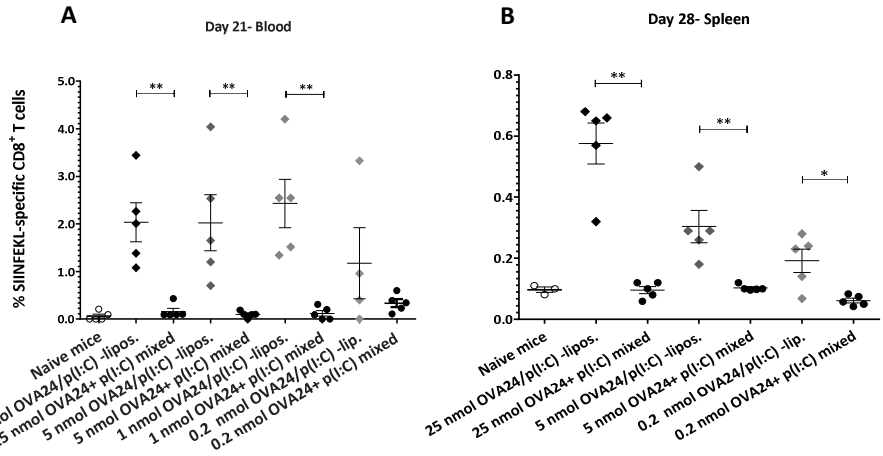

Figure 3: Antigen-specific CD8⁺ T cell responses following intradermal vaccination of C57BL/6 mice on day 0 and day 14 with different SLP doses of poly(I:C)-adjuvanted formulations, as monitored in blood on day 21 (A) and in spleens on day 28 (B). *P* value calculated with one-tailed Mann Whitney test, significant data with $p < 0.005$ presented as ** and data $p < 0.05$ presented as *. Each dot represents the response of an individual mouse (results represented as mean ± SEM; n=5). Control mice and groups of mice immunised with 1 nmol of vaccine that are missing from panel B were sacrificed one day later for the need of the assay presented in the supplemental figure 2).

The potency of our SLP-liposomal vaccine was firstly investigated in a dose-response *in vivo* setting. Using SLP doses ranging from 0.2 to 25 nmol, mice were immunised intradermally with either OVA24/poly(I:C)-loaded liposomes or OVA24+poly(I:C) mixed. The antigen-specific CD8⁺ T cell responses were monitored in blood (day 21) and spleens (day 28) *ex vivo*. Our adjuvanted liposome vaccines were capable of inducing strong T cell responses which were clearly detectable *ex vivo* without the need of additional re-stimulation *in vitro* (figure 3 and supplementary figure 4). Irrespective of the dose used, the liposomal OVA24/poly(I:C) formulations proved their superiority to the free ones

with regard to the induction of antigen-specific T cells. The systemic $CD8^+$ T cell frequencies in blood one week after the booster injection (figure 3A) that were induced by the three highest doses of liposomal vaccines were comparably high, showing that the peptide liposomal-loaded vaccine is significantly (at least 25-fold) more effective than the SLP mixed with poly(I:C) and delivered in solution. At day 28 (two weeks after the booster injection) the $CD8^+$ T cell response in blood has declined to lower levels, but the superiority of the liposome vaccine is still evident (supplementary figure 1). At this time, mice were sacrificed and their spleens were analysed, again showing significantly higher frequencies of OVA-specific $CD8^+$ T cells induced by the liposomal vaccine in a dose-dependent fashion (figure 3B).

Using the intermediate dose of 5 nmol, a number of SLP formulations were tested *in vivo* following the same immunisation schedule as previously for the dose-response experiment. Six days after the prime immunisation the poly(I:C)-adjuvanted OVA24-loaded liposomes showed detectable OVA-specific T cell responses, which further improved especially 21 days after prime (i.e., 7 days after boost) immunisation, while the free peptide mixed with poly(I:C) did not elicit any significant response within this timeline (figure 4), similar to our previous observation (figure 3).

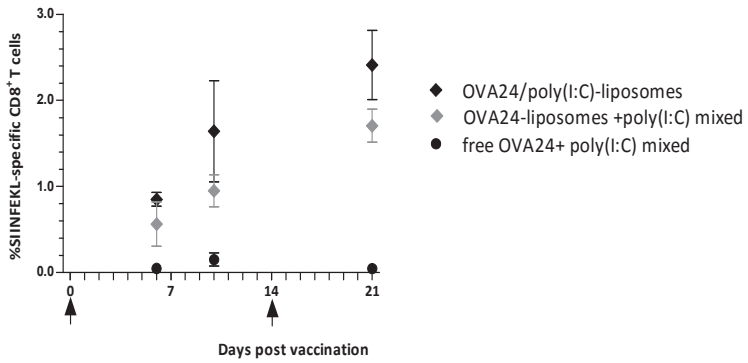

Figure 4: Induced T cell response within the immunisation schedule: Ova24/poly(I:C)-loaded liposomes induce high frequencies of OVA-specific $CD8^+$ T cells in C57BL/6 mice that were vaccinated i.d. with formulations containing 5 nmol of OVA24, on day 0 and 14 (indicated by arrows). The graph depicts the percentages of OVA-tetramer (TM-SIINFEKL) in blood (results represented as mean ± SEM; n = 5) as function of time.

On day 21, the OVA-specific $CD8^+$ T cell responses in blood in mice immunised with OVA24-liposomal formulations, OVA24/poly(I:C)-liposomes and OVA24-liposomes + poly(I:C) mixed, were significantly enhanced, leading to a T cell induction frequency of about 2% (figure 5A), irrespective of the administration route that was used

(see supplementary figure 2 for the responses induced by the subcutaneous administration route). A similar trend was observed 28 days' post vaccination in splenocytes, although here the differences between groups were not significant (figure 5B).

From the above results we can conclude that for an efficient antigen-specific $CD8^+$ T cell immune response, incorporation of the SLP peptide in the liposomal delivery system appears crucial (figure 4-5): unlike liposomal OVA24, free OVA24 mixed with poly(I:C) or with poly(I:C) and empty liposomes did not induce any significant measurable $CD8^+$ T cell response. In contrast, poly(I:C) significantly enhanced the induced T cell response elicited by the OVA24-loaded liposomes, no matter whether the TLR3 ligand was incorporated or mixed with the liposomes, as measured in blood (figure 4 and 5A) and in splenocytes (figure 5B). Altogether, the above results suggest that the efficacy of poly(I:C) adjuvanted OVA24-loaded liposomes to induce T cell immunity is clearly superior to that of free OVA24 mixed with poly(I:C).

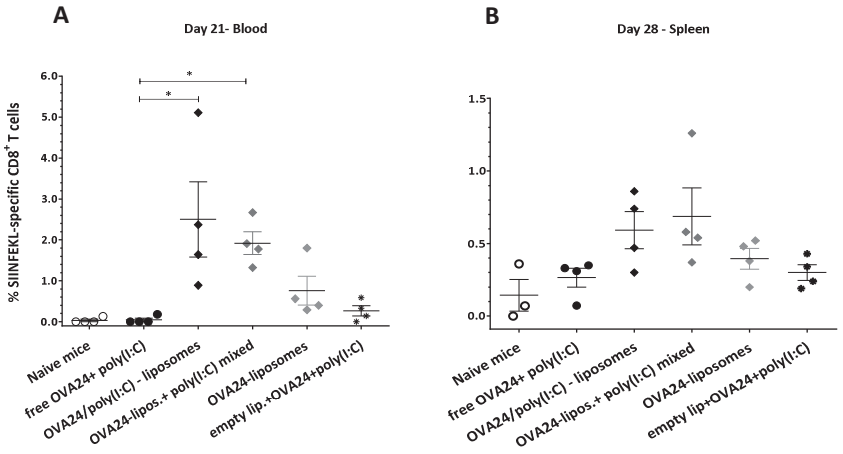

Figure 5: Antigen-specific $CD8^+$ T cell responses in blood on day 21 (A) and in splenocytes on day 28 (B) following intradermal vaccination with 5 nmol of OVA24 formulations on day 0 and day 14. The experiment was performed three times with comparable results. Data analysed with Kruskal- Wallis w/Dunn's multiple comparison post-test, and p value calculated with non-parametric Mann Whitney test. Significant data with a $p < 0.05$ presented as * (results represented as mean ± SEM; n=4).

While monitoring of T cell responses with TM-SIINFEKL allowed us to assess the capability of the formulations to expand antigen-specific $CD8^+$ T cells, it does not give insight into their functionality. Therefore, flow cytometric analysis was used to also determine the killer cell lectin-like receptor G1 (KLRG1) expression on the surface of the

induced CD8⁺ T cells as a measure of potential effector functionality [30,31]. KLRG1 is expressed on antigen-specific memory T cells that are fully capable of responding with effector functions to antigen encounter [30,31]. The percentages of the effector - memory induced cells defined by the expression of KLRG1, within the antigen-specific T cell population, were in all SLP dosages significantly higher for the liposomal

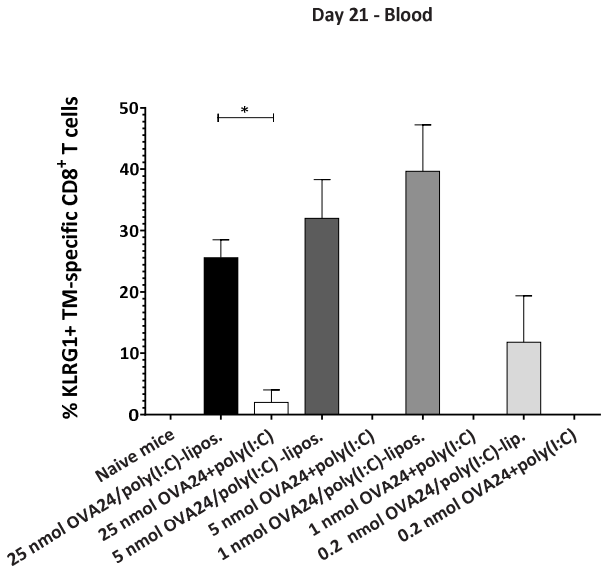

Figure 6: Induction of KLRG1⁺ effector- memory CD8⁺ T cells within the population of SIINFEKL⁺- specific CD8⁺ T cells (depicted in figure 3A). The bar graphs depict the mean percentages (+SEM) at day 21. *P* values calculate with one-tailed Mann Whitney test, significant data with a p <0.05 presented as * (n=5).

formulations (no obvious dose-dependent effect was observed) compared to the soluble SLP poly(I:C)-adjuvanted formulations, which did not induce detectable KLRG1 expression (figure 6 and supplementary figure 5). These data demonstrate the functional effectiveness of adjuvanted liposomal OVA24 formulations.

Induction of CD8⁺ cytotoxic T cells in vivo

An *in vivo* cytotoxicity assay was performed in order to assess target cell-specific killing potency of the induced CD8⁺ T cells. Intradermal immunisation of mice with 5 nmol OVA24-liposomes combined with poly(I:C) led to a strong cytotoxic activity (figure 7), in contrast to vaccination with the mixture of free OVA24 and poly(I:C) (figure 7). In detail, immunisation with OVA24/poly(I:C)-liposomes led to a killing capacity up to 80% when delivered intradermally, while the same formulation delivered subcutaneously showed

a less effective killing activity, up to 40%. Moreover, intradermally administered OVA24-liposomes mixed with poly(I:C) led to a weaker cytotoxicity efficiency of around 50%. In summary, these results demonstrate that there is an administration route effect with regard to the killing capacity of our vaccine, and that the poly(I:C) adjuvanticity depends on the way it is delivered with the liposomes. Interestingly, the observed cytotoxic activity of the induced T cells was maintained for at least 2 weeks after the boost immunisation (day 29) in mice receiving an even lower dose (1 nmol SLP) of the liposomal vaccine (see supplementary figure 3), confirming the efficacy of the SLP liposomal vaccine based on the observed frequencies of SIINFEKL-specific $CD8^+$ T cells at day 28 (figure 5).

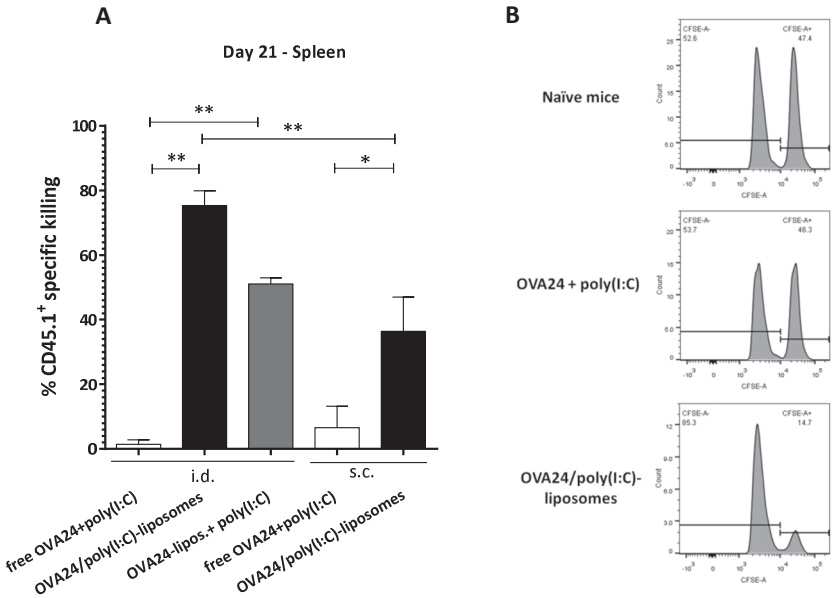

Figure 7: *In vivo* cytotoxicity assay. The normalised mean percentages (±SEM) of the killing activity are depicted based on the percentages of transferred $CD45.1^+$ that could be detected against the control -naive mice. Mice immunised with 5 nmol of OVA24 vaccine intradermally (i.d.) or subcutaneously (s.c.) (the last two bars), monitored one week after the boost immunisation. The bar graphs depict the mean percentages (+SEM) at day 21 (A). Data analysed with 1-way ANOVA w/Tukey's multiple comparison test and *p* value calculated with one-tailed Mann Whitney test. Significant data with $p < 0.0005$, presented as ***, $p < 0.005$ as ** and $p < 0.05$ as * (n=5). Representative histograms of CFSE-labelled positive target cells (right peak= peptide pulsed and left peak = control) (B).

The capability of the induced CD8⁺ T cells to produce effector cytokines, such as IFN-γ and TNF-α, is an indication of their functional effectiveness. IFN-γ is vital for protection and therapy against intracellular pathogens as well as tumours. Mice vaccinated with liposomal formulations of the peptide with poly(I:C) showed a higher efficiency to induce CD8⁺ T cells that produce IFN-γ compared to the adjuvanted free OVA24 in PBS (figure 8A). A fraction of these IFN-γ producing T cells was also positive for TNF-α (figure 8B). This simultaneous production of both IFN-γ and TNF-α cytokines shows the functional capacity of the SLP-liposomal formulations regarding the induction of antigen-specific effector CD8⁺ T cells.

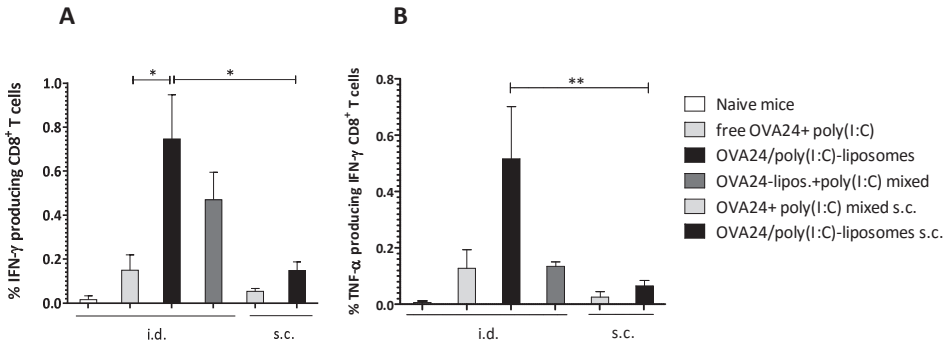

Figure 8: Intracellular cytokine analysis. Blood of immunised mice was stimulated overnight *ex vivo* with SIINFEKL minimal peptide (OVA8) so that cytokine production will be amplified at detectable levels. CD8⁺ T cells producing IFN- γ (A) and CD8⁺ T cells producing both IFN-γ and TNF-α (B). Data analysed with 1-way ANOVA w/Tukey's multiple comparison test and *p* value calculated with one-tailed Mann Whitney test. Significant data with a $p < 0.005$ presented as ** and $p < 0.05$ as * ($n = 4$).

Discussion

The design of safe and effective therapeutic cancer vaccines for human use remains an urgent but unmet medical need. Liposomes can be a promising delivery system, able to promote the uptake of the loaded antigen by DCs and elicit an anti-tumour immunity after vaccination. In this study we showed that poly(I:C) adjuvanted-DOTAP-based liposomes loaded with a SLP can be a successful vaccine candidate for the induction of a functional $CD8^+$ CTL response that is required for tumour eradication.

The superior immunogenicity of our OVA24-loaded DOPC: DOTAP liposome formulation can be attributed to the adjuvant nature of cationic liposomes. It has been reported that DOTAP or other cationic liposomes promote a vaccine-induced anti-tumour immune response, more efficiently than anionic or neutral liposomes [32], since they can enhance DC activation and maturation and the subsequent T cell priming. This ability is due to their lipid structure: cationic lipids, such as DOTAP with a quaternary ammonium group are more potent than lipids with a tertiary ammonium group [33]. Priming of DCs by antigen-loaded DOTAP-based liposomes induces the generation of reactive oxygen species (ROS), which in turn leads to the trigger of various signalling pathways, such as extracellular-signal-regulated kinase (ERK) and p38, and consequently the production of cytokines, chemokines and expression of co-stimulatory molecules [34].

It has been reported that liposomes consisting only of DOTAP were more efficient for the delivery of an HPV E7-derived peptide and that incorporation of an inert lipid could reduce the anti-tumour activity of the liposomes [33]. However, in most cases the role of a "helper" neutral lipid has been shown to be essential for the stability of the formulation and the antigen delivery [35]. The unsaturated zwitterionic lipid DOPC is known to possess such a role, contributing to the stability of the DOTAP-based loaded formulation by the physical interactions of the two phospholipids (salt bridges formation) and alterations on the membrane fluidity that improves the stability as well as the encapsulation efficiency [35].

The high surface charge density in cationic liposomal vaccines, apart from the improved adjuvant effect, can also be crucial for the loading and entrapment of the therapeutic agent, in our case the OVA24 peptide. OVA24 loading in the liposomes proved to be challenging mainly due to the peptide's nature: it is neither very hydrophilic nor very lipophilic; it does not easily dissolve in aqueous solutions and is insoluble in many organic solvents, such as chloroform. Preparation and encapsulation procedure (mechanism of SLP loading) had to be optimised, in order to achieve a high peptide

loading efficiency. In an attempt to find the most suitable formulation, liposomes of various lipid compositions were prepared, most of them yielding low loading efficiencies, especially when liposomes consisted of only neutral lipids or positively charged lipids in a low molar ratio (results not shown). This suggests that the peptide entrapment in the DOPC: DOTAP formulation is highly dependent on electrostatic interactions between the positive charged liposomes and the negatively charged peptide (at physiological pH). Indeed, decreasing the pH below OVA24's isoelectric point (pI 4.3) led to a substantial drop in loading efficiency, and the loading efficiency of the SLP in neutral or negatively charged liposomes was very low (unpublished data).

In this study we showed that a cationic liposomal formulation loaded with a SLP and combined with poly(I:C) is more potent than the free equivalent vaccine for the induction of a $CD8^+$ T cellular immune response. In addition, the induced T cell responses that were observed upon administration of the liposomes were much higher compared to other vaccine systems that have been used for SLP delivery, such as Montanide, even when the dose was reduced more than 25 times. SLP-loaded liposomes can promote the entry of the peptide into the cytoplasm, their fragmentation by the proteasome and the access of the peptide fragments to the MHC class I proteins that will eventually result in a CTL response [36]. The liposomal formulations and controls of OVA24 and TLR ligand were delivered intradermally. Yet, subcutaneous injection was used as a control as the most commonly used administration route. It is known that the administration route can influence the nature of the induced T cell response [37,38] and the targeted APC subsets [39]. After intradermal injection, liposomes can form an antigen depot at the injection site, which facilitates a prolonged antigen loading of cross-presenting specialised DCs (such as $CD8\alpha^+$) almost exclusively in the local draining lymph nodes [40], whereas upon subcutaneous immunisation the antigen more likely is taken up by DCs at the injection site and subsequently transported to lymph nodes, leading to a less efficient T cell activation. Interestingly, OVA24/poly(I:C)-loaded liposomes seem to have a significant efficacy to induce a specific T cell immune response after 21 days, regardless of the administration route used. However, intradermal administration appeared to be superior to the subcutaneous route regarding the effectiveness of the SLP-specific induced T cells, as indicated by cytokine production and killing capacity. It is known that activated T cells amplify their reactions via the secretion of cytokines, such as IFN-γ that plays a key role in the induction of cellular immunity. Additionally, the detection of high and simultaneous production of IFN-γ and TNF-α by $CD8^+$ T cells in mice immunised intradermally with the poly(I:C)-adjuvanted OVA24-liposomes indicates the functional properties of the induced immune response*:* the simultaneous production of IFN-γ and TNF-α is a functional capacity of effective vaccine-induced $CD8^+$ T cells [41-43].

Furthermore, the efficacy of our liposomal vaccine has also been proven via its capacity to induce cytotoxic specific $CD8^+$ T cells capable of killing target cells *in vivo*. Cancer vaccines loaded with peptides and co-delivered with poly(I:C) have been reported to elicit tumour antigen-specific cytotoxic T cell responses *in vivo* or even suppress the tumour growth [44]. While poly(I:C) free in PBS with OVA24 (with or without empty liposomes) did not enhance the peptide's immunogenicity, when combined with loaded liposomes the adjuvant effect was pronounced. Although the SLP-specific $CD8^+$ T cell priming was improved in mice immunised with the adjuvanted liposomes irrespective of the way the poly(I:C) was delivered (encapsulated or mixed), we observed a significant difference between the OVA24/poly(I:C)-loaded liposomes and the OVA24-liposomes + poly(I:C) mixed formulation, regarding the functionality of the induced T cells. The OVA24/poly(I:C) co-encapsulated liposomal formulation appeared to be more effectively regarding the production of IFN-γ and TNF-α cytokines from the activated T cells and more effective in inducing killing capacity. This finding is likely due to the enhanced poly(I:C) internalisation by the liposomes and the promoted interaction between the TLR3 and its ligand in the endosomes [45, 46].

Conclusion

Therapeutic vaccination with overlapping SLPs has been proven superior to vaccination with minimal peptide epitopes for the induction of long-term effector $CD8^+$ T cell immune responses. The features of cationic liposomes, including safety, storage stability and versatility with regard to the accommodation of antigens as well as adjuvants with variable physicochemical properties, make them promising for vaccine delivery. Our data clearly show the superior efficacy of our cationic SLP-loaded liposomal formulation adjuvanted with poly(I:C) compared to free SLP, and its potential to be used as an effective therapeutic vaccine against cancer.

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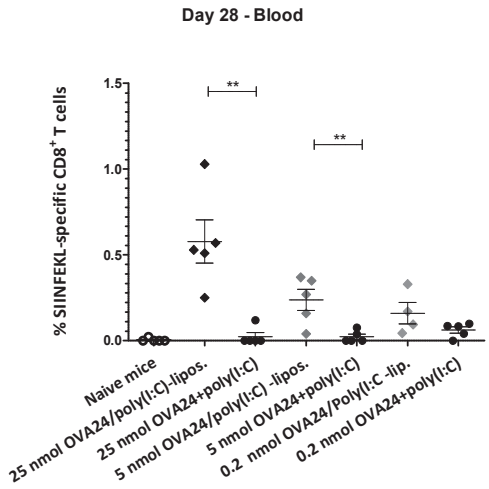
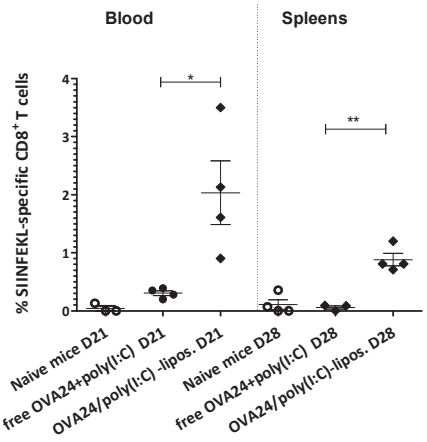
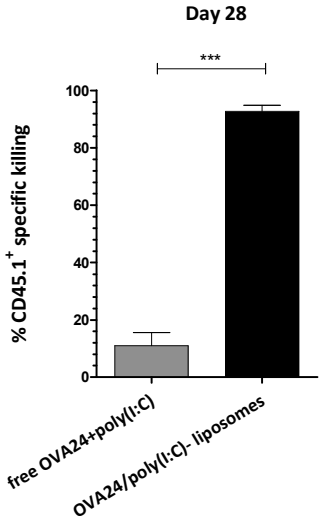
Supplementary data



Supplementary figure 1: Antigen-specific $CD8^+$ T cell responses following intradermal vaccination of C57BL/6 mice on day 0 and day 14 with different SLP doses of poly(I:C)-adjuvanted formulations, as monitored in blood on day 28. *P* value < 0.005 is represented as **. Each dot represents the response of an individual mouse (n=5).



Supplementary figure 2: Antigen-specific $CD8^+$ T cell responses in blood on day 21 and in splenocytes on day 28 following subcutaneous vaccination with 5 nmol of OVA24 formulations on day 0 and day 14. The experiment was performed three times with comparable results. *P* values calculated with *t* test, ** p <0.005 and * p < 0.05, (n=4).



Supplementary figure 3: *In vivo* cytotoxicity assay. The normalised mean percentages (±SEM) of the killing activity are depicted based on the percentages of transferred CD45.1^{+} cells that could be detected against the control -naive mice. Mice immunised intradermally with 1 nmol of OVA24 vaccine monitored two weeks after the boost immunisation. The bar graphs depict the mean percentages (+SEM) at day 28. *P* value < 0.0001, presented as ***.

Chapter 3

Adjuvanted cationic liposomes loaded with synthetic long peptides: an effective vaccine formulation for melanoma treatment in mice

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Abstract

In this study, the potency of peptide-loaded cationic liposome formulations for cancer immunotherapy was investigated. Cationic (DOTAP/DOPC) liposomes were loaded with three well-defined molecules, crucial to raise effective T cell responses: two synthetic long peptides (SLPs), OVA24 and OVA17, harbouring an ovalbumin-derived cytotoxic (CTL) and T helper (T_H) epitope, respectively, and a potent TLR ligand immunostimulator. The SLPs were introduced either co-encapsulated in the same particle or loaded in separate liposomes, and their effect on the induction of antigen-specific T cells was studied, also when combined with different TLR ligands. The obtained nanoparticles were positively charged, rather monodisperse and had a size of approximately 140 nm. The loaded compounds' functionality was evaluated *in vitro*, based on their capacity to present the CTL epitope to antigen-specific T cells and activate dendritic cells (DCs) via the encapsulated TLR ligand. The efficiency of our vaccines *in vivo* and the effect of the loaded compounds on the priming of naive T cells were assessed by intradermal immunisation in mice. Strong *in vivo* induction of antigen-specific $CD8^+$ and $CD4^+$ T cells, functional cytokine production by the induced T cells and *in vivo* cytotoxicity against specific target cells were observed. Finally, our adjuvanted-SLPs-loaded liposomal formulations were able to significantly control outgrowth of established melanoma tumours upon therapeutic vaccination in mice, in contrast to adjuvanted free SLPs, pointing out the potential of this liposomal formulations as powerful vaccines for cancer immunotherapy.

Keywords: cancer immunotherapy, cationic liposomes, DOTAP, synthetic long peptides, TLR ligands, vaccine delivery

Introduction

The design of safe and effective therapeutic vaccines for immunotherapy of cancer in humans remains an unmet medical need. Therapeutic vaccination against cancer requires, in most cases, the induction of cytotoxic $CD8^+$ and specific T_H1 helper $CD4^+$ T cells [1], through the activation of dendritic cells (DCs), as the most well-equipped antigen presenting cells (APCs) [2-5]. In order for vaccines to optimally activate functional $CD8^+$ cytotoxic T cells, the exogenous antigen has to be cross-presented on MHC class I molecules by specialised DC subsets, located in the lymphoid tissues or in vaccination sites such as in skin [6]. Similarly, specific T helper cell activation by antigen presentation in MHC class II molecules is required for optimal expansion of tumour-killing $CD8^+$ T cells. Therefore, optimally designed vaccines have to be targeted preferably to these DC subsets, so that after processing of the antigen, the fragmented peptides will be efficiently presented to antigen-specific T cells [7,8]. In addition to that, as a vaccine antigen the use of overlapping synthetic long peptides (SLPs), containing all the potential MHC class I and II epitopes, has been proven superior to the use of proteins or minimal peptide epitopes [9,10] and effective in clinical studies [11].

The majority of the clinical trials for cancer immunotherapy have been based on the use of Montanide ISA-51, an incomplete Freund's adjuvant (IFA)-like water-in-oil emulsion, known for its serious local side effects [12,13]. As an alternative to Montanide, we propose liposomes as a promising antigen delivery system, able to enhance antigen uptake by DCs and the subsequent initiation of cell-mediated anti-tumour immune responses upon vaccination [14-18]. Cationic liposomes have been reported to be more immunogenic than neutral or anionic ones [19,20]. Recently we also showed that a poly(I:C)-adjuvanted DOTAP-based liposomal formulation, loaded with OVA24, a SLP containing solely a CTL epitope (SIINFEKL), and the TLR ligand poly(I:C), shows promise as a therapeutic cancer vaccine formulation. This liposomal SLP formulation, without any active targeting molecules, appeared to be very effective regarding the induction of functional cytotoxic T cells, compared to the free equivalents [21]. In this study, we analysed the potency of our OVA24-loaded DOTAP-based cationic formulation by incorporating a second SLP containing a T_H epitope, the OVA17, when also combined with different TLR ligands as therapeutic cancer vaccines. We showed here that tumour-specific T helper responses were crucial for tumour control by effector $CD8^+$ T cells and that liposomes in which both SLPs were co-encapsulated in the same particle together with poly(I:C) was the most effective formulation for activation of functional cytotoxic T cells *in vivo.* Finally, the potency of the cationic SLP(s)-loaded liposomes as a therapeutic vaccine in an aggressive melanoma tumour model was investigated.

Materials and Methods

Materials

The ovalbumin-derived SLP OVA24 [DEVSGLEQLESIINFEKLAAAAAK], including the CTL epitope SIINFEKL, and the short peptide OVA8 [SIINFEKL] were produced and purified at the GMP facility of the Clinical Pharmacy and Toxicology Department at the Leiden University Medical Center [22]. The ovalbumin-derived SLP OVA17 [ISQAVHAAHAEINEAGR], including the helper T_H-epitope AAHAEINEA, was produced in the Immunohematology and Blood Transfusion Department of the Leiden University Medical Center. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and the TLR ligands (poly(I:C), Pam3CSK$_4$, and CpG) and their labelled analogues (rhodamine for poly(I:C) and Pam3CysK$_4$, and FITC for CpG) were obtained from InvivoGen (Toulouse, France). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, Netherlands), 100 IU/ml penicillin and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionised water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ water). Phosphate buffer was composed of 7.7 mM $Na_2HPO_4 \cdot 2\ H_2O$ and 2.3 mM $NaH_2PO_4 \cdot 2\ H_2O$, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Darmstadt- Germany) before use. Phosphate-buffered saline (PBS), which was used for all the *in vitro* and *in vivo* assays was purchased from B. Braun (Meslungen, Germany).

Mice

Female C57BL/6 (H-2^b) mice were purchased from Charles River (L'Arbresle, France) and congenic CD45.1 (Ly5.1) mice were bred at the Leiden University Medical Center animal facility and used at 8-14 weeks of age according to the Dutch Experiments on Animal Act, which serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe. All performed animal experiments were approved by the animal experimental committee of Leiden University.

Liposome preparation

Cationic liposomes loaded with OVA24 were prepared by using the thin film dehydration-rehydration method, as described previously [21]. Briefly, DOTAP and DOPC (molar ratio 1:1) in chloroform, were mixed in a round-bottomed flask to reach a concentration of 10 mg lipid per ml of final liposome dispersion. The formed dry film was rehydrated either with filtered MQ water (2 ml, pH 5.5 – 5.8) for non-loaded (empty) liposomes, or with 2 ml of a solution of 1 mg/ml OVA24 in ACN/H_2O 1:1 (v/v) (OVA24-loaded liposomes). For the liposomes loaded with OVA17 or both OVA24 and OVA17, the aqueous solution of the SLPs was first adjusted to a pH of about 8.5. For poly(I:C) or CpG loaded liposomes, the ligand (including 0.5% fluorescently-labelled equivalent) in a total concentration of 200 µg/ml was added dropwise to the dispersion (so after the lipid film was rehydrated with the SLP solution). For liposomes loaded with the lipophilic $Pam3CysK_4$, the TLR ligand was dissolved in chloroform together with the lipids, before the dry film formation. After the lipid film hydration, the liposome dispersion was snap-frozen in liquid nitrogen, followed by overnight freeze-drying. Dehydrated-rehydrated liposomes were generated by gradually adding 10 mM PB, pH 7.4, to the freeze-dried lipid cake. The liposomal mixture was vortexed well during the rehydration steps and the resulting dispersion was kept at room temperature for 1 h.

Sizing of the obtained multilamellar vesicles was performed by high-pressure extrusion at room temperature using a Lipex extruder (Northern Lipids Inc., Canada) and concentration of peptide-loaded liposomes was performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa) as described previously [21].

Liposome characterisation

Average diameter (Z_{ave}) and polydispersity index (PDI) of the liposomes were determined by dynamic DLS using a Zetasizer (NanoZS, Malvern Ltd. UK). The same instrument was used for zeta-potential determination by laser Doppler electrophoresis. For these measurements liposome samples were diluted 100-fold in 10 mM PB, pH 7.4.

Peptide loading efficiency was determined by extracting OVA24 and OVA17 from the liposomes by using a modified Bligh-Dyer method [21] and applying a UPLC method. In detail, 5 µl of the water-methanol extract was injected onto a reversed-phase UPLC column C18-1.7µm (2.1 x 50 mm) (Acquity UPLC, Waters, Milford MA, USA) by using an Acquity UPLC system from Waters. The mobile phases, 100% MQ water with 0.1% TFA (Solvent A) and 100 % ACN with 0.1% TFA (Solvent B), were applied in a linear gradient

from 0-95 % solvent B over 5 min at a flow rate of 0.37 ml/min. Detection of the peptide(s) was achieved by measuring the absorbance at 220 nm through the ultraviolet system detector (Acquity UPLC TUV detector) and peptide concentrations were determined based on a calibration curve with known OVA24 and OVA17 concentrations (0.5 – 500 μg/ml).

The amount of TLR ligands in the liposomes was determined by measuring the fluorescence (ex. 546/em. 576 nm for the rhodamine-labelled poly(I:C) and $Pam3CysK_4$, and ex. 495/em. 520 for the FITC-labelled CpG analogues) in collected non-solubilised (i.e., intact) liposomal samples and the PB filtrates containing the free TLR ligands. A calibration curve of each fluorescently labelled compound was made, ranging from 7.81 ng/ml to 1000 ng/ml, and all samples were measured by using a fluorescence micro plate reader (Tecan, Salzburg, Austria).

DC maturation evaluated by flow-cytometric analysis of CD80 surface marker

Immature D1 cells ($5x10^4$ cells/well) (i.e., a long-term growth factor-dependent dendritic cell line derived from spleens of C57BL/6 mice) were incubated in 96-well round-bottomed plates at 37°C in supplemented IMDM with SLP-loaded liposomes or soluble SLP formulation at different concentrations as described previously [21]. Briefly, after overnight incubation at 37°C the cells were stained in staining buffer (PBS + 0.1% (w/v) bovine serum albumin (BSA) + 0.05% (w/v) sodium azide) for 30 min. with fluorescently labelled FITC antibody specific for the mouse CD80 DC surface marker (eBiosciences). 7-Aminoactinomycin D (7AAD) (Life Technologies) was used for the exclusion of dead cells and data acquisition of samples was done with a BD LSR II flow cytometer. The data were analysed by using the FlowJo software (Tree Star).

In vitro MHC class I antigen presentation

Immunogenicity of the SLP formulations was initially tested in an *in vitro* read-out, with regard to their efficiency to activate immature DCs and present SIINFEKL to the $CD8^+$ antigen/OVA- specific B3Z T cells, leading to their activation. Immature D1 cells were incubated in 96-well flat-bottomed plates at 37°C in supplemented IMDM with SLP-loaded liposome formulation or plain SLP (with or without TLR ligands) in PBS at different concentrations. The stability of the liposomes did not change when diluted in PBS, as assessed by DLS analysis. After 2.5 hours the plates were washed three times and subsequently T cell reporter hybridoma B3Z cells ($50x10^5$/well) were added followed by overnight incubation at 37°C. Chlorophenol red-β-galactopyranoside (CPRG) was used

as lacZ substrate in cell lysates and colour conversion was measured by detecting absorbance at 590 nm.

Immunisation of mice

Mice were immunised by intradermal injection of the formulations in the abdominal skin area [23]. All formulations were prepared on the day of injection. Vaccination dose was based on the OVA24 SLP concentration, 1 nmol (2.5 µg) of peptide in a total volume of 30 µl, and immunisations were performed on day 0 (prime immunisation) and on day 14 (boost injection). Vaccinations with adjuvanted liposomes included a dose of 0.5 – 1.0 µg of a TLR ligand. During the *in vivo* studies, blood samples were obtained from the tail vein at different time points.

Analysis of antigen-specific $CD8^+$ and $CD4^+$ T cell responses by flow cytometry

Staining of the cell surface was performed on blood samples after red blood cell lysis. Cells were stained in staining buffer for 30 min with allophycocyanin (APC)-labelled tetramer-OVA8 (TM-SIINFEKL) and fluorescently labelled antibodies specific for mouse CD3 (BD Biosciences, Breda, the Netherlands), CD4, CD8 and the killer cell-lectin-like receptor G1 (KLRG1) (eBiosciences). 7-Aminoactinomycin D (7AAD) (Life Technologies) was used for the exclusion of dead cells.

Overnight intracellular cytokine analysis of PBMCs was performed after incubating the cells with 2 µM of OVA8 and 2 µM of OVA17, in presence of brefeldin A (7.5 µg/ml) (BD Biosciences). The next day the assay was developed as described [21].

In vivo cytotoxicity assay

Splenocytes from naive congenic $CD45.1^+$ mice were lysed and split into two equal parts. Cells were labelled with CFSE and adoptively transferred intravenously in previously immunised recipient C57BL/6 mice in a volume of 200 µl in PBS as described [21]. Two days after the cell transfer (day 24), mice were sacrificed, spleens were isolated and single cell suspensions were analysed by flow cytometry. Specific killing (SK) was calculated according to the following equation:

$$\mathrm{SK} = \left\{ 1 - \frac{\left[\frac{\text{CFSE high}}{\text{CFSE low}}\ \text{vaccinated mice}\right]}{\left[\frac{\text{CFSE high}}{\text{CFSE low}}\ \text{naive mice}\right]} \right\} \text{x } 100\ \%$$

Tumour regression experiment

B16-OVA melanoma cells were cultured at 37°C with 5% CO_2 in IMDM containing 8% foetal calf serum + 2 mM glutamine and 100 IU/ml penicillin in the presence of 1 mg/ml geneticin (G-418) (Life Technologies), non-essential amino acids (10X; Life Technologies) and 1 mM sodium pyruvate (Life Technologies).

On day 0, mice were injected subcutaneously in the flank with $1x10^5$ tumour cells in 100 µl PBS. On day 9, when tumours were palpable (> 8 mm^3), mice were split into groups with similar tumour size and were vaccinated, as described above. Non-immunised mice injected with tumour cells were used as a negative control.

The mice were weighed three times a week and the size of their tumour was measured in three dimensions by using a calliper. Mice having a tumour size > 2000 mm^3 were sacrificed for ethical reasons.

Statistical analysis

The significance of differences in the *in vitro* and *in vivo* assays were evaluated by GraphPad Prism 5 (GraphPad) software, by using an analysis of variance (ANOVA) at a 0.05 significance level, followed by Tukey's and non-parametric Mann Whitney post-test. The significance of differences between the survival curves was calculated with the log-rank (Mandel-Cox) test.

Results

Cationic liposomes successfully loaded with two SLPs and an adjuvant

Previously we have shown that following the dehydration-rehydration lipid film method, cationic DOTAP-based liposomes loaded with the SLP OVA24, harbouring the CTL epitope SIINFEKL, can be successfully formed [21]. In other studies, we have also reported that for effective SLP-based cancer vaccines inclusion of both a tumour-specific CTL and a T_H epitope is essential [22,24]. Therefore, in the present study we included a second SLP (OVA17), harbouring an ovalbumin-derived T_H epitope, in our liposomal formulation to enhance the induced $CD8^+$ T cell response through the potential activation of antigen-specific $CD4^+$ T cells.

As we reported for OVA24, entrapment of OVA17 in the liposomes seems to be highly dependent on electrostatic interactions between the liposomes and the peptide (at physiological pH) [21]. In order to encapsulate and possibly achieve the highest loading efficiency of OVA17, which has an isoelectric point (pI) of 6.0 (higher than the pI of OVA24, ca. 4.25), the optimal conditions were determined by studying systematically the effect of the pH and the initial SLP concentration, before the hydration step, on the loading efficiency. The highest loading efficiency for both SLPs, when separately encapsulated in liposomes at an initial concentration of 1 mg/ml, was achieved at a pH above their isoelectric point (pI): around 8.5 (Table I; Figure 1). To examine whether the same optimisation conditions can be applied for the co-encapsulation of the two SLPs, the pH of the SLP solution was adjusted at the fixed value of 8.5 and different initial concentrations of both SLPs were tested (Figure 1).

As a result, when 1 mg/ml of each SLP was initially used, cationic liposomes co-encapsulated with two SLPs were successfully prepared in a reproducible way; at a pH of 8.5 both peptides are expected to be strongly negatively charged, which improves the electrostatic interactions with the positively charged liposomes and yields the highest loading efficiency (LE) for both SLPs.

Table I: % of loading efficiencies (LE) of OVA24 and OVA17 in separate liposomes at different pH values

pH [a]	*3.5*	*4.25*	*6.0*	*7.5*	*8.5*	*9.5*
			SLP LE (%) in liposomes			
OVA24	48	50	50	55	62	40
OVA17	5	5	18	24	30	20

[a] The adjusted pH value of the stock SLP solution at the moment of dry lipid film hydration. Data are averages of LE values of 10 individual batches.

The SLP(s)-liposomes obtained following the above-described optimised protocol had an average diameter that varied from 127 nm (OVA24-liposomes) to 155 nm (OVA17-liposomes) (Table II). Although the presence of OVA24 did not change the liposome size significantly compared to empty liposome formulations, the liposomes with OVA17, with or without OVA24, were slightly larger and less monodisperse (PDI > 0.2).

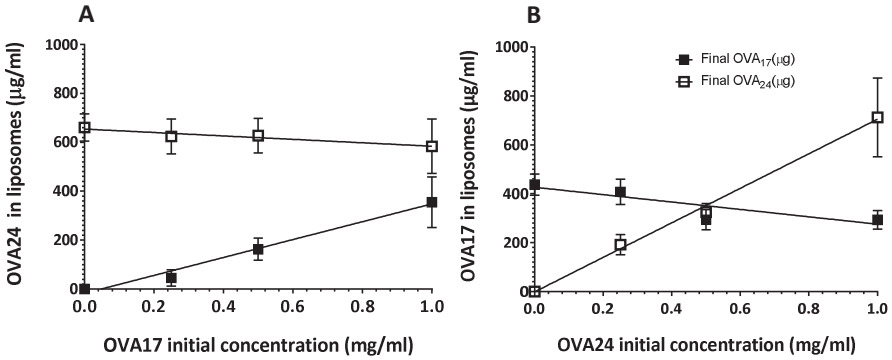

Figure 1: Loading of OVA17 and OVA24 in liposomes as function of (A) adjustable initial OVA17 concentration with the initial OVA24 concentration fixed at 1 mg/ml and (B) as function of adjustable initial OVA24 concentration with the initial OVA17 concentration fixed at 1 mg/ml (B). The pH of the SLP stock solutions was adjusted at 8.5. Data are averages values of at least 3 individual batches (mean ± SD; n ≥ 3).

The (positive) zeta-potential was practically independent of the loading of any of the components. The loading efficiency (LE) of OVA24 and OVA17 in non-adjuvanted liposomes when 1 mg/ml of each SLP was used, was about 50 % and 25%, respectively, resulting in a final OVA24/OVA17 ratio of about 2 (w/w); ratio similar to that of the mixture of the two peptides separately encapsulated in the liposomes (Table II).

Regarding the physicochemical characteristics of the adjuvanted formulations (initially loaded with 1 mg of OVA24 and OVA17), co-encapsulation of poly(I:C) led to a significant increase in size and PDI of the OVA24/OVA17-liposomes (ca. 209 nm, PDI >0.3), but not of any of the other formulations. In contrast, co-encapsulation of $Pam3CSK_4$, which is likely incorporated in the lipid bilayer, led to a reduction of the liposome size (ca. 128 nm). When poly(I:C) was co-encapsulated the loading efficiency of the SLP(s) remained similar, but when poly(I:C) was replaced by the lipophilic $Pam3CSK_4$ the encapsulation of both peptides was reduced significantly. Co-encapsulation of CpG in the liposomes did not alter the physicochemical properties of the particles; however, it reduced significantly the encapsulation of OVA17, but not that of OVA24 (Table II).

Overall, our data show that after the peptide loading optimisation studies, two different ovalbumin-derived SLPs were efficiently loaded, with or without TLR ligand in a reproducible way. Although co-encapsulation of some of the compounds affected the loading efficiency and/or the size of the liposomes, these effects were relatively minor and reproducible.

Table II: Physicochemical properties of the liposomal formulations used for immunological characterisation in this study.

				LE (%)		
	Z_{ave} diameter (nm)	PDI	ZP (mV)	OVA24	OVA17	TLR ligand
Empty liposomes	*133±10*	*0.15±0.02*	*32±1*	*NA*	*NA*	*NA*
OVA24-liposomes	*127±10*	*0.14±0.02*	*27±2*	*55±10*	*NA*	*NA*
OVA17-liposomes	*155±5*	*0.23±0.01*	*32±1*	*NA*	*28±7*	*NA*
OVA24/OVA17-liposomes	*146±8*	*0.27±0.03*	*25±2*	*50±10*	*25±5*	*NA*
OVA24-lip. + OVA17-liposomes	*154±5*	*0.28±0.05*	*25±1*	*55±10*	*29±7*	*NA*
OVA24-lip. + poly(I:C)	*135±5*	*0.19±0.05*	*30±3*	*55±10*	*NA*	*NA*
OVA24/poly(I:C)-liposomes	*145±3*	*0.17±0.02*	*28±2*	*56±6*	*NA*	*50±4*
OVA24/OVA17-lip. + poly(I:C)	*149±1*	*0.24±0.02*	*29±1*	*45±10*	*25±5*	*NA*
OVA24/OVA17/poly(I:C)-liposomes	*209±10*	*0.35±0.01*	*26±2*	*54±6*	*25±3*	*60±5*
OVA24/OVA17/Pam3CSK$_4$-liposomes	*128±10*	*0.19±0.02*	*25±1*	*41±4*	*19±5*	*40±5*
OVA24/OVA17/CpG liposomes	*159±5*	*0.18±0.02*	*28±1*	*60±10*	*16±2*	*70±5*

Data are average ± SD of at least 4 independent batches. SLP-loaded liposomes were prepared with 1 mg/ml initial SLP concentration, yielding a final OVA24/OVA17 ratio of ca. 2 (w/w); adjuvanted liposomes were loaded initially with 200 µg/ml of TLR –ligand.
Z_{ave} = Z-average particle diameter in nanometres, *PDI* = polydispersity index, *ZP* = zeta-potential, *LE* = loading efficiency, *NA* = not applicable.
The "+" denotes mixture of compounds and the "/" denotes co-encapsulation of compounds.

In vitro MHC class I antigen presentation

As shown before, OVA24-loaded cationic DOTAP-liposomes are able to promote DC uptake and processing of the SLP antigen for MHC class I presentation, and subsequent activation of SIINFEKL-specific $CD8^+$ T cells (B3Z cells), as compared to the free antigen [21]. In this study, liposomal formulations including two SLPs (OVA24 and OVA17), which contain epitopes that are presented in MHC class I and MHC class II, were similarly incubated with immature DCs. Improved activation of $CD8^+$ T cells was observed when liposomes were used compared to free peptides, indicating efficient processing and presentation of OVA24 SLP by the DCs. This improved $CD8^+$ T cell activation by the OVA24-liposomes was apparent in the absence of the OVA17 SLP, but also when OVA17 was either co-encapsulated (OVA24/OVA17-liposomes) or loaded in different liposomes

(OVA24 lip.+OVA17 lip.) (Figure 2A). Therefore, as expected, the presentation of the CTL epitope (derived from OVA24) to the B3Z T cells was independent of the presence of the Th epitope SLP. In addition to that, TLR ligands did not negatively influence the induced T cell activation by the SLP- liposomes. The hydrophilic poly(I:C), CpG or the lipophilic Pam3CSK$_4$, co-encapsulated or mixed with SLP-liposomes, were compared with their free equivalent mixtures with SLPs (Figure 2B) and all liposomal SLP formulations showed comparable efficacy to activate CD8$^+$ T cells, regardless of the presence and the way the TLR- ligand and the SLPs were included in the formulation (mixed or co-encapsulated).

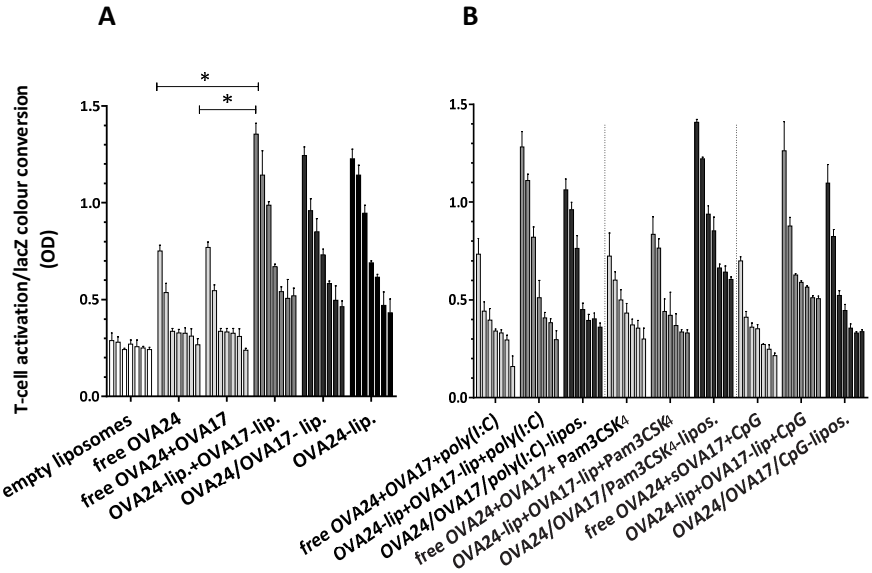

Figure 2: Activation of SIINFEKL-specific B3Z CD8$^+$ T cells after overnight culture with DCs pulsed for 2.5 h with titrated amounts of OVA24 (4.0-0.0625 µM, 2-fold dilutions) formulations (A) without and (B) with TLR ligand. Empty liposomes were diluted to lipid concentrations corresponding to those of the SLP-loaded liposomes. Graphs depict T cell activation based on the optical density (OD) at 590 nm measured after colour conversion of cell lysates after addition of CPRG (lacZ substrate). *p<0.05, evaluated with one-way ANOVA with Tukey's post hoc test and calculated with Mann-Whitney non-parametric test. The "+" denotes mixture of compounds and the "/" denotes co-encapsulation of compounds

In vitro DC maturation

The TLR ligands in the liposomes, as immunostimulatory signals, can result in an efficient activation of DCs, which leads to an increased expression of co-stimulatory molecules and production of cytokines, like IL-12 stimulating the CD8$^+$ T cell activation [21]. The hydrophilic poly(I:C) and CpG stimulators for the endosomal TLR3 and TLR9, respectively, were loaded most likely in the aqueous compartment of the liposomes.

In contrast, the rather lipophilic TLR2/1 ligand Pam3CSK$_4$ is likely located in the lipid bilayer. The bioactivity of the liposomal-loaded poly(I:C), Pam3CSK$_4$ and CpG was analysed *in vitro* by measuring the expression of the co-stimulatory marker CD86 on the surface of DCs incubated with the formulations, and the IL-12 secretion in the supernatant of these cultures (data not shown). The graphs in Figure 3 show that for poly(I:C) and CpG, encapsulation in liposomes did not diminish their ability to activate DCs, as they induced similarly high expression of the CD86 marker as induced by the free TLR ligand alone or mixed with the free SLPs (free OVA24+OVA17).

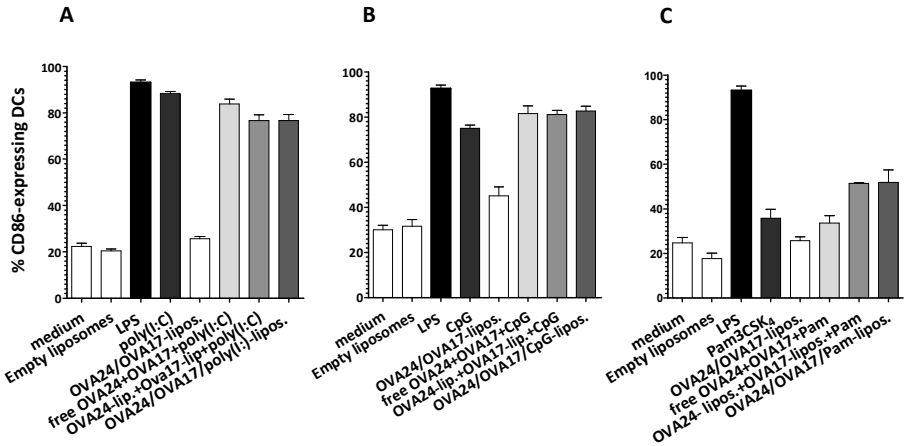

Figure 3: *In vitro* DC activation measured as CD86 expression by flow-cytometric measurement of DCs incubated overnight with SLPs-formulations (and controls) at 37°C. Graphs present the frequencies of activated DCs after their incubation with Poly(I:C) (1.0 µg) (A), Pam3CSK$_4$ (0.7 µg) (B) and CpG (1.2 µg) (C) adjuvanted formulations. Samples of medium, empty liposomes and OVA24/OVA17-liposomes (same lipid content as the adjuvanted ones) illustrate the background of the assay (no detectable DC maturation). The "+" denotes mixture of compounds and the "/" denotes co-encapsulation of compounds

Characterisation of the T cell responses induced in vivo

The ability of our cationic liposome formulations to induce cell-mediated immune responses was tested *in vivo,* with poly(I:C) selected as a TLR ligand adjuvant for a direct comparison with our previous studies [21].

Firstly, the number of SIINFEKL-specific CD8$^+$ T cells induced in blood was assessed by a flow-cytometric analysis. In mice immunised intradermally twice with the mixture of the separately loaded liposomes (OVA24-lip+OVA17-lip.) a high percentage of antigen-specific CD8$^+$ T cells was detected, which was elevated compared to the frequency in mice immunized with OVA24-liposomes only, suggesting that OVA17-mediated help improved the CD8$^+$ response. The response was even higher in mice immunised with the poly(I:C)-adjuvanted formulation (OVA24-lip.+OVA17-lip.+poly(I:C)) (Figure 4A).

Liposomes co-encapsulated with both SLPs (OVA24/OVA17-lipos.) showed a lower potency to induce CD8⁺ T cells than the mixture of OVA24-lip+OVA17-lip; the response was similar to that in mice immunised with liposomes including only OVA24 (OVA24-liposomes). The level of T cell activation increased when poly(I:C) was included in the formulation, either mixed with the SLP-liposomes (OVA24/OVA17-lip.+poly(I:C)) or co-encapsulated with the peptide antigens in the same liposomes (OVA24/OVA17/poly(I:C)-lip.).

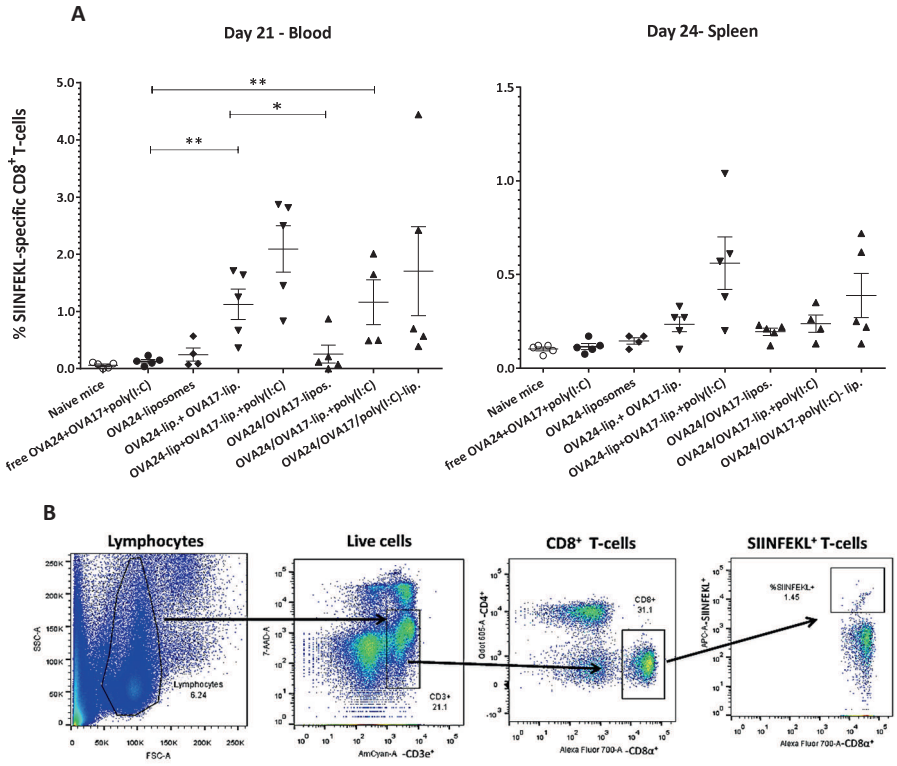

Figure 4: Antigen (OVA24)-specific CD8⁺ T cells responses in blood (day 21) and in splenocytes (day 24) following intradermal immunisation with 1 nmol of OVA24 on day 0 and 14 (A). Representative gating strategy for detection of SIINFEKL-specific CD8⁺ T cells by flow cytometry using specific MHC class I tetramers (B). The experiment was performed twice with comparable results. * p<0.05, ** p<0.01 calculated with by one-tailed non-parametric Mann-Whitney test.

Secondly, the phenotype and functional capacity of the liposome-induced T cells was analysed. Blood samples from the immunised mice were re-stimulated with MHC class I- and class II-binding peptides *ex vivo* and the percentages of CD8⁺ and CD4⁺ T cells producing IFN-γ only, or IFN-γ and TNF-α simultaneously, were assessed by an intracellular cytokine staining analysis (Figure 5). All SLP-containing liposomal formulations, including the mixture of the separately loaded SLP-liposomes (with and

without poly(I:C)) as well as the adjuvanted co-encapsulated SLP particles induced high percentages (> 1.0 %) of cytokines-producing $CD8^+$ T cells (Figure 5A & B). In addition to that, in blood of mice immunised with the OVA24-lip.+OVA17-lip+poly(I:C) formulation, high levels of IFN-γ and TNF-α -producing $CD8^+$ T cells were detected even in the *ex vivo* non-re-stimulated blood samples, demonstrating the potency of this vaccine (data not shown). Moreover, we were able to detect functional cytokine-producing $CD4^+$ T cells in all OVA17-containing liposomal formulations (Figure 5C & D). Result showing that OVA17 retains its functionality when co-encapsulated with OVA24 and leads to an improved $CD8^+$ T cell cytokine- response in all liposomal formulations.

Therefore, liposomal formulations including both SLPs, either co-encapsulated or loaded in separate particles, appeared to be more potent compared to the free SLPs, to induce not only antigen (OVA24)-specific $CD8^+$ T cells, but also antigen (OVA17)-specific $CD4^+$ T cells, thereby enhancing the strength and the quality of the induced T cell response.

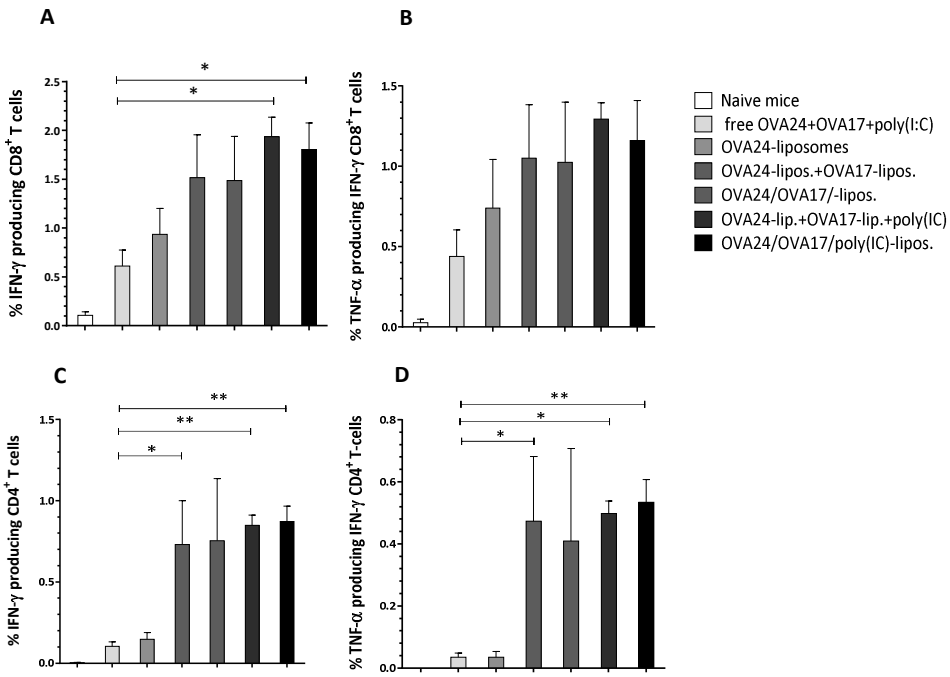

Figure 5: Intracellular cytokine analysis in blood of immunised mice at day 21 which was stimulated *ex vivo* overnight with the minimal SIINFEKL CTL epitope and OVA17 T_H peptide. Plots show $CD8^+$ T cells producing IFN-γ (A); $CD8^+$ T cells producing both IFN-γ and TNF-α (B); $CD4^+$ T cells producing IFN-γ (C); and $CD4^+$ T cells producing both IFN-γ and TNF-α (D). * $p<0.05$, ** $p<0.01$ calculated by one-tailed Mann-Whitney test. The "+" denotes mixture of compounds and the "/" denotes co-encapsulation of compounds.

Thirdly, the most potent formulations presented in Figure 5 were evaluated for their capability of inducing cytotoxic $CD8^+$ T cells, able to kill adoptively transferred target cells. The *in vivo* cytotoxicity assay presented in Figure 6 showed a strong cell-specific killing capacity by the activated $CD8^+$ T cells in mice immunised with SLP-containing liposomes. More specifically, vaccination with the mixture of separate SLP-loaded liposomes resulted in strong killing activity above 80% and when poly(I:C) was included in the formulation, mixed or co-encapsulated with the SLPs, the cytotoxic activity was even further enhanced.

Similar results were obtained in mice vaccinated with the formulation where the poly(I:C) was replaced by CpG in the mixture of the SLP separately loaded in liposomes (supplementary figure 1). In that case, the improved killing capacity induced by the liposomes was highest in mice having received the OVA24-lip.+OVA17-lip.+CpG vaccine, but lower when all compounds were co-encapsulated (OVA24/OVA17/CpG-lipos.) (supplementary figure 1B). Thus, the strength of the cytotoxic activity correlated with the $CD8^+$ T cell priming frequency detected after vaccination with the respective SLP-liposomes and TLR ligands (supplementary figure 1A). A similar effect was observed for the Pam3CSK$_4$-loaded formulations, although the adjuvant effect in that case was less pronounced, since the number of the induced SIINFEKL-specific T cells was significantly lower than that induced by CpG-adjuvanted liposomes.

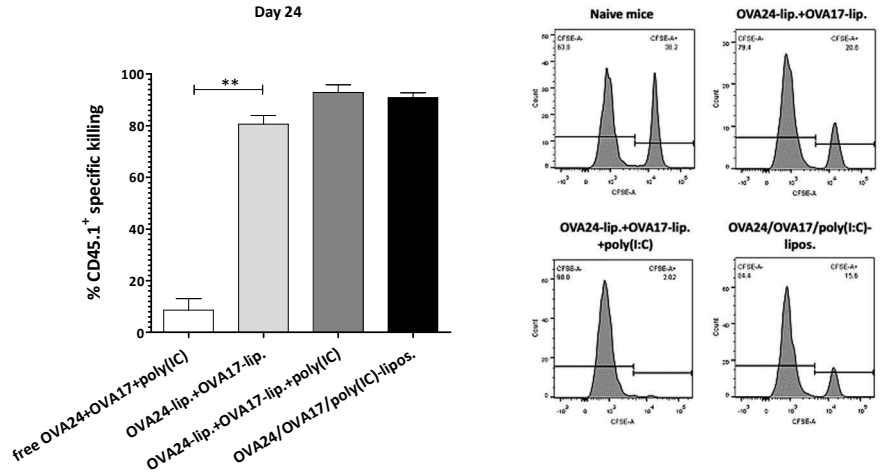

Figure 6: *In vivo* cytotoxicity assay. The mean percentages of the killing activity of OVA formulations are presented based on the frequency of the transferred CD45.1$^+$ specific target cells that could be detected in splenocytes of mice immunised with 1 nmol of OVA SLPs-loaded formulations. Bar graph shows the mean percentages of cells killing (+SEM) on day 24. ** p<0.005 calculated with by one-tailed Mann-Whitney test. Representative histograms of CFSE-labelled target cells: right peak=peptide pulsed and left peak= control.

To conclude, our data indicate that the immunogenicity of SLP-loaded liposomes can be enhanced with the incorporation of different TLR-immunostimulants, such as poly(I:C), CpG or $Pam3CSK_4$. However, adjuvanted formulations with poly(I:C) tend to be more versatile since the ligand in the presence of the T_H SLP OVA17, can be either co-encapsulated with the SLPs or be simply mixed before immunisation without compromising the strength and the quality of the induced T cell response.

Vaccine-mediated regression of established melanoma tumours

Our poly(I:C)-adjuvanted SLP-liposomal formulations induced high frequencies of $CD8^+$ T cells, functional $CD4^+$ helper cells and developed a high cytotoxic activity, as described above. Therefore, we investigated their potential as therapeutic anti-tumour vaccines in an established melanoma model (Figure 7). Nine days after the subcutaneous B16-OVA tumour cell inoculation, when tumours had reached a clear palpable size, mice were injected twice with the liposome vaccines intradermally in a two-week interval. Over a period of 60 days, all mice in groups immunized with poly(I:C)-liposomal formulations were able to control the outgrowth of the established tumours, whereas non-immunised mice or mice immunised with the free compounds (OVA24+OVA17+ poly(I:C)) had to be sacrificed within three weeks when the progressively growing tumours reached the humane endpoint of 2000 mm^3 tumour size. In detail, mice vaccinated with the separate OVA24 and OVA17 liposomal mixtures and poly(I:C) or the poly(I:C)-co-encapsulated SLPs (OVA24/OVA17/poly(I:C)-lip.) showed a substantial tumour regression with a delay of outgrowth of two to four weeks. No adverse side-effects were observed at the injection spot. Additionally, when the OVA24/OVA17/poly(I:C)-lip. formulation was delivered intradermally at the tail base instead of the abdominal area, a significant three to five weeks' delay in outgrowth was observed. Two out of eight mice were even cured of these aggressive tumours, indicating that vaccination effectiveness can be dependent on location and drainage of the injection site.

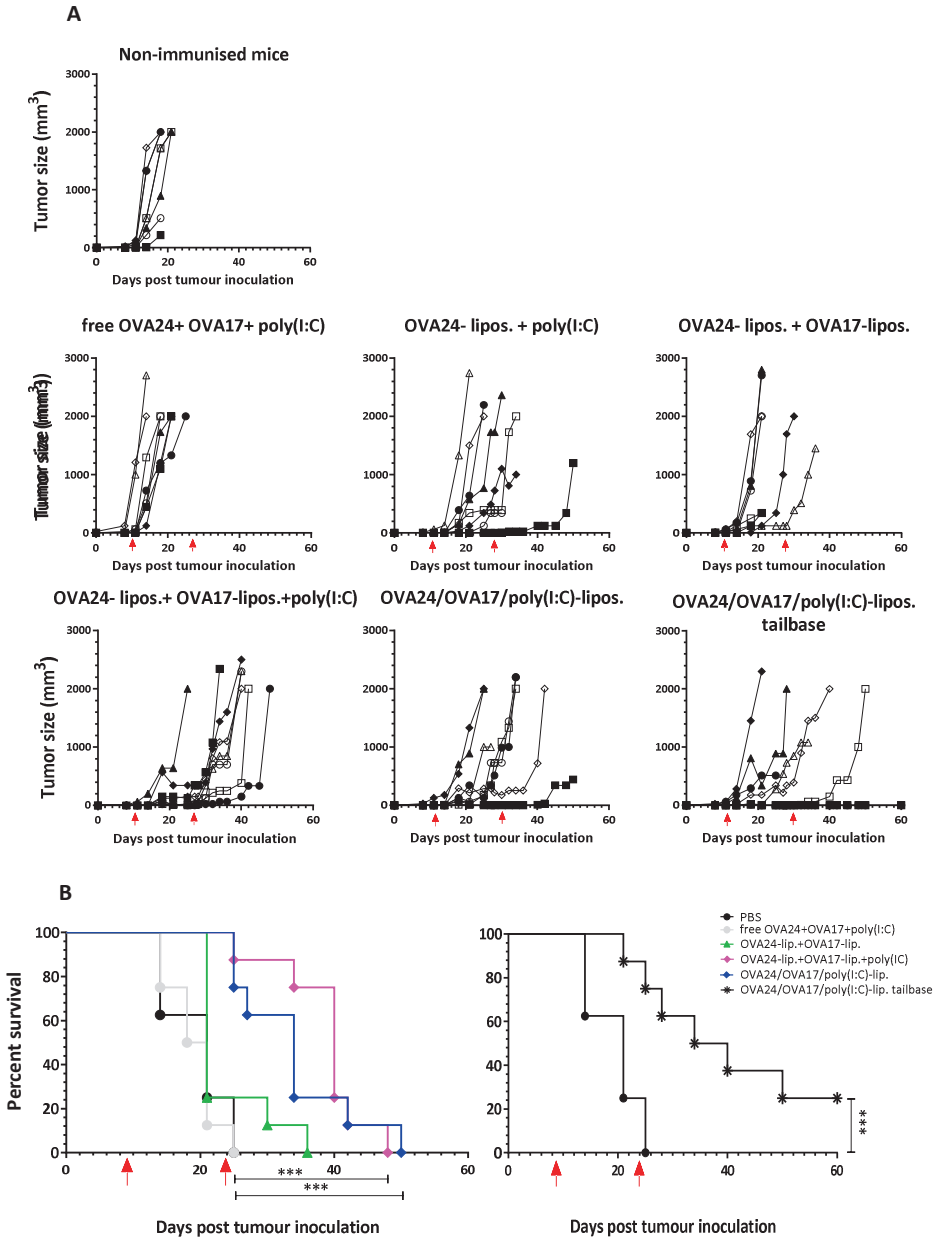

Figure 7: Tumour outgrowth in a therapeutic melanoma model, in mice immunised i.d. twice (as the red arrows indicate) with different OVA SLPs formulations, after subcutaneous injection on day 0 with $1x10^5$ B16-OVA cells. Tumour sizes in individual mice (A) and survival plots (left: i.d. abdominal skin vaccinations, right: tail base i.d. vaccination) (B) are presented. The curve of the two OVA SLPs+ poly(I:C) liposomal formulations are significantly different from the free OVA SLPs+ poly(I:C), *** P< 0.0001 calculated with the log-rank (Mandel-Cox) test. The experiment was performed twice with comparable results.

Discussion

For the development of a safe and effective therapeutic vaccine the choice of an appropriate delivery system and immune stimulator is critical. We showed previously how a poly(I:C)-adjuvanted liposomal formulation can be a powerful candidate for the induction of a strong and long-lasting cytotoxic cell-mediated immune response against OVA24, a relatively poorly immunogenic SLP antigen derived from the ovalbumin model protein [21]. In this study, we further investigated the potential of cationic liposomes, including a second SLP harbouring a T_H OVA epitope and comparing three defined TLR ligands poly(I:C) (TLR3), CpG (TLR9) or Pam3CSK$_4$ (TLR2), in order to optimise the formulation for effective tumour eradication. With respect to that, we have previously also shown that effective tumour vaccines require both tumour-specific CTL as well as tumour-specific T_H epitopes [22,24].

We and others have reported that the processing of SLPs by DCs makes them more efficient than both minimal peptide epitopes and whole proteins for the induction of a T cell-based immune response [9,25-27]. Moreover, clinical trials with overlapping SLPs of HPV16 E6 and E7 oncoproteins have shown their effectiveness in Montanide ISA-51 formulations [12]. Here, by inclusion of the OVA17 SLP in the OVA24-loaded liposomes we aimed to improve our formulation, enhancing the induction of antigen-specific $CD4^+$ T cells [28]. However, optimisation of the peptide loading was needed to ensure the highest possible encapsulation efficiency of both SLPs. As a result, the optimal conditions were determined by systematically studying the effect of pH of the SLP solutions before the dehydration step and the SLP concentrations on the respective loading efficiencies.

Furthermore, in liposomes prepared with different ratios of the initial peptides concentration (0-1.0 mg/ml) a competition between the two SLPs was observed, which appeared to greatly favour OVA24. This may be due to not only its lower pI, but also the higher number of acidic amino acid residues in the sequence, which makes it more negatively charged and can therefore allow it to have stronger interactions with the positively charged liposomal bilayer. In addition, OVA24 is considered to be less hydrophilic (based on the GRAVY hydropathicity scale) than OVA17. Depending on its configuration, the localization of OVA24 within the liposomes (e.g., partitioning between the aqueous compartment and the lipid bilayer) can be different from that of OVA17, which may contribute to its higher encapsulation efficiency. Taking into account that the initial OVA24/OVA17 concentration ratio of 1/1 (mg/ml) yields a final (w/w) ratio of 2, we prepared all the liposomes used for the described *in vivo* studies, although it is not

really known which is the optimal ratio between the CTL and T_H- containing SLP for therapeutic vaccination purposes.

Regarding the loading of the TLR ligands in the liposomes, the hydrophilic, negatively charged poly(I:C) and CpG are expected to be localised in the aqueous layer of the formulation; however, poly(I:C) can be also detected on the lipid bilayer, as it has been reported for liposomes formed by using the double emulsion method, since the positively charged ammonium head groups of the lipids interact with the negatively charged phosphate groups of poly(I:C) [15]. Both compounds have a negative charge at pH 8.0-9.0 which causes them to strongly interact with the cationic bilayer of the liposomes. In contrast, $Pam3CSK_4$ seem to compete with the peptide reducing its LE and/or its functionality compared to poly(I:C) or CpG, observation that might be attributed to its lipophilic nature. Altogether, the results of the liposome characterisation indicate not only that it is feasible to load two SLPs, either separately or co-encapsulated, into liposomes, combined with a TLR ligand, but also that the TLR ligands can be replaced and the peptide ratios can be adjusted to the desired final concentrations, making the method useful for more precise tailoring depending on the needed formulation. Our data suggests that it is not necessary to co-encapsulate separate peptide antigens in one liposome particle and this can be an advantage for better control and easier adjustment of peptide ratios in multiple long peptide vaccines.

We showed here that vaccination with cationic liposomes loaded with a second SLP covering an OVA T_H epitope, greatly increased the percentage of functional T cells producing IFN-γ and TNF-α. This finding is in line with the reported increase in expression of co-stimulatory molecules and effector cytokines after delivery of a T_H and a CTL epitope (due to the cognate stimulation of the specific $CD4^+$ T cells by T_H peptide-loaded MHC class II molecules displayed by DCs) followed by communication between CD40 and CD40 ligand (CD40L), which enhances the production of effector and memory $CD8^+$ T cells [29, 30]. Interestingly, the "help" by the presence of OVA17 in the formulation was effective independently of the way the SLP was formulated: co-encapsulated with OVA24 or separately loaded in liposomes and mixed shortly before administration.

In mice immunised with adjuvanted liposomal vaccines, the effect of the OVA17 on the expansion of T cell numbers seemed to be overshadowed by the presence of poly(I:C); however, in mice vaccinated with the mixture of the OVA24- and OVA17-liposomes, high frequencies of cytokine-producing $CD8^+$ and $CD4^+$ were detected, leading to a high killing capacity (above 80%) against the transferred target cells, similarly to the poly(I:C)-included formulations. The ability of our OVA24+OVA17- containing cationic liposomes

to directly activate cytokines-producing $CD8^+$ and $CD4^+$ T cells, is in line with reported observations according to which the simultaneous production of IFN-γ and TNF-α is an indication of the functional capacity of vaccine-induced $CD8^+$ T cells and induction of effector-memory cells, which is considered crucial for combating cancer [31,32].

Besides, delivery of a TLR ligand can aid the development of strong T cell memory, mediated by increased DC maturation and enhanced production of cytokines involved in activating $CD8^+$ T cells [33]. Vaccines containing the hydrophilic poly(I:C) (TLR3 ligand) or CpG (TLR9 ligand) induced high frequencies of effector-memory cells ($KLRG1^+$) after vaccination in non-tumour bearing mice, which indicates the superiority of these formulations to inhibit tumour out-growth when they are used in an *in vivo* therapeutic setting [31]. We have also previously shown that OVA24/poly(I:C)-loaded cationic liposomes can efficiently induce $KLGR1^+$ -expressing $CD8^+$ T cells [21].

Furthermore, despite the observation that the presence of the T_H epitope in the formulation seems to be sufficient for the initiation of a strong T cell-mediated immunity, for tumour eradication the delivery of a TLR ligand, such as poly(I:C), appeared to be important: using an aggressive melanoma model, the outgrowth of established tumours was better controlled in mice vaccinated with the poly(I:C)-adjuvanted liposomes, which survived for almost 2 weeks longer than mice treated with the non- adjuvanted liposomal formulations. In addition to that, when the liposomal formulation was delivered intradermally at the tail base, two out of eight mice were cured completely of their established melanoma tumours. Whereas the liposomes' processing mechanism after intradermal immunisation is still unknown, the poly(I:C)-adjuvanted liposomes appear to enhance most likely the targeting of the most specialised cross-presenting DCs (such as $CD8\alpha^+$ DCs). Combined with our previous findings [21] where intradermal immunisation appeared to be superior to subcutaneous immunisation for the induction of cytotoxicity, this observation suggests a difference in the formulation' ability to target cross-priming DCs, such as DCs locating in the lymph nodes; via not only different administration routes, but also injection sites, indicating a more effective drainage to relevant lymph nodes through the tail base area. Consequently, in cancer patients the choice of the vaccination site near functional draining lymph nodes in this active immune therapy approach may be crucially important.

Conclusions

Therapeutic vaccination against cancer, although not yet established as a standard treatment, is a potential approach to eradicate established tumours. Here we showed the improved efficacy of DOTAP-based SLP- and TLR ligand-loaded cationic liposomal formulations, compared to the free equivalent SLP vaccine. In particular, poly(I:C)-adjuvanted DOTAP-liposomes, loaded with two SLPs harbouring a model CTL- and a T_H-epitope, showed a strong ability to induce specific and functional T cells, and a superior capacity when used as a therapeutic vaccine in an aggressive melanoma tumour model. Their versatility regarding the loading of different antigens and TLR ligands makes them a very promising platform for the treatment of cancer or chronic infectious diseases.

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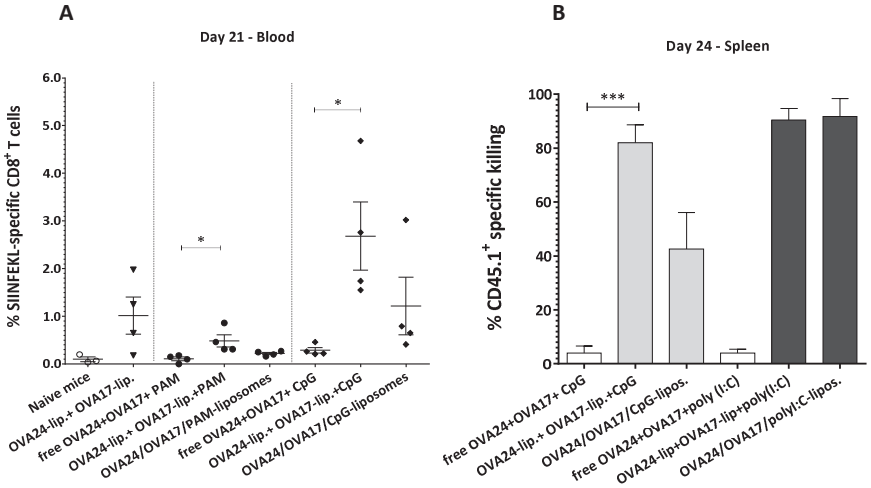
Supplementary data



Supplementary figure 1: Antigen-specific $CD8^+$ T cells in blood on day 21 in $Pam3CysK_4$ and CpG-adjuvanted liposomes (A) and *in vivo* cytotoxicity in mice immunised with CpG-adjuvanted formulations compared to poly(I:C)-loaded liposomes on day 24 (B) in mice immunised with formulations including 1 nmol OVA24. * $p<0.05$ and *** $p<0.001$ evaluated by one-way ANOVA with Tukey's post hoc test and calculated with Mann-Whitney non-parametric test.

Chapter 4

Synthetic long peptide-based vaccine formulations for induction of cell mediated immunity: a comparative study of cationic liposomes and PLGA nanoparticles

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Abstract

Nanoparticulate formulations for synthetic long peptide (SLP)-cancer vaccines as alternative to clinically used Montanide ISA 51- and squalene-based emulsions are investigated in this study. SLPs were loaded into TLR ligand-adjuvanted cationic liposomes and PLGA nanoparticles (NPs) to potentially induce cell-mediated immune responses. The liposomal and PLGA NP formulations were successfully loaded with up to four different compounds and were able to enhance antigen uptake by dendritic cells (DCs) and subsequent activation of T cells *in vitro*. Subcutaneous vaccination of mice with the different formulations showed that the SLP-loaded cationic liposomes were the most efficient for the induction of functional antigen-specific T cells *in vivo*, followed by PLGA NPs which were as potent as or even more than the Montanide and squalene emulsions. Moreover, after transfer of antigen-specific target cells in immunised mice, liposomes induced the highest *in vivo* killing capacity. These findings, considering also the inadequate safety profile of the currently clinically used adjuvant Montanide ISA-51, make these two particulate, biodegradable delivery systems promising candidates as delivery platforms for SLP-based immunotherapy of cancer.

Keywords: cellular immune response, synthetic long peptides, TLR ligands, cationic liposomes, PLGA nanoparticles

Introduction

Peptide-based vaccine formulations offer several advantages over protein-based vaccines, as peptides can be easily synthesized and characterized, and are generally more stable [1] and better processed [2] than whole proteins. Synthetic peptides derived from tumour- associated antigens (TAAs) have attracted considerable interest as a basis for cancer vaccines, and vaccination with synthetic long peptides (SLPs), containing all the CTL and T_H epitopes of a TAA, has been applied in mouse models with superior efficacy to protein antigen [2] or minimal MHC class I restricted epitopes [3,4]. In contrast to short peptides, SLPs cannot bind directly to MHC molecules, but have to be taken up and processed by DCs like regular pathogens, inducing a stronger immune response, owing to the activation of both $CD4^+$ and $CD8^+$ T cells [4-6]. However, peptides alone are poorly immunogenic and need to be combined with adjuvants such as immune modulators and/or delivery systems in order to properly activate the innate and adaptive arms of the immune system [1].

Over the past few years, delivery systems that elicit strong immune responses, such as nano-emulsions and particulate delivery systems have been extensively studied. These include MF59 (Novartis) and AS03™ (GlaxoSmithKline), squalene-based oil-in-water emulsions, which have been approved in Europe for use in the Fluad® and Pandemrix™ influenza vaccines, respectively [7]. Despite the efficacy of these emulsions as influenza vaccine adjuvants, and though some degree of T_H1 responses have been observed, still they lack the ability to stimulate strong T cell responses [8]. Montanide (ISA-51, Seppic) water-in-oil (w/o) emulsions have shown to elicit CTL responses in clinical studies, and have been applied to formulate SLPs in several clinical therapeutic cancer vaccination trials [9-15]. However, the use of Montanide has some important limitations, such as non-biodegradability, limited long-term stability, poorly defined release properties, suboptimal efficacy, and in some cases induction of local adverse side effects [16,17]. Therefore, alternative delivery systems for SLP-based vaccines are highly needed.

Studies have shown that peptide-based vaccines may benefit from particulate delivery systems that mimic the size and structure of a pathogen, facilitating uptake by DCs and increasing cross-presentation of the peptide [18-20]. Importantly, they can harbour multiple vaccine components and be actively or passively targeted to DCs, also shaping the induced immune response via specific receptors, such as toll-like receptors, by incorporation of TLR ligands. Among several particulate delivery systems, both liposomes and polymeric particles have been widely studied.

We have previously studied the application of poly-(lactic-co-glycolic-acid) (PLGA) NPs [21] and cationic liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [22] for the encapsulation of a 24-amino acid-long SLP (referred to as OVA24) harbouring the CTL epitope SIINFEKL of ovalbumin (OVA). Encapsulation of this SLP in PLGA NPs led to a significant enhancement of MHC class I antigen presentation and $CD8^+$ T cell activation compared to free SLP *in vitro* [21]. The SLP-specific $CD8^+$ T cell frequency induced *in vivo* by a liposomal SLP formulation containing poly(I:C) showed a 25-fold increase compared to poly(I:C)-adjuvanted free SLP. Furthermore, intradermal immunisation of mice with SLP-liposomes combined with poly(I:C) led to a strong cytotoxic activity, in contrast to vaccination with a mixture of free SLP and poly(I:C) [22].

In this study, considering the different physicochemical properties that cationic liposomes and PLGA NPs have, we further investigated the potential of both systems in a direct comparative study. For that purpose, we studied the co-delivery of two SLPs containing the CTL (OVA24) and the T helper (T_H, OVA17) epitopes of OVA together with poly(I:C) and $Pam3CSK_4$, a TLR3 and TLR2/1 ligand, respectively, in comparison to the clinically used adjuvants Montanide ISA-51 and SWE, a squalene oil-in-water emulsion. OVA24/OVA17-loaded PLGA NPs and liposomes with or without the TLR ligands were characterized for particle size, zeta-potential, and for peptide and TLR loading efficiencies. The obtained formulations were assessed *in vitro* and *in vivo* for their potency to induce $CD8^+$ and $CD4^+$ T cell immune responses. The observed T cell immune responses induced by our particulate formulations were superior to the ones observed with the emulsions (Montanide ISA-51 or SWE), with the liposomal formulation outperforming PLGA NPs. These findings reinforce that particulate systems are promising delivery vehicles for clinical application in cancer immunotherapy.

Materials and Methods

Materials

The ovalbumin-derived SLP OVA24 [DEVSGLEQLESIINFEKLAAAAAK], including the CTL epitope SIINFEKL, and the short peptide OVA8 [SIINFEKL] were produced and purified at the GMP facility of the Clinical Pharmacy and Toxicology Department at the Leiden University Medical Center [5]. The ovalbumin-derived SLP OVA17 [ISQAVHAAHAEINEAGR], including the helper T_H-epitope AAHAEINEA, was produced in the Immunohematology and Blood Transfusion Department of the Leiden University Medical Centre. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and the TLR ligands (poly(I:C) and Pam3CSK$_4$) with their labelled analogues (rhodamine and fluorescein) were obtained from InvivoGen (Toulouse, France). Resomer® RG 502H was purchased from Boehringer Ingelheim (Ingelheim, Germany). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) was obtained from Biosolve BV (Valkenswaard, the Netherlands), PVA 4-88 (31 kDa) was purchased from Fluka (Steinheim, Germany). Sodium hydroxide was purchased from Boom (Meppel, Netherlands). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, Netherlands), 100 IU/ml penicillin and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionized water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ water). Montanide ISA-51 was purchased from Seppic SA (Paris, France). Squalene oil-in-water emulsion (SWE) contained 3.9% (w/v) squalene, 0.5% (w/v) Tween 80 and 0.5% (w/v) Span 85 in 10 mM citrate buffer pH 6.5 and it was manufactured by the Vaccine Formulation Laboratory of the University of Lausanne. Phosphate buffer was composed of 7.7 mM $Na_2HPO_4.2H_2O$ and 2.3 mM $NaH_2PO_4.2H_2O$, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, which was used for all the *in vitro* and *in vivo* assays was purchased from B. Braun (Meslungen, Germany). All other chemicals were of analytical grade and all aqueous solutions were prepared with milli Q water.

Mice

Female C57BL/6 (H-2^b) mice were purchased from Charles River (L'Arbresle, France) and congenic CD45.1 (Ly5.1) mice were bred at the Leiden University Medical Centre animal facility and used at 8-14 weeks of age according to the Dutch Experiments on Animal Act, which serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe.

Liposome preparation

Cationic liposomes loaded with SLPs were prepared by using the thin film dehydration-rehydration method, as described previously [22]. Briefly, DOTAP and DOPC (1:1 molar ratio) in chloroform were mixed in a round-bottomed flask to reach a concentration of 10 mg total lipid per ml of final liposome dispersion. The formed dry film was rehydrated with 2 ml of 1 mg/ml OVA24 and/or OVA17 in ACN/H_2O 1:1 (v/v); for the liposomes loaded with both OVA24 and OVA17, the aqueous solution of the SLPs was first adjusted to pH 8.5. For poly(I:C)-loaded liposomes, the ligand (including 0.5% fluorescently-labelled equivalent) in a total concentration of 200 μg/ml was added dropwise to the dispersion, while for the Pam3CSK_4-loaded liposomes, the TLR ligand was dissolved in chloroform together with the lipids, before the dry film formation. After the lipid film hydration, the liposome dispersion was snap-frozen in liquid nitrogen, followed by overnight freeze-drying. Dehydrated-rehydrated liposomes were generated by gradually adding 10 mM PB, pH 7.4, to the freeze-dried lipid cake. Liposomes were down-sized by high-pressure extrusion at room temperature using a Lipex extruder (Northern Lipids Inc., Canada) and concentration of peptide-loaded liposomes was performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa) as described previously [22].

PLGA NPs preparation

Nanoparticles loaded with OVA24 and/or OVA17 and/or TLRLs were prepared by using a double emulsion with solvent evaporation method [21]. In brief, 50 mg of PLGA dissolved in 1 ml of dichloromethane, with or without 0.25 mg Pam3CSK_4 (and 0.1% Pam3CSK_4 Rhodamine-labelled), were emulsified under sonication (30 s, 20 W) with 1.4 mg OVA24, 1 mg OVA17, 1 mg poly(I:C) (and 0.1% Poly(I:C) fluorescein-labelled, dissolved in 50% ACN in 0.25 mM NaOH + 400 μL HEPES pH 8.0). To this first emulsion (w1/o), 2 ml of 1% PVA solution were added immediately, and the mixture was emulsified again by sonication (30 s, 20 W), creating a double emulsion (w1/o/w2). The emulsion was then added dropwise to 10 ml of extraction medium (0.3% w/v PVA)

previously heated to 40°C under agitation, to allow quick solvent evaporation, while stirring, which was continued for 1 h. The particles were then collected by centrifugation for 15 min at 15000 g at 10°C, washed, resuspended in deionized water, aliquoted and freeze-dried at -55°C in a Christ Alpha 1-2 freeze-dryer (Osterode am Harz, Germany) for 12 hours.

Liposome and PLGA NP characterization

Average diameter (Z_{ave}) and polydispersity index (PDI) of the formulations were determined by dynamic light scattering (DLS) using a Zetasizer (NanoZS, Malvern Ltd. UK). The same instrument was used for zeta-potential determination by laser Doppler electrophoresis. For these measurements, liposome samples were diluted 100-fold in PB, pH 7.4. Peptide loading efficiency was determined by extracting OVA24 and OVA17 from the liposomes using a modified Bligh-Dyer method and applying a UPLC method, as described previously [22].

For the PLGA NPs, the Z-average size, polydispersity index and zeta-potential were measured after the freeze-dried NP were resuspended in 1 mM HEPES pH 7.4 to a final concentration of 10 mg PLGA/ml. Peptides' loading efficiency was determined by measuring the peptide content of digested particles by reversed phase HPLC, as described previously [21].

Loading efficiency of poly(I:C) and $Pam3CSK_4$ was calculated by fluorescence detected with an Infinite® M 1000 Pro (Tecan, Switzerland) microplate reader (excitation/emission wavelengths: 492 nm/518 nm for fluorescein and 549 nm/566 nm for rhodamine).

Montanide ISA-51 and SWE emulsions preparation and characterization

Preparation of Montanide ISA-51 emulsion was performed by diluting the SLPs, $Pam3CSK_4$ and poly(I:C) in PBS and mixing with Montanide ISA-51 water-in- oil for 30 min in a 1:1 (v/v) ratio, using a vortex mixer. The squalene-based formulation, SWE, was prepared as previously described [23,24]. For the loading of the SLPs and adjuvants, the SWE was diluted with vaccine medium to 2% (v/v) squalene to the same ratio and mixed gently for 10s prior to immunisation.

In vitro MHC class I antigen presentation

Immature D1 cells were incubated in 96-well flat-bottomed plates at 37°C in supplemented IMDM with SLP-loaded formulations or plain SLP (with or without TLR

ligands) in PBS at different concentrations. After 2.5 hours T cell reporter hybridoma B3Z cells ($50x10^5$/well) were added and the mixture was incubated overnight at 37°C. Chlorophenol red-β-galactopyranoside (CPRG) was used as lacZ substrate in cell lysates and colour conversion was measured by detecting absorbance at 590 nm.

Immunisation of mice

Mice were immunized with SLP-loaded formulations or free peptides, OVA24 and OVA17 (with or without TLR ligands), by subcutaneous injection in the tail base. All formulations were prepared on the day of injection. Vaccination dose was based on the OVA24 SLP concentration, 1 nmol (2.5 µg) of peptide in a total volume of 100 µl, and immunisations were performed on day 0 (prime immunisation) and on day 14 (boost injection). Vaccinations with adjuvanted vaccines included a dose of 0.5 – 1.0 µg of a TLR ligand. During the *in vivo* studies, blood samples were obtained from the tail vein at different time points.

Analysis of antigen-specific $CD8^+$ and $CD4^+$ T cell responses by flow cytometry

Staining of the cell surface was performed on blood samples after red blood cell lysis. Cells were stained in staining buffer for 30 min with allophycocyanin labelled tetramer-OVA8 (TM-SIINFEKL) and fluorescently labelled antibodies specific for mouse CD3 (BD Biosciences), CD4, CD8 (eBiosciences). 7-Aminoactinomycin D (Life Technologies) was used for the exclusion of dead cells.

Overnight intracellular cytokine analysis of PBMCs was performed after incubating the cells with 2 µM of OVA8 and 2 µM of OVA17, in presence of brefeldin A (7.5 µg/ml) (BD Biosciences, Breda, the Netherlands). After the overnight cells incubation the assay was developed as previously described [22].

In vivo cytotoxicity assay

Splenocytes from naive congenic $CD45.1^+$ mice were lysed and split into two equal parts. Cells were labelled with CFSE and adoptively transferred intravenously in previously immunized recipient C57BL/6 mice in a volume of 200 µl in PBS as described [22]. Two days after the cell transfer (day 24), mice were sacrificed, spleens were isolated and single cell suspensions were analysed by flow cytometry. Specific killing (SK) was calculated according to the following equation:

$$\mathrm{SK} = \left\{ 1 - \frac{\left[\frac{\mathrm{CFSE\ high}}{\mathrm{CFSE\ low}}\ \mathrm{vaccinated\ mice}\right]}{\left[\frac{\mathrm{CFSE\ high}}{\mathrm{CFSE\ low}}\ \mathrm{naive\ mice}\right]} \right\} \mathrm{x}\ 100\ \%$$

Results

Characterisation of adjuvanted SLP-loaded liposomes and PLGA NPs

We have previously shown that effective tumour vaccines require the inclusion of both CTL and T_H epitopes [5,25]. In this study, a 24-mer SLP covering a CTL epitope and a 17-mer covering a T helper epitope of ovalbumin, designated as OVA24 and OVA17, respectively, were used as model antigens to study the effect of co-encapsulation of these SLP adjuvanted with poly(I:C) and $Pam3CSK_4$ (TLR3- and TLR2-ligands, respectively) in liposomes and PLGA nanoparticles (NPs). Our main objective was the direct comparison of the immunogenicity of the different systems, relative to that of a squalene- oil-in-water emulsion (SWE) used at preclinical stage studies and the clinically Phase I/II used Montanide ISA-51 water-in-oil emulsion.

SLP-loaded liposomes were prepared by adjusting the dehydration-rehydration method so that the highest loading of both SLPs in the liposomes was achieved. As we reported for OVA24 [22], entrapment of OVA17 in the liposomes seems to be also dependent on electrostatic interactions between the cationic liposomes and the peptide. OVA17 has an isoelectric point (pI) of 6.0 (higher than the pI of OVA24, ca. 4.25) and the highest loading efficiency for both SLPs, when separately loaded or co-encapsulated in liposomes, was achieved at a relatively high pH of about 8.5, i.e., above their isoelectric point (pI). At this pH, both peptides are expected to be strongly negatively charged, favouring the electrostatic interactions with the positively charged liposomes and yielding the highest loading efficiency for both SLPs.

The obtained SLP-liposomes had an average diameter that ranged from 147 nm (only SLPs-loaded liposomes) to 180 nm (OVA24/Pam/poly(I:C)-loaded liposomes). Liposomes with poly(I:C) or poly(I:C) combined with $Pam3CSK_4$ were larger and less monodisperse (PDI > 0.2). The positive zeta-potential was about 26 mV, independent of the formulation (Table I). The loading efficiency of OVA24 and OVA17 in unadjuvanted liposomes was about 46 % and 20%, respectively, and practically independent of the co-encapsulation of poly(I:C) and $Pam3CSK_4$ (Table I), suggesting that there is no competition between the TLRLs and the two peptides.

PLGA NPs were prepared by a double emulsion with solvent evaporation method described by Silva et al. [21]. Irrespective of the type of the loading, PLGA NPs were negatively charged, with a zeta-potential ranging from -11 to -14 mV (Table I), with a final particle size varying from 260 to 360 nm and a PDI below 0.3. The loading efficiency varied between 21 - 30% for OVA24, 31 – 36% for OVA17, 65 – 75% for $Pam3CSK_4$ and

53 – 73% for poly(I:C). We have previously shown the importance of the pH of the inner aqueous phase for the effective encapsulation of peptides in PLGA NPs and how crucial low burst release is in order to induce a cellular response [21]. Therefore, both OVA24 and OVA17 SLP were formulated at pH 8.0 to achieve optimal encapsulation and a relatively low burst release of circa 30% within 24 h (data not shown).

Table I: Physicochemical properties of SLP (+TLR ligand)-loaded formulation

				LE (%)			
	Z_{ave} diameter (nm)	PDI	ZP (mV)	OVA24	OVA17	TLR ligand Pam3CSK$_4$	TLR ligand Poly(I:C)
OVA24/Pam/poly(I:C) liposomes	180 ±10	0.29 ±0.03	26 ±3	56 ±5	NA	40 ±5	60 ±6
OVA24/OVA17 liposomes	147 ±10	0.21 ±0.02	25 ±2	46 ±7	20 ±5	*NA*	
OVA24/OVA17/Pam/poly(I:C) liposomes	175 ±20	0.27 ±0.05	26 ±2	46 ±5	20 ±5	40 ±5	55 ±8
OVA24/OVA17/poly(I:C) liposomes	173 ±20	0.28 ±0.05	27 ±2	42 ±10	20 ±3	*NA*	60 ±4
OVA24/Pam/poly(I:C) PLGA	260 ±19	0.19 ±0.02	-14 ±1	26 ±3	*NA*	67 ±7	60 ±2
OVA24/OVA17 PLGA	355 ±13	0.24±0.02	-14 ±1	30 ±10	36 ±6	*NA*	
OVA24/OVA17/Pam/poly(I:C) PLGA	357 ±45	0.22 ±0.02	-14 ±2	21 ±8	31 ±5	65 ±7	73 ±5
OVA24/OVA17/poly(I:C) PLGA	350 ±23	0.22 ±0.02	-14 ±3	29 ±10	35 ±6	NA	70 ±8
OVA24/OVA17/Pam/poly(I:C) ISA-51	500 ±20	0.25 ±0.04	42 ±5	*NA*	*NA*	*NA*	
OVA24/OVA17/Pam/poly(I:C) squalene (SWE)	136 ±15	0.15 ±0.00	-20 ±3	*NA*	*NA*	*NA*	

Data are averages ± SD of at least 3 independent batches
Z_{ave} average particle diameter in nanometres, *PDI* polydispersity index, *ZP* zeta-potential, *LE* loading efficiency, *NA* not applicable

Altogether, the data presented in table I shows that the model SLPs, OVA24 and OVA17, can be efficiently and reproducibly loaded in liposomes and PLGA NPs, also in presence of up to two TLR ligands.

In vitro SLP cross-presentation

The immunogenicity of the SLP formulations was first tested *in vitro*, based on their efficiency to activate immature DCs and cross-present the processed SIINFEKL-harbouring peptide (OVA24) to the $CD8^+$ antigen-specific (B3Z) T cells leading to their activation. Immature DCs were incubated with liposomes and PLGA NPs including both SLPs and both TLR ligands. Improved concentration-dependent activation of $CD8^+$ T cells was observed when particles were used compared to free peptides (OVA24+OVA17+Pam+poly(I:C)). Moreover, although PLGA NP plots consistently show

a larger dose-response effect, liposomes seem to be more effective in antigen presentation at lower concentrations (Figure 1).

Furthermore, the B3Z assay suggests efficient processing and presentation of OVA24 SLP by the DCs, irrespective of the presence of the OVA17 SLP (Figure 1). In addition to that, the incorporation of the TLR ligands poly(I:C) and Pam3CSK$_4$ did not significantly affect the *in vitro* T cell activation by the SLP-loaded particle formulations, as expected (Figure 1).

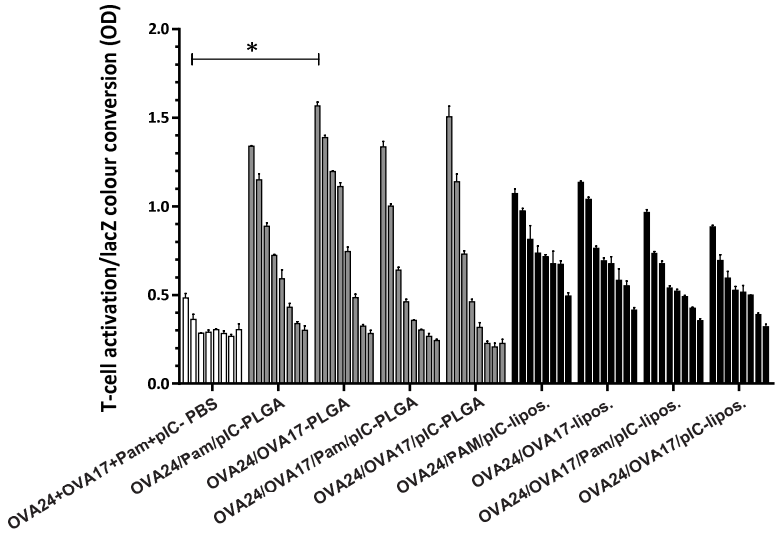

Figure 1: *In vitro* OVA-specific CD8$^+$ T cell priming. SIINFEKL-specific B3Z T cell activated after overnight co-culture with DCs and titrated amounts (4.0-0.0625 µM, 2-fold dilution series) of SLPs/TLR ligands-loaded formulations. Graphs show T cell activation based on the optical density (OD) at 590 nm after colour conversion of cell lysates after addition of CPRG (lacZ substrate). *p<0.05, evaluated with one-way ANOVA with Tukey's post hoc test and calculated with Mann-Whitney non-parametric test. Pam = Pam3CSK$_4$; pIC = poly(I:C)

In vivo induction of CD8$^+$ T cells

We evaluated the capacity of our particulate formulations to induce cell-mediated immune responses *in vivo* after subcutaneous vaccination at the tail base of mice, an administration route that appeared to enhance drainage to the lymphatic system in a more efficient way compared to subcutaneous delivery in the flank (unpublished data). The *in vivo* vaccine potency of liposomes and PLGA NPs was directly compared with that of the Montanide ISA-51 and SWE adjuvants.

In blood of mice immunized twice with SLP(s)-loaded liposome or PLGA NP formulations, on day 21 a high percentage of antigen-specific $CD8^+$ T cells (above 1% of the total $CD8^+$ T cell population) was detected in most groups, whereas in the Montanide ISA-51 or SWE groups this percentage remained below 0.5% (Figure 2A). In detail, it appeared that the OVA24/Pam/poly(I:C)-liposomes were more potent than the OVA24/Pam/poly(I:C)-PLGA NPs, expanding the percentages of antigen-specific $CD8^+$ T cells to about 6% and 2%, respectively.

Incorporation of the T_H epitope-SLP (OVA17) did not seem to improve the $CD8^+$ T cell proliferation induced by the PLGA NP formulations, while the addition of at least one TLR ligand, such as poly(I:C), seemed to be essential for T cell activation.

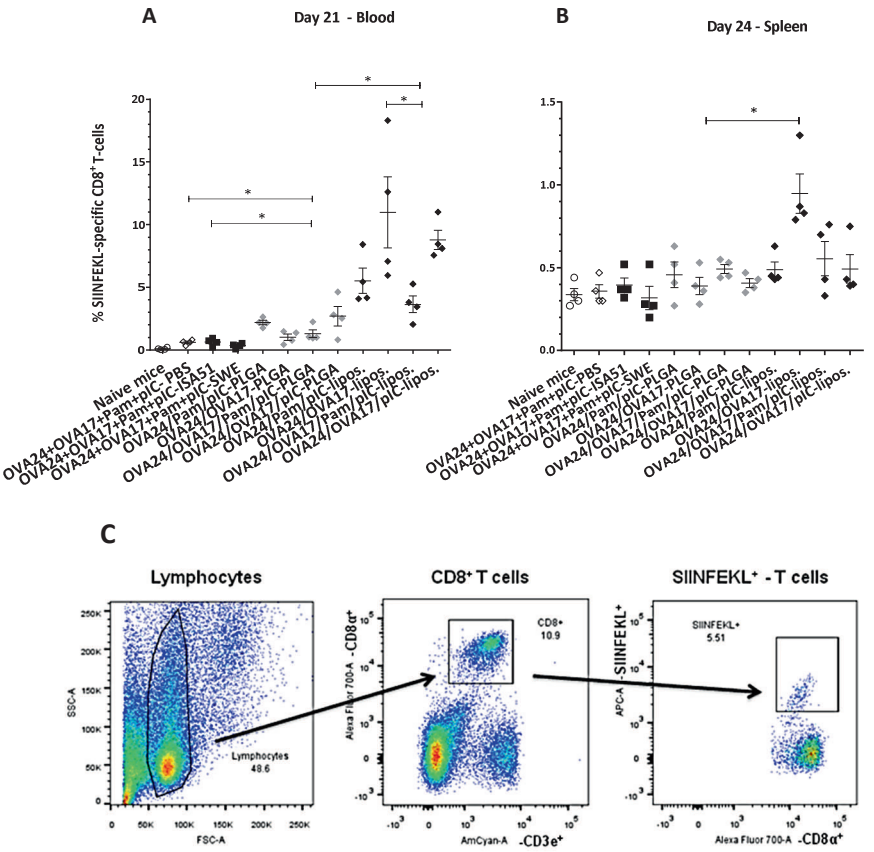

Figure 2: OVA24-specific $CD8^+$ T cell responses in blood (Day 21) and in splenocytes (Day 24) following subcutaneous immunisation with 1 nmol of SLPs on day 0 and 14 (A and B). Representative gating strategy for detection of SIINFEKL-specific $CD8^+$ T cells by flow cytometry using specific MHC class I tetramers (C). * $p<0.05$, ** $p<0.01$ calculated with by one-tailed Mann-Whitney test. ISA51= Montanide; Pam = $Pam3CSK_4$; pIC = poly(I:C)

On the other hand, incorporation of the two SLPs in the liposomes (OVA24+OVA17) seemed to be sufficient for the induction of the highest frequency of $CD8^+$ T cells (> 5 %) in blood of vaccinated mice, compared to all other liposomal groups (Figure 2A). Incorporation of poly(I:C) to the formulation with the two SLPs did not seem to further increase the number of the induced T cells in blood, while co-encapsulation of the lipophilic $Pam3CSK_4$ seemed to result in lower numbers of antigen-specific T cells. On day 24 in the analysed splenocytes of vaccinated mice, the *ex vivo* (non-restimulated) T cell responses in most groups were comparably low, except for the OVA24/OVA17-liposomes group, which showed a $CD8^+$ T cell frequency of about 1% (Figure 2B).

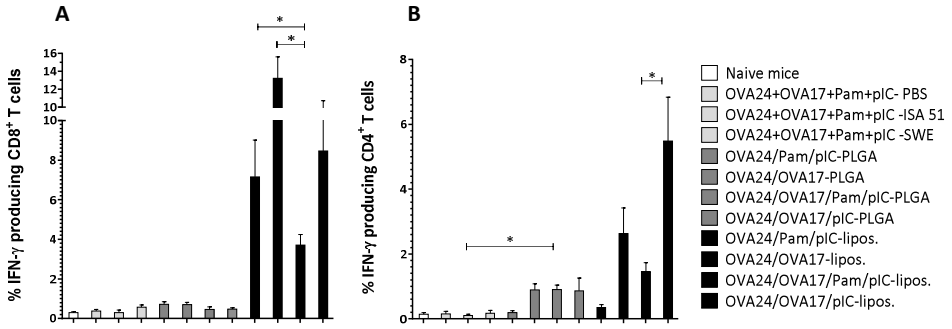

Figure 3: Intracellular cytokine analysis after immunization with OVA SLP-loaded particles. Blood of immunized mice at day 21 was stimulated *ex vivo* overnight with the minimal SIINFEKL epitope and OVA17. Plots show percentages (+SEM) of $CD8^+$ (A) and $CD4^+$ (B) T cells producing interferon gamma (IFN-γ). * $p<0.05$, calculated with by one-tailed Mann-Whitney test. ISA51 = Montanide; Pam = $Pam3CSK_4$; pIC = poly(I:C)

In vivo cytokine and cytotoxicity induction

Next to the T cell expansion potency of the vaccine formulations also the cytokine-producing functionality of the induced T cells was analysed. Thus, blood samples from the immunized mice were re-stimulated with MHC class I and class II binding peptides (short SIINFEKL and OVA17) *ex vivo* and the percentages of $CD8^+$ and $CD4^+$ T cells producing interferon gamma (IFN-γ) or IFN-γ and TNF-α simultaneously (data not shown), were assessed by intracellular cytokine staining.

In re-stimulated blood samples from mice immunized with PLGA NPs loaded with OVA24, $Pam3CSK_4$ and poly(I:C) (OVA24/Pam/polyI:C-PLGA) a higher percentage of cytokine-producing $CD8^+$ T cells was detected (ca. 0.7%) in comparison to the Montanide ISA-51 formulation (ca. 0.3%). This frequency did not increase after loading of OVA17 in the formulation, while all PLGA NPs showed a stronger induction of IFN-γ-producing

$CD4^+$ T cells (ca. 0.8 %) in comparison with the Montanide ISA-51 and SWE emulsions (ca. 0.1 %) (Figure 3B).

Mice vaccinated with liposomes loaded with both SLPs, with or without the two TLR ligands (OVA24/OVA17/Pam/poly(I:C)-liposomes and OVA24/OVA17-liposomes, respectively) showed an at least eight-fold higher efficiency to induce functional IFN-γ-producing $CD8^+$ T cells, as compared to the Montanide ISA-51- and SWE formulations (Figure 3A). As also observed in the SIINFEKL-specific $CD8^+$ T cell induction analysis (Figure 2), incorporation of poly(I:C) and $Pam3CSK_4$ into liposomes did not increase the number of induced T cells, although still much higher than the free SLP and TLR ligands. Although $Pam3CK_4$ is known to improve SLP vaccination by itself [25], its incorporation into the liposomes seems to have a inhibitory effect on the vaccination efficacy of the cationic liposomes, suggesting a possible change in the liposomes properties which influences the particles targeting.

Altogether, all liposomal formulations appeared to be significantly more efficient than any of the PLGA formulation tested (Figure 3). Furthermore, apart from $CD8^+$, functional $CD4^+$ T cells were detected in all groups treated with OVA17 (+poly(I:C))-containing liposomal formulations as well as PLGA NPs, indicating that OVA17 retains its functionality when co-encapsulated with OVA24 (Figure 3) and pointing out the importance of poly(I:C) presence for induction of antigen-specific $CD4^+$ T cells.

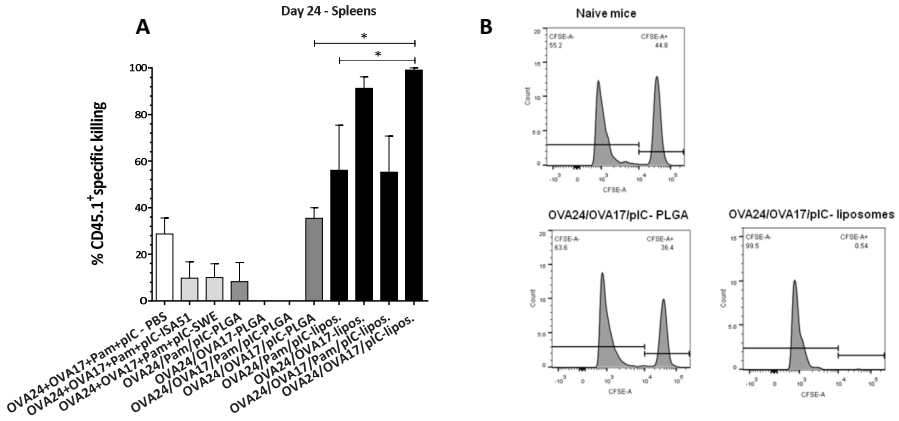

Figure 4: *In vivo* cytotoxicity against SIINFEKL-presenting target cells. The mean percentage of the killing activity of each SLP formulation is presented based on the frequency of the transferred $CD45.1^+$ cells that could be detected in splenocytes of mice immunized twice with the different formulations containing 1 nmol of OVA24. Bar graphs show the mean percentages (+SEM) of killed cells on day 24 (A). Representative histograms of CSFE-labelled positive target cells (right peak=SLP pulsed and left peak=negative control) (B). * $p<0.05$ calculated with by one-tailed Mann-Whitney test. ISA 51= Montanide; Pam = $Pam3CSK_4$; pIC = poly(I:C)

Finally, to test the effectiveness of our particulate vaccines to induce a strong functional cell-mediated immune response, the cytotoxic capacity of the induced $CD8^+$ T cells was tested in an *in vivo* cytotoxicity immunoassay, based on the killing of SIINFEKL-loaded target cells which were injected intravenously on day 22 in immunised mice (Figure 4).

Incorporation of poly(I:C) into PLGA NPs including both SLPs (and not $Pam3CSK_4$) appeared to be crucial for activation of $CD8^+$ T cells with a cytotoxic activity up to 40%, four times as high compared to the emulsions (Figure 4). In splenocytes of all mice vaccinated with liposomes a high killing capacity above 60% was detected. Mice immunised with liposomes containing both SLPs (OVA24/OVA17-liposomes) showed maximal *in vivo* cytotoxicity with or without poly(I:C).

According to the functional data of both the intracellular cytokine production and cytotoxicity assay, we can conclude that liposomal formulations loaded with the SLPs are superior to both PLGA NPs and Montanide ISA-51 or squalene-SLP-contained emulsions, when a rather low dose of SLPs (≤ 1 µg of SLP) is used like in the current study. Moreover, it appeared that the inclusion of an adjuvant ($Pam3CSK_4$ or poly(I:C)) in the liposomal formulation might not be necessary for the priming of a T cell immune response, since solely the presence of the T_H epitope seems to facilitate a T_H1-type pro-inflammatory immune reaction which is essential for effective therapeutic vaccines, to support a robust and long-lasting anti-tumour $CD8^+$ T cell response.

Discussion

There is a growing interest in therapeutic vaccination against cancer. The identification of tumour associated antigens (TAAs) has allowed the development of novel therapeutic strategies resulting in tumour regression. However, fine-tuned vaccines are required to reach the optimal potency and eventually replace the suboptimal formulations currently used. A major advantage of using particulate vaccines, such as liposomes and PLGA NPs, is their modularity. By tuning their physicochemical properties like size, charge and hydrophobicity, not only the stability of the particles and their antigen release pattern can be improved, but also the amount of antigen uptake by DCs, and priming of DCs towards cross-presentation, resulting in a more effective T_H1 type CTL response, required for cancer immunotherapy.

In this study we showed the successful co-encapsulation of two SLPs (OVA24 and OVA17) and two TLR ligands (poly(I:C) and $Pam3CSK_4$) in two different particulate delivery systems. The SLP-loaded and adjuvanted PLGA NPs and cationic liposomes were rather different in size and surface charge since PLGA NPs are negatively charged and liposomes are positively charged, while their different chemical nature is expected to influence the SLPs/TLR ligands localization and *in vivo* release profiles. Considering all possible differences, their potential for the induction of a cell-mediated immunity was investigated in comparison with two other systems, Montanide ISA-51, a water-in-oil emulsion, and SWE, a squalene-based oil-in-water emulsion.

As we showed here, co-delivery of a T_H antigen with a CTL epitope increases the expression of effector cytokines. This is most likely due to the stimulation of the MHC molecules displayed by DCs: CD40/CD40L ligation plays an important role in the activation of DCs and is a crucial stimulus for $CD4^+$ T_H-based $CD8^+$ T cell priming [26,27]. In addition, incorporation of a TLR ligand to the formulation, such as poly(I:C), can promote the active targeting and shape the immune response towards a more CTL--restricted manner. It has been shown that poly(I:C) stimulation of $CD8a^+$ DCs in mice led to successful cross-priming of $CD8^+$ T cells [28], improved the survival of $CD4^+$ T cells and produced functional $CD8^+$ memory even in the absence of $CD4^+$ T cells [29]. These findings are in line with our data which presented an improved functionality of the activated T cells by formulations where poly(I:C) is present. In contrast, inclusion of $Pam3CSK_4$ in the formulations did not further improve the induced immune response. This may be due to its lipophilic nature, resulting in localization of the $Pam3CSK_4$ lipopeptide in the lipid bilayer of the liposomes, or the polymeric matrix of the PLGA NPs, which might negatively affect the functionality of the TLR ligand, and thereby its

immunogenicity. With regard to the PLGA NPs, unadjuvanted PLGA NPs are considered to have very low immune-stimulating properties [30,31]. The formulation including both SLPs and poly(I:C), but not Pam3CSK$_4$, was the most promising for the induction of functional and cytotoxic T cells. Although the effect of Pam3CSK$_4$ on the induced immune response in PLGA NPs was not as pronounced as in liposomes, a negative influence by its presence was also observed, suggesting a change in the PLGA NPs which leads to a less efficient formulation or a different targeting that does not favour the induction of a T cell-based immune response. With respect to that, although we did not measure antibody levels in Pam3CSK$_4$-immunised mice, the trigger of an anti-bacterial humoral response rather than a cellular one could be possible through Pam3CSK$_4$–mediated signalling of the extracellular TLR1/2.

In this study we showed that for SLPs-based vaccines, cationic liposomes appeared to be the most potent delivery system, followed by PLGA NPs. It has been reported by van Duikeren et al. [32] that the efficacy of a therapeutic vaccine formulation can be predicted based not only by the levels of specific T cells, but also on the activation status of the antigen-specific T cell responses that are elicited when applied in non-tumour bearing mice. According to this, high frequencies of antigen-specific CD8$^+$ T cells with functional properties, such as production of effector cytokines combined with a strong killing capacity, can correlate with the therapeutic efficacy of the vaccine formulation that initiates this response. In our study, the SLP-loaded PLGA-based formulations induce relative high numbers of SIINFEKL-specific T cells, but these CD8$^+$ T cells are not strong IFN-γ –producers and they present a poor killing activity against target cells. In contrast, the strong killing capacity (> 80%) that the two liposomal formulations showed, combined with the high levels (5-10 %) of specific T cells induced can be used as an indication of their efficacy as therapeutic cancer vaccines to achieve protection.

It is known that the size of particulate adjuvants is crucial for their adjuvant activity and the immunogenicity difference observed in this study between liposomes and PLGA NPs may be partly attributed to the different particle sizes. In general, APCs are able to take up different particles ranging from the size of viruses (20-300 nm), bacteria (0.5-2 µm) up to whole cells, which can be bigger than 10 µm. Upon vaccination, small particles (10-150 nm) can easily penetrate the extra-cellular matrix (ECM) and be quickly transported into the lymph nodes [33] where they will interact with lymph node resident DCs [34]. Moreover, particulate systems with a size below 200 nm, such as the liposomes used in this study, will likely be taken up by DCs more efficiently than bigger particles [35], which are more prone to be recognized by macrophages and other scavenger immune cells, leading to a poorer T cell activation capacity [31,36]. In addition, it was suggested that

smaller particles (20-150 nm) are naturally taken up by endocytosis, resulting in cellular immune response, while larger particles, such as PLGA NPs used here, are more likely to be phagocytosed, leading to a predominantly humoral immune response [35].

Furthermore, the particles' size combined with the most efficient administration route has also an impact on antigen uptake and therefore can affect the efficacy of immunotherapy. Particles larger than 150 nm cannot be efficiently transported via the lymphatic system as mentioned above, and a percentage of the administered particles will be trapped in the tissue, creating a depot. As a general rule, the preferred route should be able to efficiently deliver the antigen to APCs and initiate a rapid T cell-based immunity. Although there are many administration routes nowadays under investigation [37], the most commonly used route remains the subcutaneous one. Subcutaneous vaccination at the tail base of mice, as the administration route selected for the *in vivo* studies presented in this study, appeared to enhance the drainage to the lymphatic system in a faster and more efficient way compared to subcutaneous delivery in the flank. With regard to Montanide ISA-51, although the exact adjuvant mechanism is not well understood, it is believed that such a water-in-oil emulsion creates a sustained release from the local antigen depot. However, a longer retention time of larger particles does not necessarily correlate with better antigen uptake and a stronger induced immune response [38]. Considering that, our data suggest that the internalization of particles, such as PLGA or liposomes, may be more important for the induction of an efficient cellular immune response than the formation of a depot.

Surface charge is another variable parameter between the particulate systems investigated in this study, since the PLGA NPs were negatively charged and the liposomes positively charged. However, it is not clear whether positively, negatively or neutrally charged particles are the best choice to induce effective T_H1-type cellular immune response. Investigations so far have revealed contradictive results. For instance, anionic PLGA NPs induced antibody responses as well as strong CTL responses and T_H1-biased cytokine release in mice and macaques [39]. Anionic PLGA particles also showed a higher accumulation in the lymph node compared to PEGylated particles of the same size [40]. However, anionic liposomes interacted with a limited fraction of human and murine DC populations [41], setting cationic liposomal formulations in favour. This outcome is in line with published research data where cationic liposomes were considered to be a very potent choice for immunotherapy [42] and we have recently reported efficient cellular response induction in vivo with DOTAP based cationic liposomes carrying synthetic long peptides antigens [22]. It was suggested that the positive charge promotes electrostatic interactions with the negatively charged cell

surface, thus interacting more efficiently with DCs and other APCs [41]. Positively charged liposomes showed the induction of a superior antigen specific cellular immune response, in comparison with negative or neutral liposomes [43]. Moreover, cationic particles are thought to have an adjuvant effect themselves [44]. It was found that cationic liposomes, but not anionic or neutral ones, can stimulate the expression of DC maturation markers such as CD80 and CD86, depending on the lipid structure, but did not lead to pro-inflammatory cytokine- or enhanced NF-κB expression, suggesting that they act independently of this pathway [45]. The partially contradicting results could be due to different formulation procedures, immunisation protocols and antigen characteristics used in reported studies. The latter may result in a charge-dependent entrapment efficiency and antigen release pattern.

The reason why liposomes appeared to be a more potent delivery system for the induction of a T cell-based immune response upon therapeutic vaccination than PLGA NPs is not yet fully clear. The nanoparticulate systems differ in several aspects, such as size, zeta potential and molecular composition, all of which may contribute to differences in biodistribution as well as activation and maturation of DCs, in favour of cationic liposomes. Once the particles have reached their APC (DCs) target, the intracellular processing of the antigen is equally crucial for the efficiency of the cross-presentation, as the ability of DCs to cross-present exogenous antigens is not intrinsic, but can depend on the route of antigen uptake and the following processed antigen's accumulation in the endolytic compartments [46]. Mechanistic studies of biodistribution, cellular uptake, DC activation and intracellular antigen processing are needed in order to gain insight into important parameters underlying the performance of both SLP-loaded formulations, cationic liposomes and PLGA NPs, which could help to further improve their properties for effective immunotherapy of cancer.

Conclusions

In this study we successfully co-encapsulated four compounds (two antigenic SLPs and two TLR ligands) in two different delivery systems, cationic DOTAP-based liposomes and PLGA NPs. In a direct comparative study, we compared the immunogenicity of the particulate formulations with that of two emulsion-based adjuvants, Montanide ISA-51 and squalene SWE. The capacity of the particulate systems to induce functional antigen-specific T cells was at least as good (PLGA NPs) or better (cationic liposomes) than that of the emulsion-based formulations. This, while also considering the unfavourable safety profile of the currently used adjuvant Montanide ISA-51, makes these particulate delivery systems attractive candidates as delivery platforms for SLP-based immunotherapy of cancer.

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Chapter 5

Potent cationic liposome formulation of synthetic peptide vaccines for immunotherapy against human Papillomavirus-16 E7-induced tumours

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Abstract

Antigen-specific immunotherapy by vaccination is a promising strategy to treat cancer. Through injection of a tumour-associated antigens and adjuvants, therapeutic vaccination aims to properly activate tumour-specific T cells. The use of synthetic long peptides (SLPs) has been previously shown to be effective in clinical trials of human papillomavirus (HPV)-induced lesions. However, optimisation for the most efficient delivery of the SLP vaccine is still required. In this study, we report the potency of an adjuvanted liposomal formulation loaded with an HPV E7-specific SLP (harbouring a cytotoxic and a T helper epitope) and poly(I:C), as a therapeutic vaccine against HPV-induced cancer. The nanoparticulate cationic liposomal formulation showed a strong *in vivo* capacity to activate cytotoxic $CD8^+$ T cells and effector cytokines-producing $CD4^+$ T cells, after intradermal immunisation in mice. The E7/poly(I:C)-liposomes were able to significantly control tumour outgrowth and even cure mice from their established E7-expressing TC-1 tumours, in contrast to mice that received a 65-fold higher dose of Montanide ISA-51-E7 emulsified formulation. In conclusion, the E7 SLP-loaded cationic liposomes adjuvanted with poly(I:C) showed promising potential to be used as a therapeutic cancer vaccine, replacing suboptimal formulations currently used in the clinic.

Keywords: cancer immunotherapy, cationic liposomes, DOTAP, synthetic long peptides, HPV -E7, vaccine delivery

Introduction

During the last few years therapeutic vaccination has gained interest as a possible strategy to treat cancer, since the role of the immune system in cancer is rapidly becoming more clear [1]. Although due to novel diagnostic and screening technologies mortality from cancer has decreased [2], and by the combined use of conventional anticancer therapies such as radiotherapy, surgery and chemotherapy large tumour masses can be efficiently removed, these strategies are often less effective in the elimination of residual cancer cells. Immunotherapies based on different approaches focusing on directing the immune system against tumour cells are currently being developed, such as the first approved therapeutic cancer "vaccine" sipuleucel-T (Provenge), which is used for the treatment of prostate cancer based on antigen-specific T cells stimulation [3-5]. Other areas of intensive research for therapeutic vaccination include melanoma, lung cancers and cervical cancers [6,7].

A recent success in reducing the mortality associated with human papillomavirus (HPV)-induced tumours and cervical cancer has been the development of preventive vaccines against infections by the high-risk dangerous types of HPV [8]. However, there is no evidence for efficacy of these vaccines against established genital lesions, since in contrast to the induction of neutralising antibodies needed in preventive vaccination, therapeutic vaccination mainly requires the induction of cell-mediated immunity. Cell types that need to be induced for a cellular response include antigen-presenting cells from the innate immune system and cells from the adaptive immune system: these are dendritic cells (DCs) and $CD4^+$ T helper (T_H) and $CD8^+$ cytotoxic T lymphocytes (CTLs), respectively [9,10]. $CD8^+$ T cells are known for their central role in response to viral infections and cancer; thus, research has been focused on strategies able to induce effective T cell-based immune responses [11], targeting the E6 and E7 oncogenic proteins as the most common targets for the development of therapeutic vaccination against HPV-induced tumours [12,13].

It has been shown that immunotherapy using synthetic long peptides (SLP), consisting of sequencies from the E7 protein of HPV16, efficiently eradicated $HPV16^+$ established tumours in mice [14]. Moreover, patients with high-grade vulvar intraepithelial neoplasia vaccinated with a mix of SLPs against E6 and E7 had clinical responses, with 47% of them showing complete regression of their pre-malignant lesions after one year [15]. However, this E6/E7 SLP-based vaccine was not efficient in end-stage $HPV16^+$ cervival cancer patients. In addition, SLP vaccines used for clinical trials have been administered so far emulsified in an incomplete Freund's adjuvant, the Montanide ISA-

51, a suboptimal formulation adjuvant with reported adverse effect; among others, local swelling, pain, redness and fever [15].

Recently, we reported encouraging preclinical results of a cationic liposomal formulation for the delivery of SLP vaccines. More specifically, a poly(I:C)-adjuvanted DOTAP:DOPC formulation, loaded with two model OVA-derived SLPs, harbouring a CTL- and a T_H-epitope, showed a strong efficiency to induce functional cytokines-producing antigen-specific T cells and a superior ability, as compared to the free adjuvanted SLP, to protect mice when used as a therapeutic vaccine in an aggressive melanoma tumour-model (manuscript submitted).

Here, we further investigated the potential of our adjuvanted DOTAP-based cationic formulation, loaded with an E7-derived SLP, including overlapping CTL- and T_H-epitopes of HPV16. The induction of the E7-specific T cell based immune response was characterised and the liposomes' capacity as a therapeutic vaccine was assessed, in direct comparison with a currently used Montanide ISA-51 SLP vaccine formulation.

Materials and Methods

Materials

The 21-mer amino-acid long SLP, GQAEPD**RAHYNIVTF**CCKSDS, harbouring both the CTL epitope RAHYNIVTF (H-2Db) (49-57) and the T_H epitope PDRAHYNIVTF (48-57) of HPV-16 E7 protein, was produced in the Immunohematology and Blood Transfusion Department of the Leiden University Medical Centre. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and poly(I:C) with its rhodamine labelled analogue were obtained from InvivoGen (Toulouse, France). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, Netherlands), 100 IU/ml penicillin and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionised water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ) and phosphate buffer was composed of 7.7 mM $Na_2HPO_4 \cdot 2 H_2O$ and 2.3 mM $NaH_2PO_4 \cdot 2 H_2O$, pH 7.4 (10 mM PB, pH 7.4). Both solutions were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, (PBS: 140 mM NaCl, 8.7 mM $Na_2HPO_4 \cdot 12 H_2O$, 1.8 mM $NaH_2PO_4 \cdot 2 H_2O$, pH 7.4), which was used for all the *in vitro* and *in vivo* assays was purchased from B. Braun (Meslungen, Germany).

Mice

Female C57BL/6 (H-2^b) mice were purchased from Harlan (the Netherlands) and congenic CD45.1 (Ly5.1) mice were bred at the Leiden University Medical Centre animal facility and used at 8-14 weeks of age according to the Dutch Experiments on Animal Act, which serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe.

Liposome preparation

Cationic liposomes loaded with the above-described E7 SLP were prepared by using the thin film dehydration-rehydration method, as described previously [16]. Shortly, DOTAP and DOPC (1:1 molar ratio) in chloroform were mixed in a round-bottomed flask to reach

a concentration of 10 mg lipid per ml of final liposome dispersion. The formed dry film was rehydrated with a solution of 1 mg/ ml E7 SLP in ACN/H_2O (1:1 v/v) containing 0.1% (v/v) TFA (v/v) (E7-loaded liposomes), after having adjusted the pH to 8.5. For poly(I:C)-adjuvanted liposomes (E7/poly(I:C)-loaded liposomes), after the lipid film was rehydrated with the SLP solution the TLR ligand (including 0.5% w/w fluorescently-labelled equivalent) was added dropwise to the dispersion in a total concentration of 200 µg/ml. Subsequently, the liposome dispersion was snap-frozen in liquid nitrogen, followed by overnight freeze-drying. Dehydrated-rehydrated liposomes were generated by gradually adding 10 mM PB, pH 7.4, to the freeze-dried lipid cake. The liposomal mixture was vortexed well during the rehydration steps and the resulting dispersion was kept at room temperature for 1 h.

Sizing of the obtained multilamellar liposomes was performed by high-pressure extrusion at room temperature using a Lipex extruder (Northern Lipids Inc., Canada). Next, the peptide-loaded liposomes were concentrated by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa) as described [16].

Liposome characterisation

Average diameter (Z_{ave}) and polydispersity index (PDI) of the liposomes were determined by dynamic DLS using a Zetasizer (NanoZS, Malvern Ltd. UK) and zeta-potential determination was done by laser Doppler electrophoresis using the same instrument. For the characterisation measurements, liposome samples were diluted 100-fold in PB.

The E7 SLP-content in the liposomes was determined by UPLC after its separation from the liposomes, by using a modified Bligh-Dyer method, as described previously [16]. The amount of liposome-loaded poly(I:C) was determined by measuring the fluorescence (excitation wavelength 546 nm/emission wavelength 576 nm) of the rhodamine-labelled analogue in collected non-solubilised (i.e., intact) samples and the PB filtrates containing the free TLR ligand. A calibration curve of the fluorescently labelled compound was made, ranging from 7.81 ng/ml to 1000 ng/ml and all samples were measured by using a fluorescence micro plate reader (Tecan, Salzburg, Austria).

Immunisation of mice for direct T cell-priming

Mice were immunised with E7-loaded liposomes or soluble E7 (with or without poly(I:C)) by intradermal injection in the abdominal skin area or the tail base. Vaccination dose

was based on the E7 concentration, 1 nmol (2.3 µg) of SLP in a total volume of 30 µl and all formulations were prepared on the day of injection. Prime and boost immunisations were performed on day 0 and 14, respectively. Vaccinations with adjuvanted liposomes included a dose of about 1 µg of poly(I:C) (0.6-1.0 µg). During the *in vivo* studies, blood samples were obtained from the tail vein at different time points.

Analysis of antigen-specific $CD8^+$ and $CD4^+$ T cell responses by flow cytometry

Staining of the cell surface was performed on blood samples after red blood cells lysis. Cells were stained in staining buffer for 30 min with allophycocyanin (APC)-labelled H-$2D^b$ $E7_{49\text{-}57}$ tetramer (TM-RAHYNIVTF) and fluorescently labelled antibodies specific for mouse CD3 (BD Biosciences), CD4 and CD8 (eBiosciences). 7-Aminoactinomycin D (7AAD) (Life Technologies) was used for the exclusion of dead cells.

Overnight intracellular cytokine analysis of PBMCs was performed after incubating the cells with 5 µg of E7 SLP, in presence of brefeldin A (7.5 µg/ml) (BD Biosciences, Breda, the Netherlands). The next day the assay was developed as described [16].

In vivo cytotoxicity assay

Splenocytes from naive congenic $CD45.1^+$ mice were counted and split into two equal parts, after lysis of erythrocytes. Cells were labelled with CFSE for 1 h at 37°C, with either 5 µM (target population) or 0.5 µM (control) final concentration. The CFSE staining reaction was blocked with 10% (v/v) foetal calf serum and the cells of the target population were pulsed for 1 h with the short RAHYNIVTF peptide in complete culture medium at 37°C. Cells were washed four times with PBS before the two fractions (target population-control) were mixed in a 1:1 number ratio and adoptively transferred intravenously in recipient previously immunised C57BL/6 mice in a volume of 200 µl in PBS. Two days after the adoptive transfer of the target cells (day 24), mice were sacrificed, spleens were isolated and single cell suspensions were analysed by flow cytometry for specific killing (SK) following the equation below:

$$\mathrm{SK} = \left\{ 1 - \frac{\left[\frac{\text{CFSE high}}{\text{CFSE low}}\ \text{vaccinated mice}\right]}{\left[\frac{\text{CFSE high}}{\text{CFSE low}}\ \text{naive mice}\right]} \right\} \text{x } 100\ \%$$

Tumour regression experiment

TC-1 tumour cells expressing HPV16-E7 proteins were cultured at 37°C with 5% CO_2 in IMDM containing 8% FCS + 2 mM glutamine and 100 IU/ml penicillin in the presence of 400 µg/ml Geneticin (G-418) (Life Technologies), non-essential amino acids, 100x (Life Technologies) and 1 mM sodium pyruvate (Life Technologies).

On day 0, mice were injected subcutaneously in the flank with $1x10^5$ tumour cells in 100 µl PBS. On day 9, when tumours were clearly palpable (> 8 mm^3), mice were split into groups with similar tumour size distribution and were vaccinated, as described above. Non-immunised mice injected only with tumour cells, were used as a negative control.

The mice were weighed three times a week and the size of their tumour was measured in three dimensions by using a calliper. Mice having a tumour size > 2000 mm^3 were sacrificed for ethical reasons.

In vivo $CD8^+$ T cell depletion

For the *in vivo* $CD8^+$ T cell depletion, the tumour-bearing mice were injected i.p. with 100 mg of the monoclonal antibody (mAb), isolated from the 2.43 hybridoma cells that specifically recognizes CD8, on days -1, 1, 7 and 14 after vaccination. The antibody was prepared and purified as described [17].

Statistical analysis

The significance of differences in the *in vitro* and *in vivo* assays were evaluated by GraphPad Prism 5 (GraphPad) software, by using an analysis of variance (ANOVA) at a 0.05 significance level, followed by Bonferroni's post-test. The significance of differences between the survival curves was calculated with the log-rank (Mandel-Cox) test.

Results

Physicochemical characterisation of E7 SLP-loaded liposomes

The E7- liposomes were prepared following the optimised rehydration-dehydration film method, as described before (manuscript submitted). The highest possible loading efficiency of the E7 SLP is expected when the peptide is negatively charged, due to electrostatic interactions between the negative SLP and the cationic liposomes. The obtained E7 -liposomes had an average size of about 160 nm, a relatively low PDI (<0.3) and a positive zeta-potential (Table I). When poly(I:C) was co-encapsulated in the formulation, the properties of the E7-liposomes did not change significantly: the liposomes showed a trend to be slightly larger, whereas the PDI and the zeta-potential remained comparable (Table I). The loading efficiency of the peptide was around 55% in unadjuvanted liposomes and 50% in poly(I:C)-adjuvanted liposomes, similar to the efficiency of the TLR ligand in the poly(I:C)-adjuvanted formulation.

Table I: Physicochemical properties of the liposomal formulations used in this study. Data are averages ± SD of at least 6 independent batches.

Formulation	Z_{ave} Diameter (nm)	PDI	ZP (mV)	E7 LE (%)	Poly(I:C) LE (%)
Empty liposomes	142 ± 5	0.15± 0.02	34 ± 4	NA	NA
E7 liposomes	160 ± 20	0.23± 0.03*	28 ± 3*	55 ± 8	NA
E7/poly(I:C) liposomes	180 ± 10**	0.25± 0.05**	28 ± 3*	50 ± 5	55 ± 4

Z_{ave}: Z-average particle diameter, PDI: polydispersity index, ZP: zeta-potential, LE: loading efficiency, NA: not applicable. Size and ZP significantly different from those of empty liposomes (evaluated by Kruskal-Wallis w/Dunn's and p calculated with Mann-Whitney non-parametric test) are indicated by *$p < 0.05$ and ** $p < 0.005$.

The overall characterisation data show that E7 and poly(I:C)-adjuvanted E7- liposomes can be prepared successfully, following the protocol established previously for the encapsulation of model OVA SLPs (manuscript submitted).

Activation of E7 SLP-specific functional $CD8^+$ and $CD4^+$ T cells in vivo

The liposomal formulations were subsequently analysed for their potency to induce an antigen-specific cell-mediated immune response *in vivo*. First, the frequency of the activated E7-specific $CD8^+$ T cells was assessed after surface staining by using a fluorescently labelled tetramer (TM) antibody (H-$2D^b$ $E7_{49-57}$ RAHYNIVTF). Naive wild-

type B16 mice were injected twice (day 0 and 14) intradermally at the abdominal area with different E7-containing formulations, and blood samples as well as splenocytes were analysed by flow cytometry.

In blood of mice immunised with E7 SLP loaded into liposomes (E7- liposomes), a high percentage (ca. 4%) of antigen-specific $CD8^+$ T cells was detected a week after the second vaccination (day 21) *ex vivo* (Figure 1A). The addition of poly(I:C) in the liposomal formulation led to an even higher number: about 7% of the total $CD8^+$ T cells were E7-specific (Figure 1A). As we have reported before for the model OVA SLP, the adjuvant effect of the poly(I:C) on the activation of $CD8^+$ T cells is present, regardless the way the TLR3- ligand is loaded in the vaccine. In the same way here, co-encapsulation of the poly(I:C) with the E7 SLP (E7/poly(I:C)-liposomes) or mixed with the E7-liposomes just before the immunisation (E7-liposomes+poly(I:C)) led to an equally strong induction of T cells.

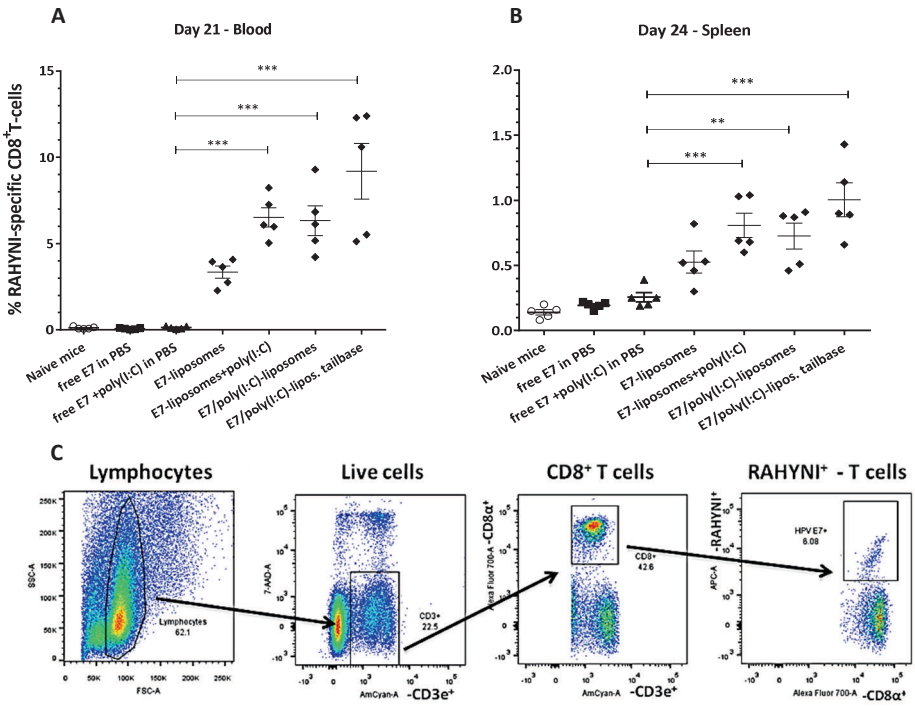


Figure 1: E7-specific $CD8^+$ T cells responses in (A) blood (day 21) and (B) splenocytes (day 24) upon intradermal immunisation of five mice per group (at the abdominal area or tail base) with 1 nmol of E7 SLP on day 0 and 14; (C) representative gating strategy for detection of RAHYNIVTF-specific $CD8^+$ T cells by flow cytometry using specific MHC class I tetramers (TM). The experiment was performed twice with comparable results. Data evaluated by one-way ANOVA with Bonferroni's multiple comparison test, ** $p< 0.001$ and *** $p<0.0001$.

Furthermore, mice immunised with the E7/poly(I:C)-liposomes intradermally on the tail base, showed a trend to have an improved frequency of activated T cells, compared to mice immunised at the abdominal area (Figure 1A). Ten days after the boost immunisation (day 24), the number of the $CD8^+$ T cells was analysed also in spleens of sacrificed mice, showing again significantly higher frequencies of antigen-specific T cells for the groups having received adjuvanted liposomal vaccines, compared to the vaccine consisting of the free compounds (free E7+ poly(I:C) in PBS) (Figure 1B).

The analysis of blood samples and splenocytes with the TM-RAHYNIVTF antibody indicated the potency of the vaccines to expand the number of primed antigen-specific $CD8^+$ T cells. In addition, we analysed their functionality and whether the vaccine (the SLP contains an overlapping CTL and a T_H epitope), could also induce E7-specific $CD4^+$ T cells. Therefore, we investigated the efficiency of our liposomal formulations to induce cytokine-producing T cells. Blood samples from immunised mice, collected on day 21 (one week after the boost immunisation), were re-stimulated *ex vivo* with the long overlapping E7-SLP and the percentages of $CD8^+$ and $CD4^+$ T cells producing effector cytokines IFN-γ and TNF-α were analysed.

Intracellular cytokine analysis showed that all liposomal formulations induced high numbers of IFN-γ-producing $CD8^+$ T cells, above 3 %, which correlates with the data of the E7-TM analysis of Fig.1. This percentage showed a two-fold increase when poly(I:C) was co-encapsulated with the SLP (E7/poly(I:C)-liposomes), but not when mixed with the E7-liposomes (E7-lipos.+poly(I:C)) (Figure 2A). Furthermore, liposome vaccination induced activation and functionality of $CD4^+$ T cells, since E7-specific T helper cells produced IFN-γ (Figure 2B and supplementary figure 1). Activation of cytokine-producing $CD4^+$ T cells underlines the development of a T_H1-oriented response, characterised by the secretion of IFN-γ and TNF-α effector cytokines.

With respect to that, a fraction of IFN-γ-producing T cells also produced TNF-α (Figure 2 B and D), while IL-2 cytokine was also produced by a significant number of $CD4^+$ T cells (Figure 2E). This indicates that specific $CD4^+$ T cells are not only expanding, but also produce different functional type 1 cytokines, amplifying in that way the induced T cell immune response.

In conclusion, the poly(I:C)-adjuvanted E7- liposomal formulations, especially the one in which SLP and adjuvant are co-encapsulated, efficiently induced a functional T cell-mediated immune response. The SLP-specific $CD8^+$ T cell- frequency induced by the liposomes showed an at least 50-fold increase over the T cell percentage induced by the poly(I:C)-adjuvanted free E7 SLP vaccine.

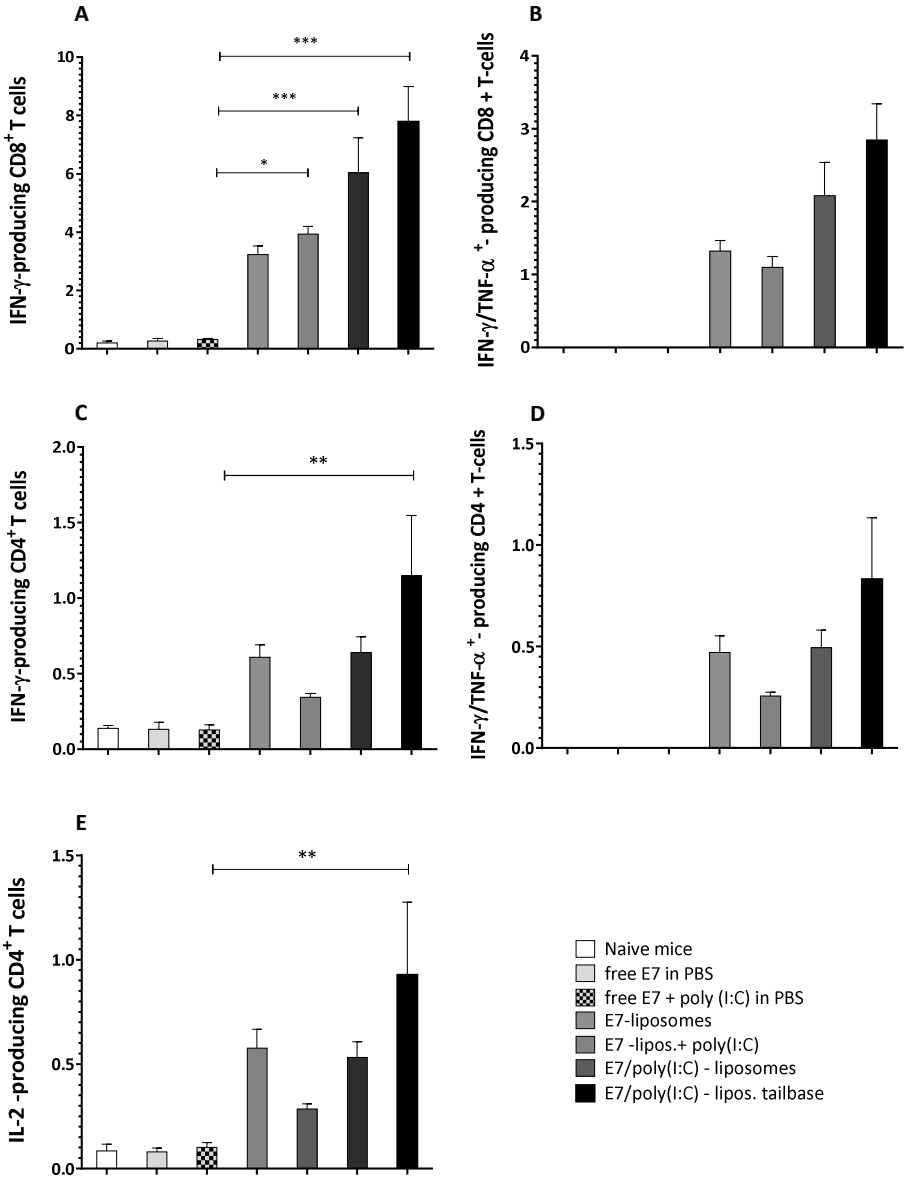


Figure 2: Intracellular cytokine analysis in blood of immunised mice at day 21. Blood samples were stimulated *ex vivo* overnight with the E7 SLP. Plots show $CD8^+$ T cells producing IFN-γ (A); $CD8^+$ T cells producing both IFN-γ and TNF-α (B); $CD4^+$ T cells producing IFN-γ (C); $CD4^+$ T cells producing both IFN-γ and TNF-α (D) and $CD4^+$ T cells producing IL-2 (E). Data shown is average of 5 mice per group ±SEM and were evaluated by one-way ANOVA with Bonferroni's multiple comparison test, *p< 0.05, ** p< 0.001 and *** p<0.0001.

Induction of in vivo CD8⁺ cytotoxicity

Both the quantity and the quality of the vaccine-specific induced T cells are components crucial for the initiation and development of a robust and long-lasting anti-tumour immune response. Using non-tumour bearing mice we assessed the quality of $CD8^+$ T cells induced by our vaccine based on their *in vivo* cytotoxic capacity. Intradermal immunization of mice with 1 nmol of E7 SLP loaded into liposomes (E7-liposomes) showed a strong killing capacity, above 70%, in contrast to vaccination with free E7 (Figure 3). Addition of poly(I:C) to the liposomal formulation (E7-lipos.+poly(I:C) did not significantly enhance the cytotoxic activity. Also, administration of the adjuvanted liposomal formulation via the tail base did only increase the strength of the induced cytoxicity up to 80%.

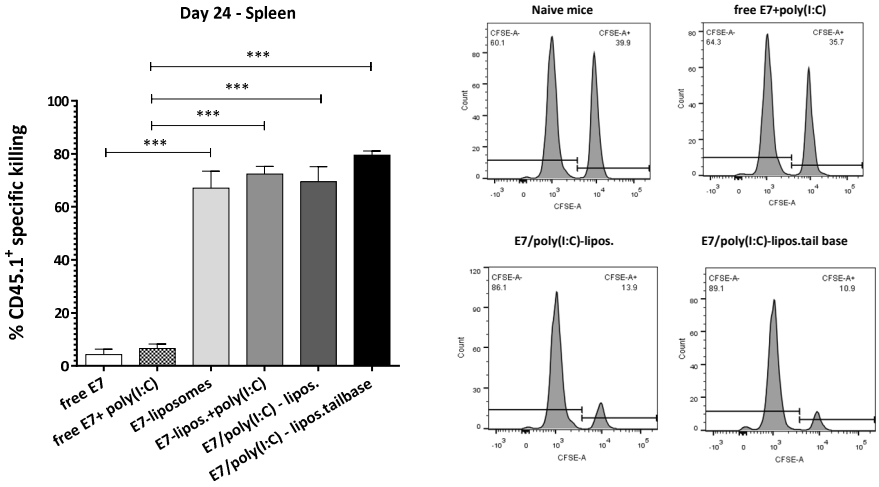
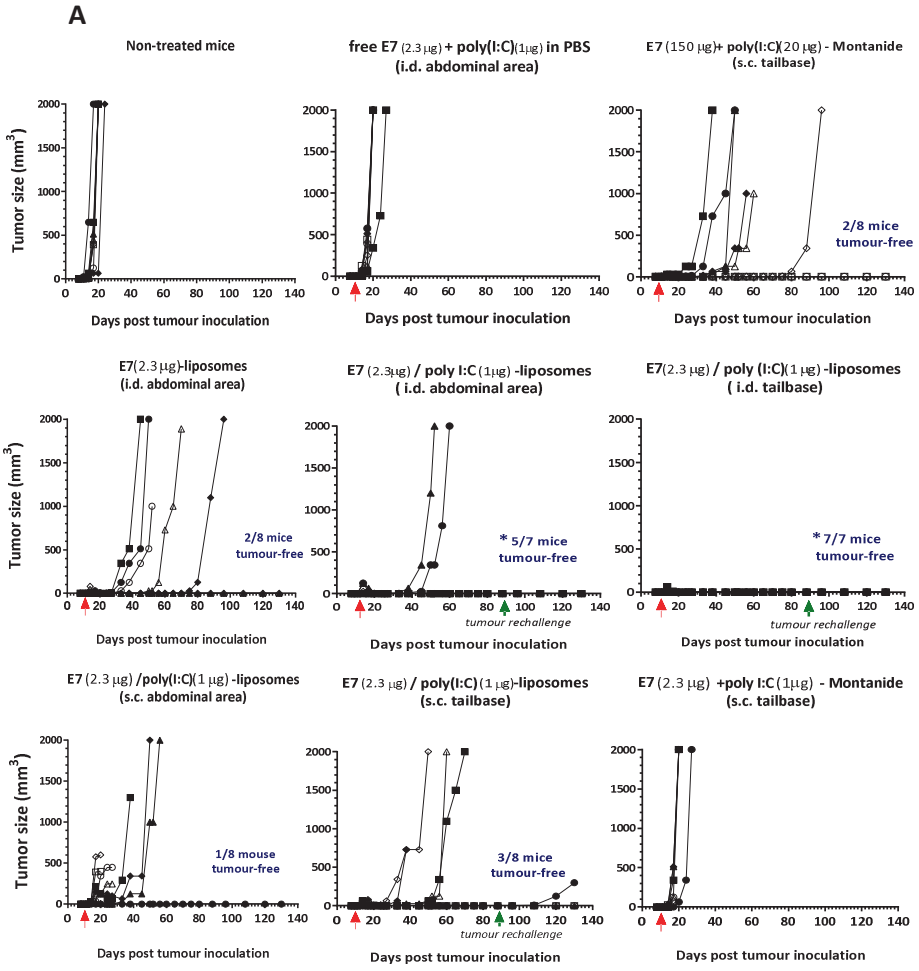


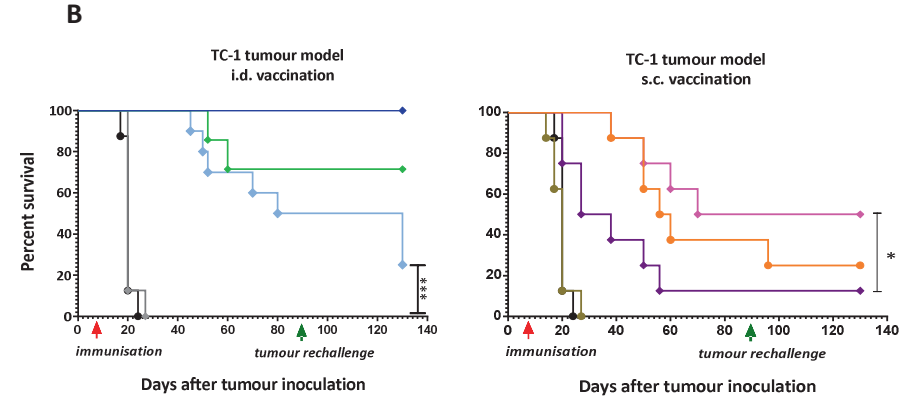
Figure 3: *In vivo* cytotoxicity against RAHYNIVTF-presenting transferred target cells. The mean percentages of the killing activity of E7 formulations are presented based on the frequency of the transferred $CD45.1^+$ specific target cells that could be detected in splenocytes of mice immunised with 1 nmol of E7 SLPs-loaded formulations. Bar graph shows the mean percentages (+SEM) on day 24. Representative histograms of CFSE-labelled target cells: right peak=peptide pulsed and left peak= control. Data evaluated by one-way ANOVA with Bonferroni's multiple comparison test, *** $p<0.0001$.

Vaccine-mediated regression of established HPV16-E7 specific tumours

The therapeutic potency of the poly(I:C)-adjuvanted E7-loaded liposomal vaccines was investigated in mice bearing (s.c.) tumours induced by the TC-1 cell line, expressing HPV16-E7 proteins. As a control, the free compounds (free E7+poly(I:C)) were used as in the above described studies, and the liposomal formulations were directly compared to the clinical "gold standard", Montanide ISA-51 mixed with a 65-fold higher dose of E7 SLP and 20-fold higher dose of poly (I:C): 150 µg and 20 µg, respectively.



*one mouse/group excluded due to death for reasons not related to the experiment set up



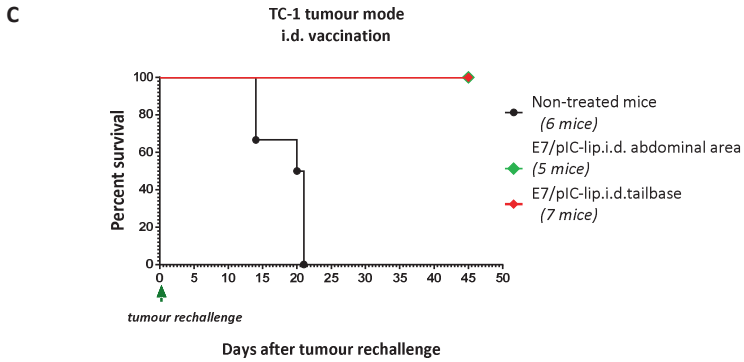


Figure 4: Tumour outgrowth in a therapeutic model, in mice immunised i.d. or s.c. at day 9 after tumour inoculation (red arrows), with different E7 SLPs formulations, after they were subcutaneously injected on day 0 with $1x10^5$ TC-1 cells. Tumour sizes in individual mice (A) and survival plots (B) are presented (left: i.d. skin vaccinations+controls, right: s.c. vaccinations+controls +Montanide groups). The curve of the E7-lip. i.d. abdominal area is significantly different from the free E7+ poly(I:C) *** p< 0.0001 and the curve of the E7/pIC-lip. s.c. abdominal area group is different from the s.c. tail base group. * p<0.05, evaluated with the log-rank (Mandel-Cox) test. The experiment was performed twice with comparable results. Green arrows in both (A) and (B) plots indicated the tumour-rechallenge time point (D85). Survival plot of mice presented in figure 4A after TC-1 tumour re-challenge. A naive group of mice was added as a control for tumourigenicity of TC-1 cells (black line) (C).

Within a period of 130 days, all mice in groups immunised with the poly(I:C)-adjuvanted E7 liposomal formulations or the standardised dose in E7-Montanide ISA-51 emulsion, were able to control the outgrowth of the established TC-1 tumours; while non-treated mice, mice vaccinated with the free compounds (free E7+poly(I:C)) or mice injected with the low dose of vaccine in Montanide had to be sacrificed within three weeks since they reached the humane endpoint of 2000 mm^3 tumour size (Figure 4). We observed a strong administration route dependent effect on the tumour outgrowth regression, with the intradermal immunisation being more efficient than the subcutaneous one. Additionally, although no significant difference was observed between mice vaccinated at the abdominal area or the tail base intradermally, the different area when mice injected subcutaneously significantly influenced the tumour regression. In other words, the same formulation (E7/poly(I:C)-lip.) was significantly more efficient when it was delivered s.c. on the tail base than on the abdominal area (Figure 4B).

More specifically, mice vaccinated with 2.3 µg of E7 in non-adjuvanted liposomes (E7-liposomes) showed a delay on the tumour outgrowth, comparable to that observed in mice injected with a 65-fold higher dose of E7 emulsified in Montanide ISA-51 and combined with a higher dose of poly(I:C) (Figure 4A). Mice immunised intradermally with the E7/poly(I:C)-liposomes showed a substantial tumour regression with 5 out of 7 mice being completely cured from their established tumours. Moreover, when the same formulation was intradermally administered at the tail base, instead of the abdominal area, 100 % of the mice were fully protected till the end of the experiment (Figure 4A)

with no adverse side-effects observed at the injection area. In the same way, when the E7/poly(I:C)-liposomal formulation was delivered subcutaneously, the tail base immunisation seemed to be more potent than the abdominal delivery (50% and 12.5% cured mice, respectively), suggesting that vaccination effectiveness might depend not only on the administration route, but also the injection site.

Importantly, in all mice immunised with liposomal formulations, irrespectively on the administration route or site, no adverse side-effect was observed at the injection spot; in contrast to the high-dose of Montanide where skin irritation was observed on the shaved tail base area of the immunised mice.

Tumour rechallenge on day 85 in mice vaccinated with E7/poly(I:C)-liposomes (i.d. abdominal area/tail base and s.c. tail base) showed that all intradermally vaccinated mice which were cured of their tumours were also fully protected by the new tumour inoculation, suggesting that these mice raised functional memory T cells to control newly injected tumour cells (Figure 4C).

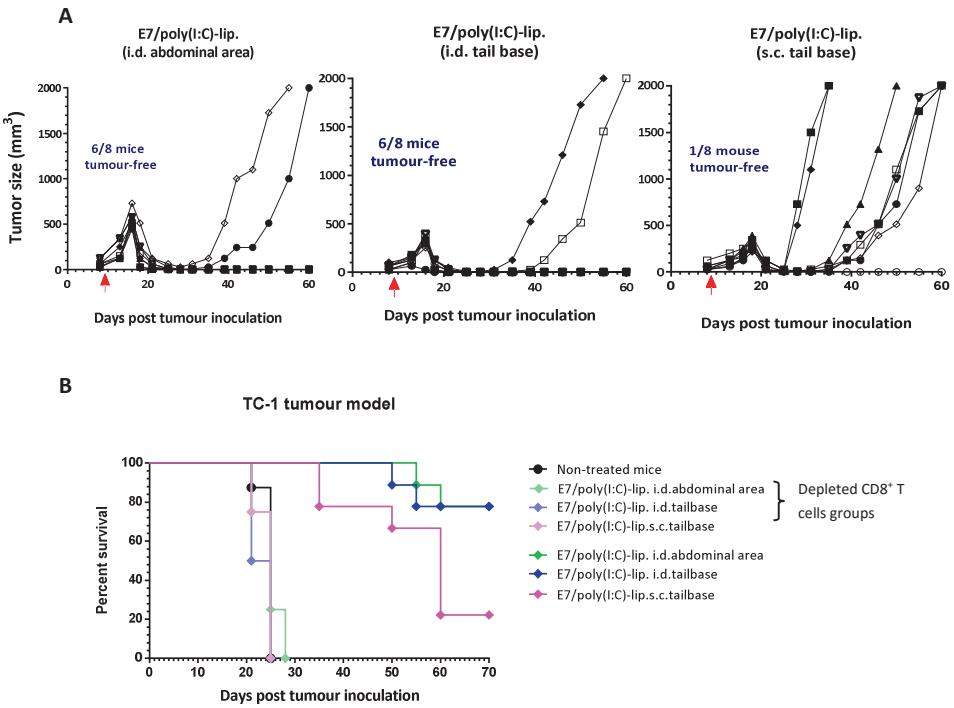


Figure 5: Tumour outgrowth in WT and mice with depleted $CD8^+$ T cells. The mice were immunised i.d. or s.c. (red arrows) with the E7/poly(I:C) formulation, after having subcutaneously injected on day 0 with $1x10^5$ TC-1 cells. Tumour sizes in individual mice (A) and survival plots of survived mice are presented compared to mice with depleted $CD8^+$ T cells (B).

Finally, in order to be able to attribute the tumour regression to the induction of a E7-tumour specific T cell mediated immunity, mice depleted from $CD8^+$ T cells were vaccinated with the E7/poly(I:C)-liposomal formulation via the most potent administration routes (i.d. abdominal area/ tail base and s.c. tail base). All mice which were depleted of $CD8^+$ T cells had to be sacrificed within three weeks due to their tumours' size reaching the humane endpoint (Supplementary figure 2), showing the crucial role of $CD8^+$ effector T cells in this system. Non-depleted mice treated intradermally, either at the abdominal area or at the tail base, showed a remarkable tumour regression of very large tumours in this experiment (Figure 5). In detail, 75% mice treated intradermally with poly(I:C)-adjuvanted E7-liposomes were cured from their established tumours, even when their size had reached a size of about 500 mm^3, pointing to the potential of cationic liposomes as a therapeutic vaccine system for SLPs.

Discussion

For therapeutic immunisation against HPV-induced cervical cancer, various vaccine modalities are under development, including recombinant live vector vaccines, nucleic acid vaccines, protein and peptide vaccines, virus-like particle vaccines (VLP), whole cell vaccines and combination vaccines [2, 9]. Vaccine formulations aimed at treatment of HPV induced carcinomas require long-lived antigen presentation of antigenic fragments by appropriately activated DCs. The efficacy of such vaccines is also highly dependent on proper adjuvants and the administration route.

Here, we investigated the potential of a liposomal vaccine for immunotherapy against HPV-induced tumours, as an alternative to Montanide ISA-51 –based formulations. Using an E7-derived 21-amino acid long SLP, cationic DOTAP-based liposomes were prepared, following the previously described protocol (manuscript submitted). The nanoparticulate liposomal vaccine showed a superior immunogenicity to induce an antigen-specific immune response, which can be ascribed to the cationic liposomal nature and especially to the presence of DOTAP [18]. In general, it has been reported that cationic liposomes can promote the activation and maturation of DCs, followed by a subsequent effective priming of T cells; in that way, they can enhance an anti-tumour immunity in a more efficient way when compared to neutral or anionic liposomal vaccines [19]. In addition, the generation of reactive oxygen species (ROS) after activation of DCs by cationic DOTAP-liposomes can be the trigger of several signalling pathways that lead to cytokines and chemokines production with co-stimulatory molecules expression [20]. In accordance with that, our E7-liposomal formulations were more effective in the production of IFN-γ and TNF-α - producing $CD8^+$ and $CD4^+$ T cells and in the induction of a high killing capacity, compared to the free E7 vaccine, even when the latter one was poly(I:C)-adjuvanted. Enhanced production of IFN-γ could also contribute to anti-HPV immunity [21], since IFN-γ associated T cell-responses are weak or absent in patients with cervical cancer [22].

Our liposomal vaccines were loaded, apart from the overlapping CTL/T_H epitopes SLP, with poly(I:C), a TLR3 ligand. Regarding the use of the E7-SLP as a peptide antigen, in contrast to full-protein and short peptide based vaccines, SLPs are known to induce $CD8^+$ T cell responses to the injected antigens, while co-administration of a helper epitope-included peptide can further enhance the immune response [23-25]. Furthermore, anti-tumour vaccines, loaded with peptides and adjuvanted with poly (I:C) have been shown to be efficient enough to elicit a tumour-specific cytotoxic T cell immunity *in vivo* and even suppress the tumour outgrowth [26]. Membrane-associated and intracellular Toll-

like receptors play an important role in controlling the innate and adaptive arms of immune response. Indeed, in our case although it seems that the presence of $CD4^+$ T_H epitope in the vaccine is sufficient for the induction of functional and cytotoxic T cells, administration of poly(I:C) appeared to be vital for the control of the established tumours in mice. Moreover, in line with our previous findings, the way poly(I:C) is formulated with the liposomal vaccines does not appear to be of significant importance for the induction of its adjuvant effect: the mixture of the ligand with the E7-liposomes or the co-encapsulation of poly(I:C) with the E7 SLP in liposomes are both strongly immunogenic vaccines, as the *in vivo* cytotoxicity assay showed. As we have tested in our model OVA loaded-liposomes, poly(I:C) interacts with the liposomes when mixed, while the size and charge of liposomes does not alter and additionally no remaining free poly(I:C) is detectable (data not shown).

Regarding the efficacy of a therapeutic cancer vaccine, $CD4^+$ T cell activation appears a key parameter in anti-tumour immunity, since through the auxiliary signals these cells provide, they can both enhance cytotoxic T cell responses and establish a robust long-term anti-tumour immunity [27,28]. With respect to HPV, sustained activation of $CD4^+$ T cells has been correlated with disease regression, while lack of $CD4^+$ T cell dependent immunity or reduced $CD4^+$ T cell functions have been associated with progressive disease [22]. Based on these statements, the $CD4^+$ T cells detected in mice immunised with liposomal formulations and their proven functionality through the production of cytokines, such as IFN-γ, TNF-α and IL-2, can be used as a first indication to predict the efficiency of these formulations when they are used as therapeutic vaccines.

The route of immunisation is another crucial factor that can greatly influence the nature of the induced T cell response [29], considering the different cell types that you can target by just changing the vaccine delivery route. The understanding of how DCs induce, regulate and maintain a T cell immunity is essential for the design of novel cancer vaccines with improved efficacy. Upon intradermal vaccination, the antigen presentation depends on the targeted APCs that transport the injected antigen towards the draining lymph nodes [28] or the recruitment of DC subsets in the local tissue or the lymph nodes for promoting the cross-presentation of the antigen [30]. Considering this, attractive cellular target for vaccination approaches can be the $CD8\alpha^+$ DCs and their equivalents in tissues, due to their enhanced ability to present exogenous antigen on MHC class I and their strong capacity to provide a robust signal 3 [27]. Furthermore, the $CD8\alpha^+$ DCs, in contrast to other DC subsets, lack for instance the expression of TLR7, whereas adjuvants recognizing TLR3, such as poly(I:C) that are expressed by $CD8\alpha^+$ DCs are expected to be more effective for their targeting [27]. Therefore, based on this we

can speculate that the administration route effect we observed upon immunization of E7-liposomes in mice with established TC-1 tumours, might be attributed to the targeting of DCs specialized in cross-presentation. Cationic liposomes formulated with the appropriate TLR ligand may be well-suited to optimally target these APCs via the intradermal route. Our results showing adequate tumour eradication obtained with two independent SLP model systems in two aggressive tumour types (TC-1 and B16 melanoma) suggest that cationic liposomes are an excellent delivery platform for peptide-based therapeutic cancer vaccines.

Conclusion

In this study, we showed that a DOTAP-based cationic liposomal formulation loaded with an HPV E7-SLP and poly (I:C), when administered intradermally, can induce efficiently a strong T cell-mediated immunity, able to control tumour outgrowth, and lead to the cure of at least 75 % of the immunized mice from their established tumours. In addition, intradermal vaccination of the liposomal formulation was more effective than the Montanide-based vaccine containing a 65-fold and 20-fold higher dose of E7 and poly(I:C), respectively, illustrating the potential of adjuvanted cationic liposomes as a delivery system for SLP-based cancer immunotherapy.

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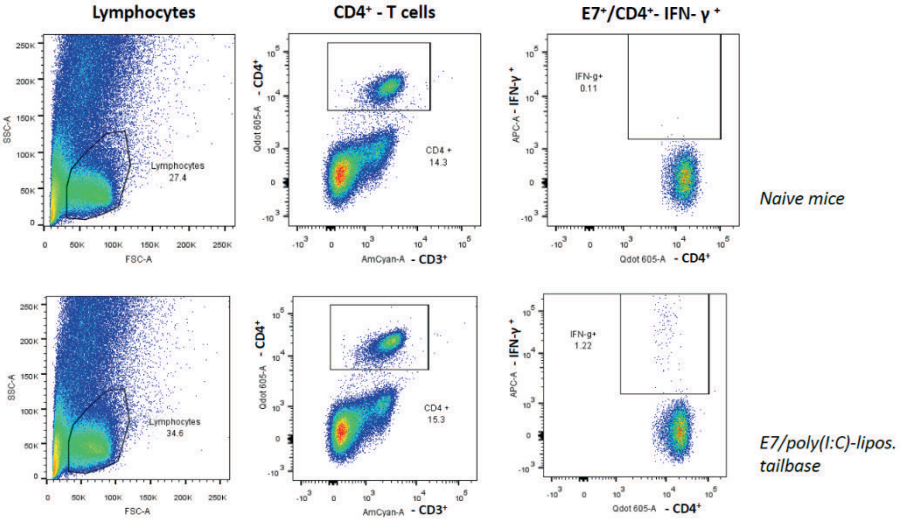
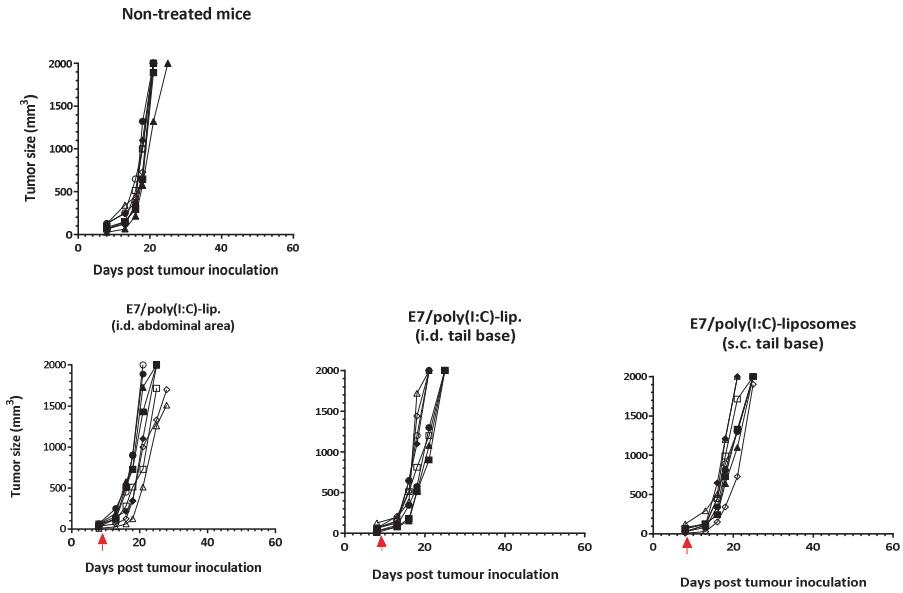
Supplementary data



Supplementary figure 1: Representative gating strategy for detection of antigen-specific IFN-γ-producing CD4⁺ T cells by intracellular cytokine staining and flow cytometric analysis in blood of immunised mice presented in figure 2.



Supplementary figure 2: Tumour out growth in mice with depleted CD8⁺ T cells (8 mice per group) immunised i.d. or s.c. with E7/poly(I:C)- liposomes (2.3 µg of E7 SLP and 1 µg of poly(I:C)).

Chapter 6

Cationic DOTAP-based liposomes: a vaccine formulation platform for synthetic long peptides with widely different physicochemical properties

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Abstract

Synthetic long peptides (SLPs) harbouring T cell epitope sequences hold great promise for cancer immunotherapy by active vaccination. However, because of their low intrinsic immunogenicity they have to be formulated with adjuvants. Cationic DOTAP-based liposomes have been shown to be suited as a delivery system including adjuvants to improve the efficiency of the SLPs. In this study, we investigated the capacity of a DOTAP:DOPC liposomal formulation to accommodate eleven 24-residue long SLPs, all harbouring the immunodominant SIINFEKL model epitope of ovalbumin, but widely differing in hydrophobicity and isoelectric point. Following a standard optimised protocol, the eleven SLPs were successfully loaded into the cationic liposomes. The obtained particles had a size below 200 nm, a low polydispersity index (< 0.3) and a positive zeta-potential (ca. 30 mV). The SLP loading efficiency was at least 50% for all the peptides, independently of their physicochemical nature. Finally, all the different SLP-loaded liposomes efficiently delivered the long peptide to immature DCs which could process the antigenic peptide and efficiently activated SIINFEKL-specific $CD8^+$ T cells *in vitro*. The improved immunogenicity of the SLP set makes liposomes a flexible and therefore promising vehicle for the delivery of SLPs.

Keywords: cationic liposomes, synthetic long peptides, SIINFEKL antigen, therapeutic vaccine, in vitro immunogenicity

Introduction

The success of therapeutic vaccination largely depends on the capacity of the vaccine to induce a strong cellular immune response able to eradicate tumours or infections [1, 2]. In order to achieve this, specific antigens have to be efficiently delivered to and processed by properly activated DCs, which generally requires the inclusion of an adjuvant in the vaccine. Adjuvants can be generally divided into immunostimulatory compounds and delivery systems [3]. Currently several delivery systems are available for use in humans, including aluminium salts, influenza virosomes, virus-like particles (VLPs), liposomes, and oil-in-water emulsions, such as MF59, AS03 and AF03 [1,4-6]. However, the majority of these adjuvants are generally applied in prophylactic settings for induction of humoral immunity and therefore are considered less suitable for therapeutic vaccination [4].

One of the most common delivery systems that have been also traditionally used for the delivery of anti-cancer drugs, such as doxorubicin or anthracycline [7,8] are liposomes; these are small vesicles consisting of, one or several, lipid bilayers enclosing an aqueous compartment [9]. By nature, liposomes are able to accommodate a wide range of molecules-antigens. The hydrophilic heads of the phospholipids cover the inner and outer surface of the liposomes, so that hydrophilic compounds will most likely be found encapsulated in the aqueous inner compartment of liposomes, protected from enzymatic degradation by proteins and enzymes. In contrast, the hydrophobic tails of the lipid bilayer will associate or adsorb lipophilic compounds [8]. Liposomes, due to their similarity to the membrane of cells, can target the loaded drug or antigen directly into the cells [10] and by adjusting their properties – such as size and surface charge - the induced immune response can be tuned towards the desired one.

Recently we reported promising preclinical results using a cationic liposomal formulation for the delivery of SLP vaccines. Briefly, a DOTAP:DOPC formulation loaded with two model OVA SLPs or an E7-derived SLP from HPV, showed a strong efficiency to induce functional cytokines-producing antigen-specific T cells and a strong efficacy, as compared to the free SLPs, to cure mice when used as a therapeutic vaccine in two independent tumour models. In addition, the direct comparison of SLP-loaded liposomes to other particulate vaccine systems, underlined their superior immunogenicity [18].

SLP-based therapeutic vaccination has been succesfull in patients with premalignant vulvar lesions caused by oncogenic HPV type 16 by using a multiple overlapping SLP vaccine covering the entire sequences of the HPV16 E6 and E7 viral oncoproteins [11].

In addition, identification of neo-epitopes in cancer types with high mutation rates like melanoma and non-small cell lung cancer by exome sequencing of tumor cells [12,13] allows the design of personalized SLP-based cancer vaccines. For both approaches a flexible and adequate vaccine delivery system is required since the identified antigenic peptide sequences will differ in their physicochemical properties.

Considering the need for multiple peptides for the design of a therapeutic cancer vaccine, in this study we investigated the potential of this cationic DOTAP-based liposomal formulation as a platform for various synthetic long peptide (SLP) antigens. The creation of an SLP library, consisting of 24-amino acid-long peptides, all of them harbouring the ovalbumin immunodominant SIINFEKL epitope, but covering different physicochemical properties, allowed us to assess whether the earlier developed liposomes are capable of accommodating SLPs independent of their pI and hydrophobicity, without compromising their potency. In that way, a first assessment on the feasibility of a “universal” liposomal-based vaccine system for the delivery of SLP vaccines will be achieved.

Materials and Methods

Materials

The NBD-(7-nitrobenzofurazan) fluorescently labelled 24-mer SLPs (Table I) including the immunodominant cytotoxic T-lymphocyte (CTL) epitope [SIINFEKL] of ovalbumin (OVA) were produced and purified at the peptide facility of the Department of Immunohematology and Blood transfusion of Leiden University Medical Center. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8 % (v/v) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 µM 2-mercaptoehanol (Sigma-Aldrich, Zwijndrecht, Netherlands) and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionised water with a resistivity of 18 MΩ.cm was produced by a Millipore water purification system (MQ water). Phosphate buffer was composed of 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-µm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, (PBS: 140 mM NaCl, 8.7 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH 7.4), which was used for the *in vitro* MHC class I assays, was purchased from B. Braun (Meslungen, Germany).

Liposome preparation

Cationic liposomes loaded with the SLPs were prepared in two ways by using the thin film dehydration-rehydration method, followed by extrusion. In detail, 1 DOTAP: 1 DOPC (molar ratio) in chloroform was mixed in a round-bottomed flask at a concentration of 10 mg lipid per ml of final liposome dispersion. A lipid film was formed by chloroform ($CHCl_3$) evaporation in a rotary evaporator for 1 hour at 37°C. For the relatively hydrophilic SLPs (hydropathicity below -0.9; see Table 1), the film was then rehydrated with a solution of 1 mg/ml SLP in ACN/H_2O 1:1 (v/v), adjusted to a pH value of about 8.5 and after mixing in the presence of glass beads the dispersion was equilibrated for 1 h at room temperature. For the preparation of liposomes loaded with the relatively hydrophobic SLPs (hydropathicity above -0.2; see Table 1), the peptides dissolved in a $CHCl_3$: MeOH: H_2O solution (60:36:4, v/v) were added to the organic lipid solution to reach a concentration of 1 mg/ml, before the formation of the dry film. Next, the liposome dispersion (of both hydrophilic and hydrophobic SLPs) was snap-frozen in

liquid nitrogen, followed by freeze-drying in a Christ alpha 1-2 freeze-dryer (Osterode, Germany) overnight. Dehydration-rehydration liposomes were generated by gradually hydrating the freeze-dried lipid cake in 10 mM PB, pH 7.4. To prepare 2 ml liposome dispersion, two volumes of 400 µl and one volume of 1200 µl PB were successively added, with intervals of 20 minutes between each addition. The mixture was vortexed well during the rehydration steps and the resulting dispersion was kept at room temperature for at least 1 h (Figure 1).

Sizing of the obtained vesicles was performed by high-pressure extrusion at room temperature, by using a Lipex extruder (Northern Lipids Inc., Canada). To obtain monodisperse liposomes, the liposome mixture was passed four times through a 400-nm pore size membrane and four times through a 200-nm pore size membrane (polycarbonate Track Etch membranes, Nucleopore Millipore, Kent, UK).

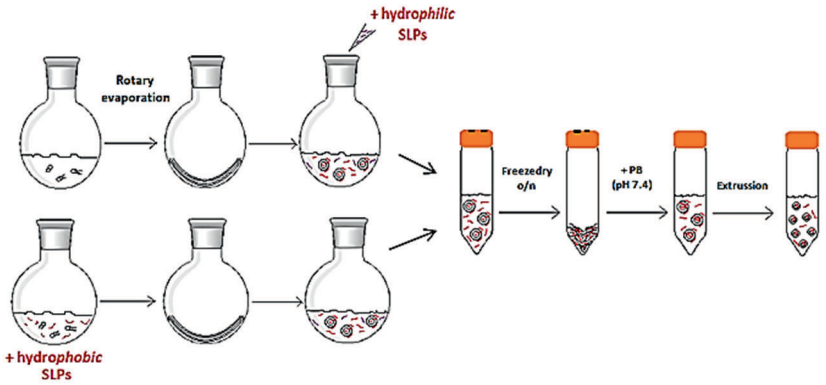

Figure 1: SLP-loaded liposomes preparation procedure. Hydrophobic SLPs were dissolved in chloroform with the lipids before the formation of the dry film, whether the solutions of hydrophilic SLPs (adjusted to a pH of 8.5) were added during the hydration of the lipid film. Overnight freeze-drying and rehydration with PB were followed by liposome sizing through filter extrusion.

Purification (removal of free SLP) and concentration of SLP-loaded liposomes were performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa): 1300 µl of the liposome dispersion were loaded in the concentrator, which was then centrifuged at 2000 rpm and 20°C for 6-7 hours, till the suspension was concentrated five-fold. The filtrate, containing the free SLP, was collected and the concentrated liposome dispersion was diluted by adding 1000 µl PB, followed again by a five-fold concentration-wash to remove most of the free peptide.

Samples of the liposome fraction and the free fractions were taken after each step for peptide content analysis (Figure 2).

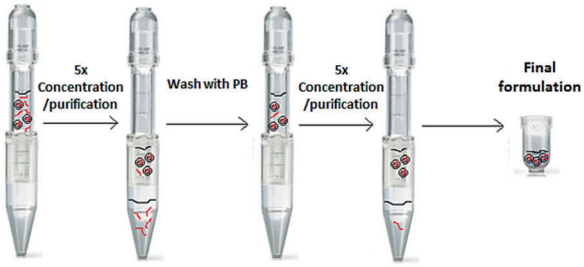

Figure 2: Purification (removal of free SLP) and concentration of SLP-loaded liposomes using VivaSpin 2 centrifugation concentrators.

Liposome characterisation

Dynamic light scattering and zeta-potential

Average diameter (Z_{ave}) and polydispersity index (PDI) of the liposomes were determined by dynamic light scattering (DLS) using a Zetasizer (NanoZS, Malvern Ltd. UK). The zeta-potential was determined by laser Doppler electrophoresis using the same device. For these measurements liposome samples were diluted 100-fold in PB.

SLP loading efficiency into the liposomes

The amount of the SLP loaded in the liposomes (t=0) was determined by measuring the fluorescence of the NBD fluorophore conjugated to the peptide (excitation wavelength 458 nm; emission wavelength 530 nm) in collected non-solubilized (i.e., intact) liposomal samples and the PB filtrates from the purification/concentration process (before wash and after wash) containing the free SLP. A calibration curve of the fluorescently labelled SLP was made and all samples were measured by using a fluorescence micro plate reader (Tecan, Salzburg, Austria).

Eight weeks (t= 8 weeks) after the first measurement of the SLP content in the liposomes, 200 µl of liposomal formulation were diluted 5x with PB buffer, followed by concentration using the VivaSpin 2 centrifugation concentrator, as previously performed. The filtrate (including free SLP) and the concentrated liposomes were collected and the loading efficiency and peptide recovery were calculated and expressed as percentage of the initial calculated loading efficiency (t=0).

In vitro MHC class I antigen (SLP) presentation

The immunogenicity of the SLP-loaded formulations was tested in an *in vitro* read-out, regarding their efficiency to activate immature DCs and present SIINFEKL to $CD8^+$ antigen specific T cells, leading to their activation. Immature D1 cells were incubated in 96-well flat-bottomed plates at 37°C in supplemented IMDM with SLP-loaded liposome formulations or plain SLPs in PBS at different concentrations. After 2.5 hours the plates were washed three times with supplemented IMDM culture medium, in order to remove excess antigen. Subsequently, T cell hybridoma B3Z cells ($50x10^5$/well) were added, followed by overnight incubation at 37°C [14]. Chlorophenol red-β-galactopyranoside (CPRG) was used as lacZ substrate in cell lysates and the colour conversion was measured by detecting absorbance at 590 nm.

Results

Using the online ExPASy ProtParam tool that allows for the calculation of various physical and chemical parameters, a series of fifteen SLPs was designed. The eleven 24-amino acid long peptides, presented in table I, cover a wide range of theoretical pIs (from 4.02 to 9.40) and the grand average of hydropathicity (GRAVY) values (from -1.208 to +0.713), representative to SLPs properties used in previous clinical studies [11]. From the theoretical pI one can judge the net charge of the peptides throughout the whole process of the liposomal preparation, while the hydropathicity factor indicates whether they are considered to be more hydrophilic or hydrophobic.

Table I: The SLP library with calculated SLPs properties based on the online ExPASy tool

	Peptide sequence NBD-G-XXXXXXXXXX-SIINFEKLAAAK	**Theoretical pI ***	**Hydropathicity (GRAVY) ****
Hydro*phili*c SLPs			
SLP 1	DEDKDKDDEEA	4.16	-1.208
SLP 2	DEEEKEGKEKA	4.82	-1.096
SLP 3	RKDDKDDKDLA	6.23	-0.963
SLP 4	RKHDHEHEHHA	7.16	-1.171
SLP 5	EDKKKSEKESA	8.34	-1.017
SLP 6	DEKRKKERELA	9.40	-1.004
Hydro*phobic* SLPs			
SLP 7	DELYDLYDELA	4.02	-0.079
SLP 8	DEGLLRHLDEA	4.90	-0.163
SLP 9	DAKHDHLLHAA	7.02	-0.104
SLP 10	LDKKLLEKELA	8.38	-0.008
SLP 11	RIDIRLIIEIA	8.59	0.713

** The isoelectric point -pI was calculated for the unlabelled peptide (11-mer varying sequence+ SIINFEKLAAAK) of which the N-terminus was not blocked, so the real theoretical pI values are expected to be slightly lower for the labelled peptide*

*** The sum of hydropathicity values of all amino acids, divided by the number of residues in the sequence. Hydrophilic proteins/peptides have values < - 1*

Four rather hydrophobic SLPs, with a pI range from 4.18 to 9.55 (supplementary table I) were excluded from the study, because of solubility problems. In an attempt to test the potential of a DOTAP-based formulation, liposomes were prepared following the dehydration-rehydration lipid film method. Hydrophilic SLPs, with a GRAVY hydropathicity value around or below -1, were loaded during the dry film rehydration, whereas hydrophobic SLPs were added to the lipid mixture before the formation of the film, since their lipophilic nature led to a by eye extensive aggregation upon mixture with aqueous solvents or physiological buffer (PB).

With regard to the encapsulation of the hydrophilic SLPs into the liposomes, the peptide's pH was adjusted at a value of about 8.5, thus above the isoelectric point (>pI) of most of the peptides where they are expected to be negatively charged. Based on our previous observations (Chapters 3-4, this thesis), this appeared to be of vital importance in order to achieve a high loading efficiency.

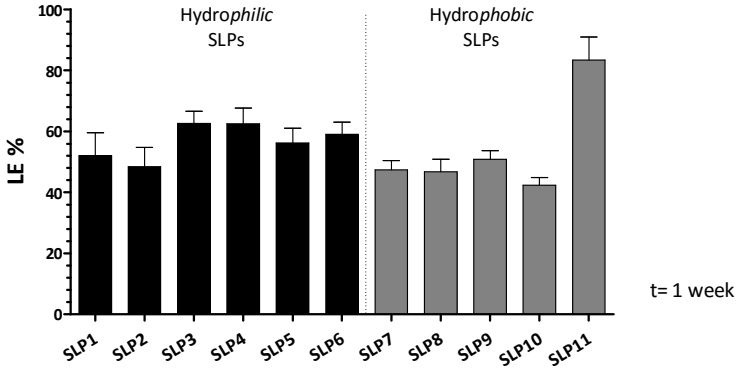

Figure 3: Loading efficiencies of hydrophilic and hydrophobic SLPs into the cationic liposomal formulation, expressed as a percentage based on the initial amount loaded (100 % LE = 1300 μg).

DOTAP/DOPC liposomes were successfully loaded with both hydrophilic and hydrophobic SLPs. Encapsulation of the SLPs yield an excellent loading efficiency above 40 % for all of the peptides, while the SLP11, the most hydrophobic SLP based on its GRAVY value, was the most readily loaded-adsorbed into the liposomes (about 80% LE) (Figure 3).

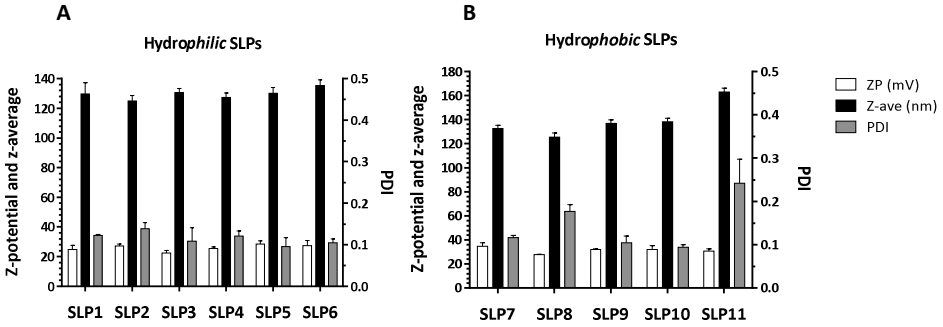

Figure 4: Physicochemical properties of the liposomal formulations loaded with (A) hydrophilic and (B) hydrophobic SLPs. Data are average ± SD of 3 independent batches. *ZP* = zeta-potential measured in mV, *Z-ave* = Z-average particle diameter in nanometres, *PDI* = polydispersity index.

Particles characterization showed that all produced liposomes had a size below 200 nm – around 130 nm – (Figure 4), a relatively low PDI (ca. < 0.3) and a positive zeta potential (ca. 30 mV). Up to at least 8 weeks of storage at 4°C, all the three parameters did not change significantly (results not shown).

The efficacy of the liposomal SLP-formulations to be engulfed by DCs and process the loaded peptide for MHC class I antigen presentation was tested in an *in vitro* immunoassay by activation of SIINFEKL-specific $CD8^+$ T cell hybridoma B3Z. Immature DCs were incubated with free SLPs and SLP-loaded liposomes, and after the removal of

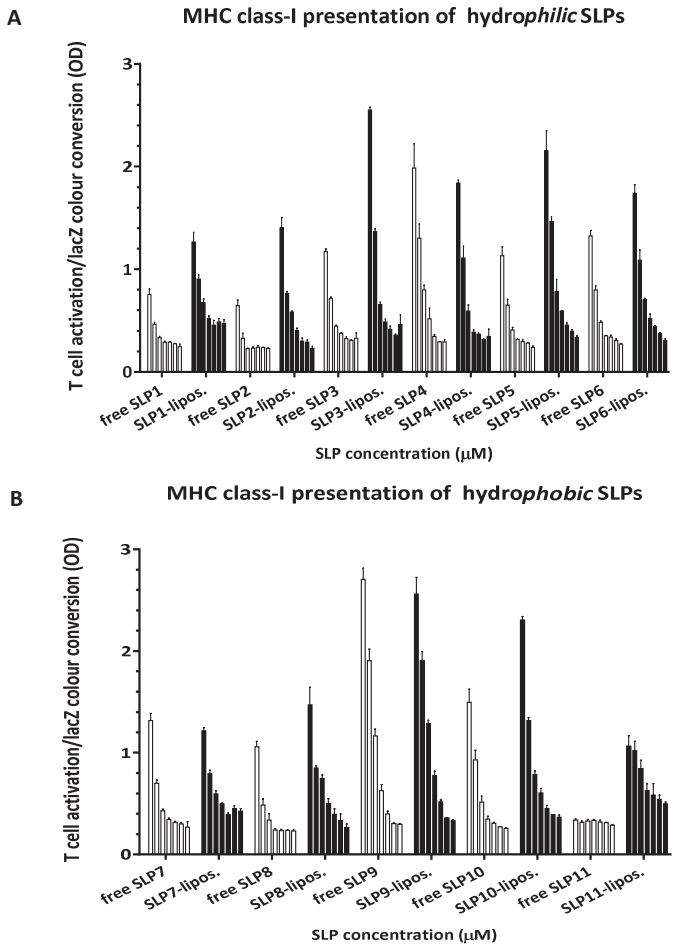

Figure 5: Activation of SIINFEKL-specific B3Z $CD8^+$ T cells after overnight culture with DCs pulsed for 2.5 h with titrated amounts of free SLPs and SLP-loaded formulations (8.0-0.125 μM). Graphs depict T cell activation based on the optical density (OD) at 590 nm measured after colour conversion of cell lysates after addition of CPRG (lacZ substrate) in samples of hydrophilic (A) and hydrophobic (B) SLPs.

the excessed antigen, B3Z cells were added to the culture for overnight incubation.

Figure 5 shows that *in vitro* activation of SIINFEKL-specific $CD8^+$ T cells by liposomes loaded with hydrophilic or hydrophobic SLPs, is similar or improved than the free peptides, showing that the antigenicity of the SLPs after their encapsulation into the liposomes has been retained; and for the lipophilic SLP 11 antigen presentation is strongly enhanced.

Finally, eight weeks after storage of the SLP-loaded liposome at 4°C, their (storage) stability was checked, by diluting the formulations 5 times in PB and then using the Vivaspin columns to separate the released free peptide from the particles. Although, for both hydrophilic and hydrophobic SLP-loaded particles, the size, PDI and z-potential remained comparable, their peptide content was reduced. More specifically after the final quantification, the SLP content in the liposomal formulations varied between 45 and 55% (Figure 6). Therefore, in all liposomes about half of the initial peptide content was still preserved after two months of storage irrespective on the peptide's nature.

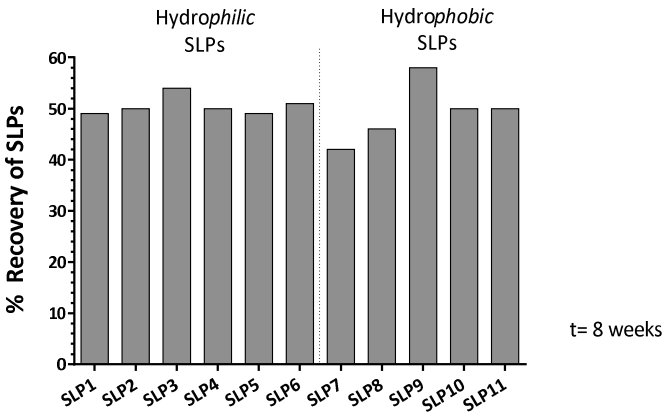

Figure 6: Recovery efficiency of hydrophilic and hydrophobic SLPs in the liposomes measured eight weeks after preparation. SLP recovery is expressed relative to the peptide loading efficiency presented in figure 3.

Discussion

In this study we investigated the potential of our DOTAP-cationic liposomal formulation for the loading of synthetic long peptide antigens and we tested their immunogenicity *in vitro*. Although liposomes have been widely used for the delivery of drugs, therapeutic agents and antigens, the development of a universal formulation for the delivery of both hydrophilic and hydrophobic compounds has not been reported yet. Using a library of 11 SLPs in total, six peptides expected to be hydrophilic and five peptides expected to be hydrophobic according to the obtained GRAVY values, the feasibility of a liposomal-based "platform" was tested.

The backbone of our vaccine delivery system is a DOTAP-based liposomal formulation. It is known that positively-charged liposomes can enhance DC maturation and subsequent T cell priming, and as a result they improve the strength of the vaccine-induced immune response, compared to negative or neutral liposomes [15]. Nevertheless, a second neutral lipid, such as DOPC in this case, with a helper role is required to preserve the stability of the formulation [16].

With regard to the loading of the hydrophilic SLPs, the standard optimized protocol for the preparation and encapsulation into liposomes procedures was followed that requires the adjustment of the SLP's solution to a pH around 8.5. Previously reported data on the DOPC: DOTAP liposomes preparation loaded with the so-called OVA24 peptide showed that a decrease of the SLP's pH below its isoelectric point led to a significant reduction of the loading efficiency [17], suggesting that the peptide entrapment in the cationic liposomes is significantly dependent on electrostatic interactions. In addition to that, the importance of the DOTAP-cationic formulation can be pointed out, since negative or neutral liposomes would negatively influence the entrapment of the hydrophilic SLPs, as we have previously observed with other SLPs.

In contrast, for the loading of the expected hydrophobic SLPs a different preparation procedure had to be followed, especially due to the lipophilic character of the peptides that did not allow their mixture with the ACN: H_2O solution. As a result, the SLPs had to be added with the lipid mixture before the dry film formation, allowing them to be readily entrapped in the lipid bilayer. In contrast, hydrophilic peptides will more likely be encapsulated in the liposomes' aqueous core and/or associated with the outer part of the lipid membrane.

Interestingly, although it would be expected that the different preparation method for the loading of the (hydrophilic and hydrophobic) SLPs would influence the

physicochemical properties of the particles, we did not observe any significant difference between the hydrophilic and hydrophobic-loaded liposomes, regarding their zeta-potential, size and polydispersity index. Furthermore, the loading efficiency did not vary significantly among the different SLPs, being around 50% for all of them, apart from the extreme hydrophobic SLP11 that yielded a LE around 80%. The reason for this improved loading efficiency that was not further investigated is not really clear; however, it could be speculated that is due to the strong hydrophobic forces between particles and the SLP11. Moreover, SLP11 showed an improved *in vitro* capacity to be processed and presented by the pulsed DCs to antigen-specific $CD8^+$ T cells compared to free SLP: SLP11 might be poorly soluble in saline buffer and therefore appears to be more antigenic when adsorbed into the liposomes. In this case, liposomes may be an advantage for extremely hydrophobic antigenic peptide sequences. The rest of the SLPs showed a comparable or better efficiency between the free and the liposomal-loaded SLP, result indicating that the functionality of the SLPs is maintained after their association with the liposomes and that their antigenicity is independent of their hydrophilic or hydrophobic nature.

Although further studies and *in vivo* data are needed to validate the immunogenicity of multiple peptide liposomes, the data support the feasibility of this platform for the delivery of SLPs with varying physicochemical properties. This is important especially for the design of a vaccine where multiple overlapping peptides are needed. As it has been previously reported, the clinical product used for the treatment of vulvar intraepithelial neoplasia consisted of 13 HPV E6/E7-derived SLPs, all of them covering a wide range of pIs (3.76 – 11.54) and hydropathicity (-1.587- +0.466) (supplementary table II), comparable to the ranges of the eleven model peptides used in our study. Provided the superior immunogenicity of liposomes compared to the Montanide ISA-51, vehicle which is used in the latter study, we propose the use of cationic liposomes loaded with SLPs as a flexible platform for therapeutic vaccination-based immunotherapy.

Conclusions

In this study, we described the efficient loading of eleven model SLPs in cationic liposomes composed of DOTAP and DOPC. The SLP-loaded liposomes were physicochemically characterised and considering the in *vitro* immunogenicity assay results we showed that the same liposomal formulation can be used for the accommodation of both hydrophilic and hydrophobic peptides, resulting in a particulate delivery system that can be used as a vaccine delivery platform for multiple antigenic peptides.

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Supplementary data

Supplementary table I: The properties of the SLPs that were excluded from the study *

	Peptide sequence NBD-G-XXXXXXXXXXX-SIINFEKLAAAK	Theoretical pI	Hydropathicity (GRAVY)
	Hydro*phobic* SLPs		
SLP 12	GSAAESASGSA	6.14	0.126
SLP 13	RDKSLKELLSA	9.53	-0.113
SLP 14	ELIDIIDIEIA	4.18	0.796
SLP 15	DLKLADLLALA	6.12	0.771

*the freeze-dried product of the above SLPs was not soluble in ACN:H_20, or $CHCl_3$: MeOH: H_2O or pure DMSO (+ TFA) even when low concentrations were tried

Supplementary table II: The properties of the 9 HPV16-E6 and 4 HPV16-E7 used in the clinic [11]

	Peptide sequence	Theoretical pI *	Hydropathicity (GRAVY) *
	Hydro*philic* SLPs		
E71-35	MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEE	3.76	-1.100
E722-56	LYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVT	3.76	-1.091
E61-32	MHQKRTAMFQDPQERPRKLPQLCTELQTTIHD	8.00	-1.250
E6109-140	RCINCQKPLCPEEKQRHLDKKQRFHNIRGRWT	10.08	-1.587
E6127-158	DKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL	11.54	-1.569
	Hydro*phobic* SLPs		
E619-50	LPQLCTELQTTIHDIILECVYCKQQLLRREVY	5.53	0.056
E691-120	YGTTLEQQYNKPLCDLLIRCINCQKPLCPEEK	6.23	-0.591
E764-98	TLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP	6.41	0.466
E685-109	HYCYSLYGTTLEQQYNKPLCDLLIR	6.73	-0.400
E743-77	GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR	6.89	-0.277
E641-65	KQQLLRREVYDFAFRDLCIVYRDGN	8.17	-0.536
E655-80	RDLCIVYRDGNPYAVCDKCLKFYSKI	8.59	-0.165
E671-95	DKCLKFYSKISEYRHYCYSLYGTTL	8.71	-0.536

*SLPs properties were calculated as previously according to the online ExPASy tool

Chapter 7

Summary

General Discussion and Perspectives

Conclusion

Summary

Therapeutic vaccination with well-defined cancer- associated antigens has shown promising clinical results. Synthetic long peptides (SLP) vaccines that include potential MHC class I and II restricted epitopes of tumour antigens have been proven superior to vaccination with protein antigens or minimal synthetic peptide epitopes for the induction of long-term $CD8^+$ T cell immune responses. Impressive clinical responses have been observed in women with human papillomavirus (HPV) 16^+ pre-malignant lesions after therapeutic vaccination with 13 overlapping SLPs derived from HPV16 E6 and E7 oncoproteins; however, end stage $HPV16^+$ cervical cancer patients did not benefit from this type of vaccine. These studies showed that SLP vaccines have functional potency when applied to pre-malignant stage patients, but need to be improved for use as a therapeutic intervention against cancer. So far, SLPs have been formulated with Montanide ISA-51, the most common vehicle used for peptide vaccinations in clinical trials; however, the use of such water-in-oil formulations has some important drawbacks and can induce local side effects. Therefore, there is an urgent need for replacement of Montanide emulsion-based formulations by more potent and safe alternatives.

The main objective of this thesis was to investigate the potential of particulate systems for the delivery of SLP vaccines and ultimately develop a particulate formulation concept for cancer immunotherapy as an alternative to Montanide ISA-51 emulsion, currently used in the clinic.

In *Chapter 2* a poly(I:C)-adjuvanted DOTAP-based liposomal formulation loaded with the model OVA-derived, 24-amino acid long SLP (OVA24) is introduced as a suitable vaccine candidate for induction of a functional $CD8^+$ T cell response, required in cancer immunotherapy. The high (positive) surface charge density of the DOTAP:DOPC (1:1 molar ratio) liposomes facilitated the loading of OVA24. Following the film hydration-rehydration method, OVA24-loaded cationic liposomes were prepared with a size of about 140 nm, a low polydispersity and a peptide loading efficiency of about 40%. Encapsulation of poly(I:C) in the formulation, with a loading efficiency of 50%, did not significantly affect the OVA24-loaded liposome properties. Immunogenicity of the developed SLP formulations was firstly assessed *in vitro*, based on their capacity to efficiently activate DCs and successfully present the processed SLP to a SIINFEKL-specific T cell-line. After intradermal immunisation in mice, the OVA24-loaded poly(I:C)-adjuvanted liposomal vaccines (independent of the way poly(I:C) was delivered, co-encapsulated with OVA24 or just mixed with the OVA24-loaded liposomes prior to immunisation) induced strong endogenous T cell responses, clearly detectable in blood without the need of additional re-stimulation *in vitro*. In addition, phenotypic characterisation of the 2% SLP-specific $CD8^+$ T cells in blood induced by the

OVA24/poly(I:C)-liposomes, revealed their functionality: these cells were functional effector cells, capable of producing IFN-γ and TNF-α cytokines and showing strong cytotoxic activity *in vivo* by killing up to 80% of transferred target cells. These findings show the functional effectiveness of the OVA24/poly(I:C)-liposomal formulation compared to the poorly immunogenic free-compounds vaccine.

In *Chapter 3*, it is shown how we further improved the potency of our developed OVA24-loaded DOTAP-based liposome formulation, by incorporating OVA17, a second SLP containing an OVA T_H epitope, which is crucial to induce optimal T cell immunity by vaccination. After optimisation of the peptide loading, which was needed to ensure the highest possible encapsulation efficiency of both SLPs, OVA17 was successfully incorporated in the formulation, either co-encapsulated in the same particle with OVA24 or loaded in separate liposomes. The generated liposomes were physicochemically characterised and the effect of the presence of OVA17 on the induction of OVA24-specific T cells was investigated, also when combined with different TLR ligands, namely CpG or Pam3CysK$_4$, in comparison with poly(I:C), the adjuvant used in *chapter 2*. Here, we showed that intradermal vaccination (at the abdominal area or the tail base) with liposomes loaded with both OVA24 and OVA17 greatly increased the percentage of the functional T cells producing IFN-γ and TNF-α, as compared to liposomes containing OVA24 alone. Interestingly, the "help" by the presence of OVA17 was effective independently of the way the SLP was delivered: co-encapsulated with OVA24 or separately as OVA17-containing liposomes mixed with OVA24-containing liposomes. Moreover, we showed the ability of the OVA24+OVA17-containing cationic liposomes to directly activate not only $CD8^+$ T cells, but also significant numbers of functional $CD4^+$ T-helper cells, able to initiate a T_H1 immune response characterised by the production of IFN-γ. In addition, inclusion of a TLR ligand (poly(I:C), CpG or Pam3CysK$_4$) in the liposomal vaccines greatly improved the frequencies of the antigen-specific $CD8^+$ T cells and their killing capacity, compared to the free-compounds vaccines. Since poly(I:C) appeared to be the most potent of the three tested TLR ligands, poly(I:C)-adjuvanted liposomal formulations were also tested for their efficiency as cancer vaccines: in a therapeutic setting using the B16-OVA melanoma model, the outgrowth of established tumours was significantly controlled in mice that received OVA24/OVA17/poly(I:C)-liposomes. These mice survived for almost 4 weeks longer than mice treated with free SLP and poly(I:C), showing the effectiveness of liposome encapsulation. Last, 2/8 mice immunised intradermally on the tail base instead of the abdominal area, were completely cured from their established tumours, suggesting an improved vaccine potency and a possibly faster lymph node drainage as compared to the intradermal immunisation at the abdominal area.

Cationic liposomal formulations loaded with two OVA SLPs and adjuvanted with a TLR ligand appeared to be very effective regarding the induction of antigen-specific $CD8^+$ T cells, without the inclusion of any targeting device. In *chapter 4*, cationic liposomes and PLGA nanoparticles, both potential candidates for the delivery of SLP vaccines, are directly compared to clinically used Montanide ISA-51 and squalene-based SWE emulsion. The particles were successfully loaded with two SLPs (OVA24 and OVA17) and two TLR ligands (poly(I:C) and $Pam3CSK_4$), generating SLP-loaded PLGA NPs and liposomes that were different in size and surface charge. Co-delivery of a T_H epitope (present in OVA17) with a CTL epitope (included in OVA24) in liposomes increased the expression of effector cytokine (IFN-γ)-producing T cells, result in line with findings presented in *chapter 3*. Furthermore, incorporation of a TLR ligand in liposomes did not seem to be crucial for induction of a strong *in vivo* cytotoxicity. In contrast, unadjuvanted PLGA NPs were poorly immunogenic. The PLGA NP formulation including both SLPs and poly(I:C) appeared to be the most efficient PLGA formulation, performing better than Montanide or squalene water-in-oil emulsion (SWE), but not liposomes. Liposomes, compared to the other particulate systems studied in this chapter, appeared to be the most promising system for the delivery of SLPs. This finding can be ascribed to the positive charge of the liposomes, their small size (compared to PLGA NPs) and/or their expected effective processing by APCs.

The study presented in *Chapter 5* is a proof-of-concept application of the research described in this thesis with a clinically relevant tumour antigen. Cationic liposomes were successfully prepared following the optimised protocol described in previous chapters, loaded with a 21-amino acid-long SLP derived from the E7 protein of HPV and adjuvanted with poly(I:C). The goal of the study was to investigate the potency of the formulation as a new therapeutic vaccine concept, in comparison with a Montanide ISA-51 emulsion-based E7 SLP formulation. The E7 SLP-loaded liposomes, compared to the free-compounds vaccine, induced high numbers of antigen-specific IFN-γ +TNF-α-producing T cells and showed a strong cytotoxic activity when target cells were transferred in intradermally (abdominal area and tail base) immunised mice, even in the absence of poly(I:C). In mice with established TC-1 tumours (cells expressing HPV16-E7 proteins) when immunised with E7 SLP+poly(I:C)-loaded liposomes, the tumour outgrowth was better controlled, and at least 75% of the mice were completely cured after a single intradermal vaccination. In contrast, from the mice immunised with E7 SLP+poly(I:C) formulated with Montanide ISA-51, with the standard (65-fold higher) SLP dose, only 25% survived within the studied timeline (whereas no effect was observed when the same (low) SLP+poly(I:C) dose as in liposomes was used with Montanide). Finally, the single intradermal immunisation of mice with the E7/poly(I:C)-liposomes was

sufficient to develop an immune response strong enough to protect mice also after a tumour rechallenge.

In *chapter 6*, we examined the general applicability of the developed liposomal formulation for the delivery of different SLPs: Eleven SLPs, all of them harbouring the model SIINFEKL epitope, but with different hydropathicities and isoelectric points, were designed. Following standardised protocols, all the SLPs were successfully loaded into the cationic liposomal formulation, yielding high loading efficiencies. The prepared liposomes were characterised for their size, polydispersity and zeta potential, and for their efficiency to activate DCs and present the processed antigen to $CD8^+$ T cells. The liposomes had an average diameter of about 200 nm, with a low PDI (< 0.3) and a positive zeta-potential. All liposomal SLPs efficiently activated DCs that subsequently presented the MHC class I epitope to SIINFEKL-specific T cells *in vitro*, indicating the feasibility of the formulation as a platform for the delivery of SLPs for cancer immunotherapy.

General discussion and perspectives

Whereas several DNA-, protein- and peptide-based therapeutic vaccines have been investigated in both preclinical and clinical trials, most of them fail to induce eradication of already established tumours. The use of nanotechnology and particulate delivery systems could play a key role in developing new strategies or improving current vaccination approaches for safer, cost-effective and easier-applied cancer immunotherapy.

In this thesis, the concept of cationic liposome-based formulations was introduced, as the backbone for improved delivery of SLPs for cancer immunotherapy. As shown in *chapter 2*, cationic liposomes are excellent nanospheres to encapsulate SLPs with intact immunological functionality. The model SLP loaded in the liposomes was very potent in inducing functional cytotoxic $CD8^+$ T cells. Even though the complexes of the MHC class I + peptide can be recognised by naive $CD8^+$ T cells directly, in order for them to become effector and cytotoxic, they require sufficient co-stimulatory signals from the activated DCs [1]. Therefore, although the reason for the enhanced immunogenicity of the developed DOTAP-based liposomes is not known, it was speculated that it is due to the proper activation and maturation of DCs. It is known that particulate delivery systems, including liposomes, can offer numerous advantages for DC-targeted vaccination in comparison with free antigens [2]; considering that the developed formulation does not include any targeting molecule or device, DCs are most likely targeted in a passive way, for instance because of the small size (< 200 nm) and/or the cationic nature of the liposomes. With respect to that, cationic liposomes are regarded more efficient than neutral or anionic ones, since their affinity with cell membranes can induce maturation of DCs after particle uptake [3]. Or, as we showed here, cationic liposomes can be excellent carriers for defined TLR ligands, such as poly(I:C), which retain their DC-maturing capacity after particle uptake. Regarding the liposomes' size, it has been suggested that small particles (~200 nm) are naturally taken up by endocytosis, resulting in a cellular immune response through DC targeting, while larger particles are more likely to be phagocytosed by macrophages, leading to a humoral immune response [4,5].

Once the particles have reached their APC (DCs) target, the intracellular processing of the antigen is equally crucial for the efficiency of the cross-presentation, as the ability of DCs to cross-present exogenous antigens is not intrinsic, but can depend on the route of antigen uptake and the following processed antigen's accumulation in the endolytic compartments [1]. The mechanisms of liposome uptake and intracellular fate of the loaded SLPs were not investigated, so future focus on mechanistic studies of DC

activation with our liposomes is needed in order to gain insight into important parameters underlying the liposomes' performance [2]. Furthermore, a systematic comparison of the DOTAP-based formulation with neutral and anionic SLP-loaded liposomes as well as liposomes of different size range will contribute to define what the requirements for enhanced immunogenicity might be.

Nevertheless, from *chapters 2* and *3*, it already became clear that the liposomes can deliver adequate amounts of SLPs into the DCs, even when the injected dose in *chapter 3* was reduced at least 25-fold, compared to the standard dose used in *chapter 2*: using a rather low dose of OVA SLP –liposomes (2.5 µg OVA24/mouse), a high frequency of SLP-specific $CD8^+$ T cells *in vivo* was achieved and functional $CD8^+$ and $CD4^+$ T cell responses were observed. Activation and proliferation of $CD4^+$ T cells is crucial for an optimal $CD8^+$ T cell response and development of $CD8^+$ memory [6], since $CD4^+$ T cells help in the differentiation of naive $CD8^+$ T cells into effector T cells through activation signals, mostly IL-2 secretion [7]. So, based on the robust $CD4^+$ responses detected, the superiority of the liposomes over free OVA SLPs as a cancer therapeutic vaccine was shown. Moreover, although the use of a T_H SLP in *chapter 3* significantly improved $CD8^+$ T cell priming, the inclusion of a TLR ligand as an adjuvant appeared to be crucial for the control of the tumour outgrowth. In both humans and mice, a large list of TLR ligands is known and all of them function as adjuvants, stimulating cross-presentation [1] and proper DC targeting and activation [3]. For the currently described studies, the TLR3-ligand poly(I:C) was the mostly used adjuvant combined with the liposomes; however, replacement of poly(I:C) with the TLR9 agonist CpG and the TLR1/2 agonist $Pam3CSK_4$, which can provide different danger signals, was also achieved. In a study by Korsholm et al., cationic DDA-liposomes combined with poly(I:C) and loaded with different antigens such as OVA, Gag p24 (from HIV), TB10.3 or H56 (from tuberculosis) and E7 (from HPV), led to high numbers of antigen-specific $CD8^+$ T cells, when administered intraperitoneally in mice [8]. In another study, Jerome et al. showed significant tumour regression and prolonged survival when mice were vaccinated intradermally with liposomes loaded with a melanoma-associated peptide from the tyrosinase-related protein 2 (Trp2) and adjuvanted with CpG [9]. Noteworthy, in contrast to several studies where it has been shown that co-delivery of the adjuvant with the antigen is beneficial [2,10], we showed that the effect of the TLR3 ligand, poly(I:C), was independent of the way it was formulated, co-encapsulated or mixed just before the immunisation. This may be explained by adsorption of the free poly(I:C) to the liposomes after mixing, which was shown to occur to some extent (>50%) (unpublished data), offering a simple way to prepare poly(I:C)-adjuvanted SLP-liposomes.

The direct comparison in *chapter 4* of cationic liposomes with PLGA NPs, both of which are potential alternatives to the clinically used Montanide ISA-51 formulation, highlighted the possibilities of particulate delivery systems as therapeutic cancer vaccines. According to the literature, PLGA NPs encapsulated with the melanoma-specific antigen gp100 [11] were efficient in inducing effector cytokines-producing T cells and protecting mice from a tumour challenge. Although in our study SLP-loaded cationic liposomes appeared to be more potent than the studied PLGA NPs, it is difficult to pinpoint the critical factors responsible for this. The two SLP-loaded delivery systems differ in their particle size, charge and adjuvant effect, parameters that can significantly influence their targeting efficiency and their potential to activate DCs. Therefore, more systematic *in vitro* and *in vivo* comparative studies with particles tuned into the desired characteristics are needed, in order to gain a better understanding about formulation factors that determine the initiation and development of the induced immune response.

Apart from a potent antigen and an efficient delivery system, the route of administration is very important for antigen presentation and, consequently, the success of a therapeutic vaccine. As a general rule, the preferred route should be able to deliver the antigen to DCs and initiate a rapid T cell-based immunity. Although there are many administration routes nowadays under investigation, the most commonly used route remains the subcutaneous (s.c.) one. In *chapter 2*, intradermal (i.d.) administration of the poly(I:C) adjuvanted SLP-cationic liposomes led to induction of $CD8^+$ T cells with a stronger killing capacity, compared to s.c. administration of the same formulation. In addition, in *chapter 5*, a single i.d. tail base vaccination of TC-1 tumour-bearing mice with a low dose of E7 SPL+poly(I:C)-liposomes led to complete clearance of the tumours in 100% of the mice. It can be speculated that liposomes after i.d. administration rapidly reach the draining lymph nodes, where they are recognised and taken up by lymph node resident $CD8\alpha^+$ DCs. Besides, the skin is a very active tissue immunologically, harbouring DC subsets such as Langerhans (LCs) and dermal DCs, known as strong inducers of a T cell-based immunity [12]. In that way, we could also speculate that after i.d. administration, the liposomes can easier target and mature local dermal DCs, which in turn more efficiently migrate to the draining nodes, as compared to s.c. administration. With respect to that, although $CD8\alpha^+$ DCs are considered to be the most well-equipped DC subset for the cross-presentation of exogenously internalised antigens on MHC class I molecules [13-16], the contribution of skin-resident DCs to the liposomes' immunogenicity cannot be excluded. *In vitro* and *in vivo* trafficking studies with fluorescently-labelled liposomes are needed to investigate the liposomes' targeting properties, while some more administration routes need to be tested. Especially for the development of a therapeutic vaccine against oncogenic HPV infections, it may be of

high importance to carefully choose the administration route and location, as the generated T cells need to travel to the genital mucosa. Regarding this, studies have shown strong induction of $CD103^+$ ($CD8^+$) T cells in the vaginal mucosa after intravaginal delivery of DNA-loaded HPV pseudoviral particles [17] and development of effector memory T cells to epithelium of the vaginal tract, crucial for the combat of HPV-associated cervical tumours [18].

The findings presented in *chapter 6* are interesting from a pharmaceutical perspective: the feasibility of the generation of non-targeted liposome-based therapeutic vaccines loaded with SLPs covering a large range of physicochemical properties can offer great opportunities, not only regarding the loading of tumour-associated SLP antigens, but also SLP antigens from infectious diseases. Yet, since the work presented here is basically development of SLP-loaded liposomal formulations and includes an *in vitro* only test checking the ability of the produced liposomes to activate DCs and present the processed SLP to activated antigen-specific T cells, *in vivo* animal work is required to evaluate the particles' potency and their potential as therapeutic vaccines.

Finally, although this thesis shows the possibilities of the developed DOTAP-based cationic liposomal formulation, there is still much to be done before this technology can be transferred from the bench to human application. First of all, the feasibility of the preparation of this formulation into a larger scale and under GMP conditions has to be tested. The long-term storage stability of the liposomes is another issue that has to be assessed. In case of insufficient stability, formulation development and optimization will have to be performed, including the feasibility of developing a lyophilized formulation.

Furthermore, the promising results observed in TC-1 tumour-bearing mice (*chapter 5*) cannot be expected to be directly translatable in human patients. For human application, the antigen has to be wisely chosen; for non-virus-induced tumours personalised liposomal therapeutic vaccines against mutated neo-antigens [19-21] that are not prone to central tolerance, might stand a higher chance of success, compared to vaccines against tumour-associated self-cancer antigens [22]. Moreover, the optimal dosage of the vaccine has to be determined and possible toxicity or side effects have to be assessed as well. The administration location for human use is another parameter to be taken into consideration: for instance, the tail base immunisation in mice is a very efficient delivery route, one could consider equivalent vaccination sites in humans, such as proximate to inguinal lymph nodes, with optimal draining to improve vaccination efficacy.

In addition, because of the immunosuppressive mechanisms in cancer environment, it can be a rather ambitious approach to use cationic liposomes as cancer monotherapy. Combination of liposomes for immunotherapy with chemotherapeutics, such as particles loaded with doxorubicin, cisplatin [23,24] or paclitaxel [25] can lead to better results, by increasing the efficacy of the treatment and even reducing possible toxicity or side-effects of the free chemotherapeutic compounds. Liposome-based therapeutic vaccines can also be combined with photodynamic therapy, where photosensitizers are used to generate reactive species after selective light exposure. Photosensitizers also can be loaded into liposomes, as has been previously described with lipid nanocapsules [26]. Last but not least, since in this research we used non-targeted liposomes for cancer immunotherapy, another rational choice for application of a combined treatment would be co-medication with agonists that take away the breaks of immune suppression, such as the checkpoint-inhibitors, CTLA-4 or PD-1, or monoclonal targeting antibodies (CD40, TGF-β etc.); approaches that have led to promising results in preclinical studies and their loading into liposomes can enhance their targeting properties.

Conclusion

Cationic DOTAP-based liposomes loaded with SLPs and poly(I:C) TLR3 ligand were proven to be powerful candidates for potential therapeutic cancer vaccines. Their ability to induce high frequencies of antigen-specific effector $CD4^+$ T_H and cytotoxic $CD8^+$ T cells, as well as their efficacy to induce immune responses able to control tumour outgrowth in aggressive tumour models were reported. These findings make cationic liposomes a very promising platform for SLP-based cancer immunotherapy. Additionally, their flexibility regarding the properties of loaded SLPs, their relative inexpensive production and the possibility to administer them via different delivery routes are all in favour for liposomal SLP-based cancer immunotherapy to become reality soon. Combination of cationic liposomes with other classical and novel cancer therapies could lead to highly effective personalized immunotherapy of cancer.

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Appendices

Nederlandse samenvatting

Curriculum vitae

List of publications

List of abbreviations

Nederlandse Samenvatting

Deel I: Inleiding

Kankerimmunotherapie door vaccinatie

Immunotherapie is een veelbelovende strategie om kanker, de meest prevalente doodsoorzaak wereldwijd, te behandelen. In tegenstelling tot conventionele behandelmethoden zoals chirurgie, chemotherapie en radiotherapie, is immunotherapie in staat om kankercellen specifiek aan te vallen, terwijl het gezonde cellen grotendeels ongemoeid laat. Hiervoor is het nodig dat een adequate immuunreactie tegen kanker opgewekt wordt. Dat vereist met name een inductie van T-cel gemedieerde immuniteit omdat T cellen in staat zijn intracellulaire tumorspecifieke antigenen te herkennen door middel van peptiden die gepresenteerd worden in *major histocompatibility complex* (MHC) moleculen. De inductie van een T-celrespons verloopt via de activatie van antigeenpresenterende cellen (APCs), voornamelijk dendritische cellen (DCs), en vervolgens MHC presentatie van tumorpeptiden aan en stimulatie van $CD4^+$ T-helpercellen en $CD8^+$ cytotoxische T cellen.Het doel van kankerimmunotherapie is dus om het immuunsysteem van de patiënt zelf te gebruiken om specifiek tumorcellen te doden en verschillende methoden gebaseerd op dit principe zijn momenteel in ontwikkeling.

Echter, de meeste pogingen tot immunotherapeutische interventie tegen tumoren waren tot nog toe teleurstellend. Mogelijke verklaringen voor het ontbreken van klinische effectiviteit zijn wellicht de geringe presentatie van antigenen, de micro-omgeving van de tumor, de tumorheterogeniteit, alsook de kinetiek en het fenotype van de geïnduceerde T-celrespons. Uit veel onderzoek blijkt dat de ontduiking van de immuunrespons door tumorcellen de effectiviteit van immuuntherapie kan beperken. Niettemin deed een recente ontwikkeling gebaseerd op T-celstimulatie gericht tegen tumorspecifiekeantigenen de hoop herleven in het wetenschappelijk veld. Dit was de behandeling met sipuleucel-T (APC8015; merknaam Provenge), toegelaten voor therapeutisch gebruik bij patiënten met prostaatkanker, waarbij autologe dendritische cellen worden beladen met prostaatantigenen gericht op de inductie van T-celresponsen tegen de tumor van de patiënt. Andere oncologische aandoeningen waarvoor intensief onderzoek naar therapeutische vaccinatie wordt verricht, zijn onder andere melanoom, longkanker en baarmoederhalskanker.

Synthetische lange-peptidevaccins en HPV-geïnduceerde tumoren

Voor wat betreft immunotherapie door middel van therapeutische vaccinatie bleken synthetische lange-peptidevaccins (SLP-vaccins) die potentiële MHC klasse I- en II-epitopen van tumorantigenen bevatten superieur te zijn voor de inductie van langdurige $CD8^+$ en $CD4^+$ T-cel immuunresponsen ten opzichte van vaccinatie met eiwitantigenen of korte peptiden met een minimaal epitoop. Indrukwekkende klinische responsen zijn waargenomen in vrouwen met door humaan papillomavirus (HPV) 16^+ veroorzaakte laesies in het pre-maligne stadium na therapeutische vaccinatie met 13 overlappende SLPs afgeleid van HPV16-E6 en -E7 oncoproteïnen. Echter, eindstadium $HPV16^+$ baarmoederhalskankerpatiënten hadden geen baat bij dit type vaccin. Deze studies toonden aan dat SLP-vaccins werkzaam zijn bij patiënten in het pre-maligne stadium, maar ook dat SLP-vaccins verbeterd moeten worden om als een therapeutische interventie tegen kanker gebruikt te worden. Tot nog toe zijn SLPs geformuleerd met Montanide ISA-51, het meest gebruikte adjuvans voor peptidevaccinaties in klinische studies. Echter, het gebruik van Montanide heeft wezenlijke nadelen en kan lokale bijwerkingen veroorzaken. Daarom is er een dringende behoefte aan een vervanging van op Montanide-emulsie gebaseerde SLP-vaccins door meer potente en veilige alternatieven.

Immunostimulatoren en liposomen

Adjuvantia kunnen grofweg ingedeeld worden in immunostimulerende moleculen en toedieningssystemen. Een belangrijke functie van immunostimulerende adjuvantia is het induceren van APC-maturatie door stimulatie van receptoren betrokken bij de aspecifieke immuniteit, zodat een (antigeen-specifieke) cellulaire immuunrespons wordt geïnduceerd en tolerantie wordt voorkomen. Idealiter is een adjuvans veilig, stabiel, biodegradeerbaar en betaalbaar, en is het in staat zijn om de wenselijke cellulaire immuunresponsen op te wekken. Momenteel zijn er maar weinig adjuvantia die goedgekeurd zijn voor gebruik in mensen. Naast colloïdale aluminiumzouten zijn dit AS04 in de Verenigde Staten van Amerika en in Europa ook influenzavirosomen, VLPs en de olie-in-wateremulsies MF59, AS03 en AF03, die voornamelijk gebruikt worden in profylactische vaccins tegen infectieziekten (voornamelijk inductie van humorale immuniteit). Deze adjuvantia worden minder geschikt geacht voor therapeutische vaccinatie tegen (HPV-geïnduceerde) kanker.

Liposomen zijn van oudsher gebruikt voor de toediening van anti-kankermedicijnen zoals doxorubicine of anthracycline, maar zij kunnen ook worden gebruikt voor de toediening van antigenen. Liposomen zijn kleine blaasjes die bestaan uit een of meerdere lipide bilagen, en kunnen afhankelijk van de bereidingswijze in grootte variëren van minder dan 100 nm tot enkele micrometers. Liposomen kunnen hydrofiele stoffen, zoals nucleïnezuren, insluiten in het waterige compartiment, terwijl lipofiele stoffen geïntegreerd kunnen worden in het liposomale membraan. Zij staan erom bekend dat zij in staat zijn om de antigenen te beschermen tegen enzymatische degradatie en daarmee de stabiliteit en biologische beschikbaarheid van ervan te verbeteren. Voordelen zijn onder andere de uitstekende biologische verenigbaarheid, biodegradeerbaarheid en het vermogen om de grootte en oppervlakte-eigenschappen te kunnen modificeren. Vanuit een immunologisch standpunt kunnen zij uiterst nuttig zijn omdat zij kunnen worden opgenomen door APCs, waarbij kruispresentatie verbeterd wordt.

Deel II: Doelstellingen van het in dit proefschrift beschreven onderzoek

De hoofddoelstellingen van dit proefschrift waren:

- Het ontwerpen van een concept-formulering voor een SLP-vaccin voor kankerimmunotherapie gebaseerd op deeltjesvormige toedieningssystemen
- Het doen van onderzoek naar kationogene liposomen als een flexibel platform voor gedefinieerde therapeutische vaccins
- Het verbeteren van de immunogeniciteit van op SLPs gebaseerde vaccins
- Het vervangen van Montanide ISA-51 door veilige, biodegradeerbare nanodeeltjes voor klinisch gebruik

Deel III: Samenvatting

In *hoofdstuk* 2 is een liposoomformulering geïntroduceerd gebaseerd op DOTAP en DOPC (1:1 molaire ratio), beladen met het van ovalbumine afgeleide 24 aminozuren lange SLP (OVA24) dat het bekende $CD8^+$ T-celepitoop SIINFEKL bevat en de immunostimulator poly(I:C). Deze OVA24-liposoomformulering is onderzocht als een geschikte vaccinkandidaat voor het induceren van een functionele $CD8^+$ T-celrespons, hetgeen nodig is voor kankerimmunotherapie. De hoge (positieve) oppervlakteladingsdichtheid van de liposomen faciliteerde het inbouwen van OVA24. Door middel van de film hydratatie-rehydratatiemethode werden OVA24-liposomen

verkregen met een grootte van ongeveer 140 nm, een lage polydispersiteit en een SLP-beladingsefficiëntie van ongeveer 40%. Inkapseling van poly(I:C) in de formulering, met een beladingsefficiëntie van 50%, veranderde de eigenschappen van de OVA24-liposomen niet wezenlijk. De immunogeniciteit van de ontwikkelde SLP-liposoomformuleringen was eerst *in vitro* geëvalueerd op hun capaciteit om efficiënt DCs te activeren en succesvol het verwerkte SLP te presenteren aan een SIINFEKL-specifieke T cellijn. Na intradermale immunisatie van muizen bleek dat het OVA24-liposoomvaccin (onafhankelijk van de manier waarop poly(I:C) was toegediend, samen ingekapseld met OVA24 of in opgeloste vorm gemengd met de OVA24-liposomen) in staat was om sterke endogene T-celresponsen op te wekken, hetgeen duidelijk te detecteren was in bloed, zonder dat daar een additionele restimulatie *in vitro* voor nodig was. Bovendien werd de functionaliteit aangetoond van de 2% SLP-specifieke $CD8^+$ T cellen in bloed, geïnduceerd door de OVA24-liposomen: deze cellen waren functionele effectorcellen die IFN-γ en TNF-α cytokines produceerden en bovendien sterke cytotoxische activiteit toonden: tot 80% van de overgebrachte doelcellen werden geneutraliseerd *in vivo*. Deze bevindingen toonden de functionele effectiviteit van de OVA24-liposomen aan, terwijl het SLP in opgeloste vorm, met of zonder poly(I:C), geringe immunogeniciteit vertoonde.

In *hoofdstuk 3* is aangetoond hoe wij de potentie van onze OVA24-liposoomformulering verder verbeterd hebben, door de integratie van OVA17, een tweede SLP dat een OVA T_H epitoop bevat, welke cruciaal is om optimale T-celimmuniteit door vaccinatie te induceren. Om verzekerd te zijn van de hoogst mogelijke inkapselingsefficiëntie van beide SLPs, OVA24 en OVA17, werd het liposoombereidingsproces geoptimaliseerd. Daarnaast werd een formulering ontwikkeld welke samengesteld was uit afzonderlijke OVA17- en OVA24-liposomen. De verkregen liposomen werden fysisch-chemisch gekarakteriseerd. Het effect van de aanwezigheid van OVA17 op de inductie van OVA24-specifieke T cellen werd bestudeerd, ook in gecombinatie met verschillende TLR liganden, namelijk CpG of $Pam3CysK_4$ in vergelijking met poly(I:C), het adjuvans dat gebruikt is in *hoofdstuk 2*. Intradermale vaccinatie (in de abdominale streek of bij de staartbasis) met liposomen beladen met zowel OVA24 als OVA17 deed het percentage van de functionele T cellen dat IFN-γ als TNF-α produceert geweldig toenemen, in vergelijking met liposomen die alleen OVA24 bevatten. Wetenswaardig is dat de “hulp” van de aanwezigheid van OVA17 effectief was, onafhankelijk van de manier waarop de SLPs waren toegediend: ingekapseld in dezelfde liposomen (OVA24/OVA17-liposomen) als OVA24, of afzonderlijk als OVA17-liposomen gemengd met OVA24-liposomen. Bovendien lieten wij zien dat de OVA24/OVA17-liposomen in staat waren om zowel

$CD8^+$ T cellen als aanzienlijke aantallen van functionele $CD4^+$ T-helpercellen van het T_H1-type, gekarakteriseerd door de productie van IFN-γ, te activeren. Bovendien deed de inclusie van een TLR ligand (poly(I:C), CpG of $Pam3CysK_4$) in de liposomen de aantallen antigeen-specifieke $CD8^+$ T cellen en hun cytotoxische capaciteit geweldig toenemen, in vergelijking met vaccinsamenstellingen met opgeloste immunostimulatoren. Omdat poly(I:C) de meest potente van de drie geteste TLR liganden bleek, werden liposoomformuleringen met poly(I:C) ook getest op hun werkzaamheid als therapeutische kankervaccins. In een therapeutische proefopzet waarbij het B16-OVA-melanoommodel werd gebruikt, werd de uitgroei van gevestigde tumoren aanzienlijk ingeperkt in muizen die OVA24/OVA17/poly(I:C)-liposomen toegediend kregen. Deze muizen overleefden bijna 4 weken langer dan muizen die behandeld werden met de SLPs en poly(I:C) vrij in oplossing. Tot slot, een kwart van de muizen die intradermaal geïmmuniseerd waren op de staartbasis waren geheel genezen van hun gevestigde tumoren, wat een verhoogde vaccinatiepotentie en een mogelijk snellere lymfeknoopdrainering suggereert, in vergelijking met intradermale immunisatie in de abdominale streek.

Kationogene liposoomformuleringen beladen met twee OVA-SLPs en met een TLR ligand als adjuvans bleken erg effectief te zijn met betrekking tot de inductie van antigeen-specifieke $CD8^+$ T cellen. In *hoofdstuk 4* zijn kationogene liposomen en PLGA-nanodeeltjes (PLGA-NPs) vergeleken met twee reeds in de kliniek gebruikte adjuvantia, Montanide ISA-51 en een op squaleen-gebaseerde water-in-olie emulsie (SWE), als adjuvans voor SLP-vaccins. De liposomen en PLGA-NPs waren succesvol beladen met twee SLPs (OVA24 en OVA17) en twee TLR liganden (poly(I:C) en $Pam3CysK_4$), waardoor SLP-beladen PLGA-NPs en lipsomen ontstonden die verschillend waren in grootte en oppervlaktelading. Gelijktijdige toediening van een T_H-epitoop (aanwezig in OVA17) met een CTL-epitoop (inbegrepen in OVA24) in liposomen verhoogde de expressie van effector cytokine (IFN-γ)-producerende T cellen, in lijn met de bevindingen gepresenteerd in *hoofdstuk 3*. Bovendien bleek dat de integratie van een TLR ligand in liposomen niet cruciaal was voor de inductie van een sterke *in vivo* cytotoxiciteit. Ongeadjuveerde PLGA-NPs waren daarentegen gering immunogeen. Van alle PLGA-NP-formuleringen was degene die beide SLPs en poly(I:C) bevatte het meest immunogeen. Deze PLGA-NP-formulering presteerde beter dan Montanide of SWE, maar minder dan liposomen. Liposomen, in vergelijking met de andere deeltjesvormige systemen bestudeerd in dit hoofdstuk, bleken het meest veelbelovende systeem te zijn voor de toediening van SLPs. Deze bevinding kan worden toegeschreven aan de positieve lading

van de lipsomen, hun kleine deeltjesgrootte (in vergelijking met PLGA-NPs) en/of hun effectieve verwerking door APCs.

De studie die gepresenteerd is in *hoofdstuk 5* is een *proof-of-concept* toepassing van het onderzoek dat beschreven is in dit proefschrift met een klinisch relevant tumorantigeen. In deze studie waren kationogene liposomen succesvol bereid op basis van het geoptimaliseerde protocol zoals beschreven in de voorgaande hoofdstukken en werden beladen met een 21 aminozuren lang SLP, afgeleid van het E7-eiwit van HPV, en met poly(I:C). Het doel van deze studie was om de potentie van de formulering als nieuw therapeutisch vaccinconcept te onderzoeken, in vergelijking met een E7-SLP-formulering gebaseerd op Montanide ISA-51-emulsie. De E7-SLP-liposomen, in vergelijking met oplossingen van vrij SLP, induceerde grotere aantallen antigeen-specifieke IFN-γ- en TNF-α-producerende cellen en vertoonde een sterkere cytotoxische activiteit op doelcellen die waren overgebracht in intradermaal geïmmuniseerde muizen, zelfs in afwezigheid van poly(I:C). Wanneer muizen met TC-1 tumoren (die HPV16-E7-eiwitten tot expressie brengen), therapeutisch gevaccineerd werden met E7-SLP en poly(I:C) beladen liposomen, werd bovendien de tumoruitgroei beter gecontroleerd en waren minstens 75% van de muizen volledig genezen na een enkele intradermale vaccinatie. In tegenstelling hiermee overleefde binnen de bestudeerde tijdsspanne slechts 25% van de muizen geïmmuniseerd met E7-SLP en poly(I:C) geformuleerd met Montanide ISA-51, met de standaard SLP dosis (65-maal hoger, terwijl geen effect was waargenomen wanneer een gelijke (lage) dosis SLP en poly(I:C) als de liposomen was gebruikt in combinatie met Montanide). Ten slotte, de enkele intradermale immunisatie van de muizen met E7-SLP-liposomen (geadjuveerd met poly(I:C)) was voldoende om een immuunrespons op te wekken die sterk genoeg was om muizen zelfs bij een herhaalde blootstelling aan tumorcellen te beschermen.

In *hoofdstuk 6* hebben we de algemene toepasbaarheid van de ontwikkelde liposoomformulering voor de toediening van verschillende SLPs onderzocht. Aangezien lange antigene peptiden per definitie allen verschillend zijn, zijn elf model SLPs ontwikkeld, welke alle het modelepitoop SIINFEKL bevatten maar verschillend waren in hydrofiliteit en isoelektrisch punt. Op basis van gestandaardiseerde protocollen werden alle SLPs succesvol ingebouwd in de kationogene liposomen, waarbij hoge beladingsefficiënties werden behaald. De bereide liposomen waren gekarakteriseerd op hun grootte, polydispersiteit en zèta-potentiaal en op hun efficiëntie van DC-activatie en presentatie aan $CD8^+$ T cellen. De liposomen hadden een gemiddelde diameter van ongeveer 200 nanometer, met een lage polydisperisteit en een positieve zèta-

potentiaal. Alle liposomale SLPs waren efficiënt in het activeren van DCs, welke vervolgens het MHC klasse-I-epitoop presenteerden aan SIINFEKL-specifieke T cellen *in vitro*. Deze resultaten illustreren de haalbaarheid van de formulering om als platform te dienen voor de toediening van multipele, heterogene SLPs voor kankerimmunotherapie.

Deel IV: Conclusie

Kationogene, op DOTAP gebaseerde liposomen beladen met SLPs en poly(I:C) zijn veelbelovende kandidaten voor potentiële therapeutische kankervaccins. De SLP-liposomen bleken in staat te zijn om in groten getale antigeen-specifieke effector $CD4^+$ T-helpercellen en cytotoxische $CD8^+$ T cellen te induceren. Daarnaast is hun werkzaamheid in agressieve tumormodellen aangetoond. Bovendien zijn de flexibiliteit betreffende het inbouwen van SLPs met diverse fysisch-chemische eigenschappen, hun relatief lage productiekosten en de mogelijkheid om ze toe te dienen via verschillende toedieningsroutes in het voordeel van de realisatiekansen op SLP-liposoomgemedieerde kankerimmunotherapie. Een combinatie van kationogene liposomen met andere klassieke en nieuwere kankertherapieën zou kunnen leiden tot een hoogst effectieve gepersonaliseerde immunotherapie van kanker.

Curriculum vitae

Eleni Maria Varypataki was born on the 23rd of September 1983 in Athens, Greece. After finishing her early education at the 2nd Lyceum in Chios Island in 2001, she started her University studies at the Agricultural University of Athens where in 2007 she obtained her Engineer's degree in Agronomy, with a specialisation in Plant Biotechnology. As part of her practical training she completed an internship in the Laboratory of Molecular Biology at the Agricultural University in Athens, on the subject "Effects of regulatory proteins and proteins involved in the expression of *Arabidopsis thaliana* HSP90 genes". In 2008 she enrolled the Master's Degree in Biotechnology at Wageningen University and Research centre (UR) in the Netherlands, where in 2010 she graduated having obtained a specialisation in Cellular/Molecular and Medical Biotechnology. During this period, she completed two internships. The first one, entitled "Regulation of the phagocyte activity by IFN-γ and its influence by a low frequency electromagnetic field exposure" was performed at the Laboratory of Cell Biology and Immunology group of Wageningen UR. Subsequently, she performed her second internship at the University Medical Center in Utrecht (UMC), where she worked on the project "Inside-out regulation of human Fc-receptors and their cross-talk relationship with integrins", in the Department of Pulmonary Diseases, Division Heart & Lung. In June 2011 she started her PhD under the supervision of Prof. Dr. Wim Jiskoot at the Division of Drug Delivery Technology at Leiden Academic Centre for Drug Research (LACDR) and Prof. Dr. Ferry Ossendorp at the Department of Immunohematology & Blood Transfusion at Leiden University Medical Center (LUMC), both at Leiden University, the Netherlands. The research results of her PhD project are described in this thesis. In June 2015, she started as a postdoctoral fellow in the group of Dr. Pål Johansen and Dr. Thomas Kündig in the Dermatology Clinic of the University Hospital of Zürich, working on the development of novel approaches for melanoma immunotherapy.

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List of abbreviations

ACN	Acenotrile
ANOVA	Analysis of variance
APC	Antigen-presenting cell
B3Z	$CD8^+$ T cell hybridoma cell line specific for the SIINFEKL epitope
BSA	Bovine serum albumin
CFSE	Carboxyfluorescein succinimidyl ester
CPG ODN	Cytosine-phosphodiester-guanine oligonucleotide motif
CTL	Cytotoxic T-lymphocyte ($CD8^+$ T cell)
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DOPC	Dioleoyl-glycero-phosphatidylcholine
DOTAP	Dioleoyl - trimethylammonium-propane
EE	Encapsulation efficiency
Em.	Light emission
Ex.	Light excitation
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
GMP	Good manufacturing practice
HLA	Human Leukocyte Antigen
HPV	Human papillomavirus
IFA	Incomplete Freund's adjuvant
KLRG1	Killer cell-lectin-like receptor G1
MHC	Major histocompatibility complex
PAMP	Pathogen-associated molecular pattern
PB	Phosphate buffer
PBMCs	Peripheral blood monocytes
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
pI	Isoelectric point
PLGA	Polylactide-co-glycolide
Poly(I:C)	Polyinosinic-polycytidylic acid
ROS	Reactive Oxygen species
SLP	Synthetic long peptide
ss-/ds- RNA	Single/double strained RNA

TAA	Tumour-associated antigens
TCR	T cell receptor
TFA	Trifluroacetic acid
TH / T_H	T helper
TLR	Toll-like receptor
Trp2	Tyrosinase-related protein 2
VLP	Virus-like particles


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