PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

Monteiro Garrido Castro e Silva, Ana Luisa

PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

Ana Luísa Silva

ISBN: 978-94-6182-632-9

The research described in this thesis was performed at the Division of Drug Delivery Technology of the Leiden Academic Centre for Drug Research and at the Leiden University Medical Center, Leiden, The Netherlands.

Cover: Bruno Silva & Ana Luísa Silva. The image on the cover was adapted from a photomicrograph of PLGA microparticles *in statu nascendi* (w/o/w double emulsion, an intermediate product of the preparation process).

Layout: Ana Luísa Silva

Printed by: Off Page, Amsterdam, The Netherlands

PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 22 december 2015
klokke 15:00 uur

door

Ana Luísa Monteiro Garrido Castro e Silva
geboren op 10 februari 1980
te Monterey, United States of America

Promotoren:

Prof. dr. W. Jiskoot
Prof. dr. F. Ossendorp

Promotiecommissie:

Prof. dr. P.H. van der Graaf, chair
Prof. dr. M. Danhof, secretary
Prof. dr. Y. van Kooyk, VU Medisch Centrum Amsterdam
Prof. dr. B. Gander, ETH Zürich, Institute of Pharmaceutical Sciences
Prof. dr.ir. W.E. Hennink, Utrecht University
Prof. dr. J.A. Bouwstra, Leiden University, LACDR

“Ever tried. Ever failed. No matter. Try Again. Fail again. Fail better.”

Samuel Beckett

Aos meus pais

Contents

Chapter 1

Introduction

Abstract

Synthetic peptides hold great promise as well-defined antigens for immunotherapy of cancer. The exact sequence and length of synthetic long peptides (SLPs) derived from cancer antigens can be designed and SLPs are readily produced in quantities suited for therapeutic vaccination. However, the formulation of SLPs for *in vivo* administration still needs to be improved. So far, SLPs have been formulated in Montanide-based water-in-oil (w/o) emulsions in (pre-)clinical trials. However, the use of Montanide as an adjuvant has some important limitations, such as: non-biodegradability; significant local side effects; poor control of release rate; lack of specific dentritic cell (DC)-activating capacity; and the presence of organic solvents (needed to dissolve the peptides prior to mixing with the adjuvant) in the final formulation. Therefore, alternative formulations containing an effective delivery system for peptide-based cancer vaccines are highly needed.

Among the numerous vaccine delivery systems, poly(lactic-co-glycolic acid) (PLGA) biodegradable particulate delivery systems are particularly interesting because they are biocompatible; can protect soluble antigens from degradation and rapid clearance once administered; allow for co-encapsulation of (multiple) antigens and adjuvants; and mimic the size and structure of a pathogen, being more efficiently taken up by DCs than soluble antigen [1, 2].

This thesis describes fundamental studies on the design and applicability in a pre-clinical setting of PLGA-based particulate formulations for the delivery of SLP-based cancer vaccines.

1. Introduction

1.1. Vaccines

Vaccination is one of the greatest achievements in the history of medicine, being the most effective biomedical intervention against infectious diseases, since it was introduced by Edward Jenner in the 18th century, who showed that humans could be protected against smallpox after inoculation with material scraped from the blisters of cowpox infected cows. The mechanism of vaccination works by activating the immune system against a specific agent, in order to rapidly recognize and remove the threat on a later encounter. The concept was further developed by Louis Pasteur, who developed rabies and anthrax vaccines by using live attenuated strains or inactivated pathogens [3]. Since then, worldwide vaccination campaigns using whole inactivated or live attenuated microorganisms have led to the eradication of smallpox and a dramatic reduction of other diseases such as polio and measles.

Traditionally, vaccines were generated empirically, without deep insight into the nature of the pathogen or the immune response, usually based on live attenuated or chemically or heat inactivated bacteria or viruses. Though live attenuated or whole inactivated pathogens have proven very effective at eliciting both humoral and cellular immune responses [4], the possibility of adverse effects associated with these vaccines, such as disease symptoms resulting from the inability of immune-compromised or elderly patients to clear the vaccine [5], unwanted inflammation or the possibility of reversion of the microorganisms to virulence [6], has raised safety concerns.

The demand for safer alternatives led to the development of subunit vaccines, which instead of a whole organism contain only specific antigenic compounds derived from it. Based on this principle, numerous subunit vaccines have been developed based on purified proteins and capsular polysaccharides obtained from the respective pathogens. Since a couple of decades, vaccines have become available or are being developed based on substances that do not require the pathogen as a production source, such as recombinant proteins, synthetic peptides, and genetic vaccines containing DNA or RNA encoding the antigen(s) of interest [7]. Examples of subunit vaccines successfully applied for widespread vaccination include hepatitis B, tetanus, diphtheria, and human papillomavirus (HPV) vaccines.

The use of individual antigens rather than whole pathogens diminishes safety concerns. However, soluble antigens often are weakly immunogenic, as they are poorly taken up and processed by antigen-presenting cells (APCs), lack danger signals, and are rapidly cleared when administered *in vivo* [8]. The generation of strong immune responses upon vaccination requires the uptake of antigens by APCs, which process them intracellularly into small peptide fragments (epitopes) and present these on their surface bound to major histocompatibility complex (MHC) class I and II molecules [9]. The identification of dendritic cells (DCs) as the most important and potent APCs of the immune system, scavenging the environment for potential pathogens and directing the immune response, has made them the main target of antigen delivery [10-12]. Activated DCs are able to cross-present antigens to antigen-specific B cells, T helper (Th) cells and cytotoxic T lymphocytes (CTLs), which are able to attack virus-infected or tumor cells [11, 12].

In order to strengthen the immune response against subunit vaccines, soluble antigens should be combined with adjuvants. An adjuvant (from the latin word adiuvare, to aid) can be any substance, such as an immunostimulatory molecule or a delivery system, that is used to enhance the efficacy of a vaccine. This can be attained by prolonging or boosting the otherwise poor immune response, or modulating it towards a specific cellular (Th1) or humoral (Th2) response. Although the clinical use of these types of adjuvants has been around for about 90 years, only a few have been approved for use in humans [13]. As prophylactic vaccines are widely administered to healthy populations, mostly young children, the safety of an adjuvant is of utmost importance [13]. The most commonly used adjuvants are aluminium salts (generally, Al(OH)3 and AlPO4), also known as alum [14]. Being the only FDA approved vaccine adjuvant for prophylactic vaccines for almost eight decades, alum has benefitted from unparalleled monopoly [13]. Alum's adjuvanticity relies mainly in depot formation from where the antigen is

slowly released in a sustained manner in particulate form; but other mechanisms have been recently discovered, such as facilitated antigen uptake by DCs and inflammasome activation through NOD-like receptor protein 3 [14-16]. Still, there are also drawbacks associated with alum. Alum promotes primarily a humoral response, i.e., antibody production, rather than a cellular response [13, 17]. Additionally, alum is known to cause local adverse effects, such as granuloma at the site of injection [13]. Over the past two decades, other adjuvants have been approved for use in the clinic, including nano-emulsions such as MF59 (Novartis), an oil-in-water emulsion containing squalene, polysorbate 80, and sorbitan trioleate used in the Fluad® influenza vaccine; AS03™ (GlaxoSmithKline (GSK)), another emulsion containing squalene, tocopherol, and polysorbate 80 used in the Pandemrix™ influenza vaccine; and AS04 (GSK) containing alum and monophosphoryl lipid A (MPLA) which is used in two licensed vaccines, Fendrix® for hepatitis B and Cervarix® for cervical cancer caused by HPV [13, 18-20]. Other vaccines such as Epaxal® or Inflexal® include virosomes [21, 22]. However, also these systems fail to stimulate strong cellular responses, required for therapeutic vaccines against intracellular pathogens or cancer.

The low immunogenicity associated with subunit vaccines makes them poorly effective against diseases that require a cellular immune response for protection, posing a challenge in designing new formulations. Incorporation of antigens into particulate systems, such as liposomes and polymeric particles, can potentially achieve a better immune response. The particulate form of these systems and their ability to co-encapsulate immune stimulatory molecules, often based on viral or bacterial structures, mimic a natural invading pathogen, being more likely taken up by DCs. These systems can be used to attain a long-lasting stimulation of DCs by sustained antigen release and to induce an adequate T-cell response. Furthermore, unlike traditional adjuvants like alum, which predominantly elicit humoral responses, particulate systems may elicit both humoral and cellular responses [23].

1.2. Dendritic cell-mediated immunity

The immune system is a network of cells that work together to protect the body from infection. These cells originate in the bone marrow and migrate to peripheral tissues to detect and eliminate potentially harmful agents [24]. The immune system can be divided into an innate and an adaptive branch, which mainly differ in response time and the level of specificity. The innate immune system is activated almost immediately after the detection of danger signals and involves the migration of phagocytic cells to the site of infection forming the first line of defense [1]. Innate immune cells recognize genetically conserved patterns, but lack antigen-specificity and immunological memory. In contrast, adaptive immunity is antigen-specific and retains memory of a previous infection. There are two branches of the adaptive immune response that contribute to the memory of a specific antigen: the humoral response, carried out by B cells and type 2 $CD4^+$ T cells; and the cellular response, which is performed by type 1 $CD4^+$ and $CD8^+$ T cells [1]. These cells differ from the innate immune cells by their antigen-specificity. APCs orchestrate the communication between the innate and adaptive

immune system [8].

APCs play an important role in inducing an adaptive immune response. APCs are a group of immune cells that are able to process and display both endogenous and exogenous antigens through the MHC molecules on their surface in a process known as antigen presentation. This process is responsible for the activation of T cells, which do not identify and respond directly to native antigens like B cells do, but can recognize processed antigen presented by MHC complexes through their T-cell receptors (TCRs). This allows detection of unexposed antigens, like those expressed by intracellular pathogens and mutated proteins in cancer cells [25].

APCs can be divided into two main categories: professional and non-professional ones. The so-called non-professional APCs include fibroblasts, thymic epithelial cells and vascular endothelial cells, and do not constitutively express the MHC class II molecules, though they can be induced to present antigens for short periods upon stimulation under specific circumstances [1, 25]. Professional APCs include DCs, which are specialized in priming of naïve T cells [24] and macrophages and B cells specialized in activating specific $CD4^+$ T cells. These cells express both MHC class I and class II molecules, being able to stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells, which differentiate into Th cells and CTLs, respectively [11, 12]. CTLs are able to eliminate virus-infected or tumor cells, while Th cells promote B cell activation, macrophage function and maturation of other T cells [1].

Different APCs have different main functions, with the efficiency of antigen uptake, processing and presentation varying accordingly. B cells' main function is to produce antibodies against specific antigens; they have limited uptake capacity, as their membrane B cell receptors are restricted to a single specific antigen [25]. As for macrophages, their primary function is to clear tissues from pathogens, having extraordinary endocytosis and degradation capacity, though poorer antigen presentation ability [25]. Conversely, DCs' main specialized function is antigen presentation, thus efficiently uptaking, storing, processing and presenting antigens to initiate a T cell immune response [26].

Depending on the nature of the antigen, either endogenous or exogenous, it will be processed and presented through MHC class I or class II molecules, respectively. Intracellular antigens such as proteins produced by viruses or tumor cells are processed in the cytosol and presented by MHC class I molecules to $CD8^+$ T cells. Extracellular pathogens can be actively internalized by pinocytosis, phagocytosis or endocytosis, being processed in the endosome and presented by MHC class II molecules to $CD4^+$ T cells [25, 27]. However, DCs also have a superior ability to process exogenous antigens and present these peptides in the MHC class I pathway, leading to the activation of antigen-specific $CD8^+$ T cells, a phenomenon that is called cross-presentation [8, 25]. It is this ability that allows them to cross-present tumor antigens and generate tumor-specific CTL responses, reason why DCs have become the main target of cancer vaccines [1, 25].

DCs are the most professional APCs, scattered throughout the body at peripheral tissues, such as the skin and mucosal surfaces, the most probable entry sites for

pathogens, functioning as the sentinels of the immune system [12, 27].

Immunostimulatory molecules activate the immune system through their interaction with specific receptors of APCs, which can recognize certain evolutionary conserved molecular motifs associated with groups of pathogens, the pathogen-associated molecular patterns (PAMPs), via membrane-bound pattern recognition receptors (PRRs) [8]. PRRs are used by DCs to detect invading pathogens and increase the efficiency of uptake and include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). As part of the innate immune system, TLRs recognize cell-wall components such as lipopolysaccharides, lipopeptides or lipoproteins associated with bacteria, or nucleic acids associated with bacteria and viruses such as double-stranded RNA fragments, acting as communicators between innate and adaptive immunity [8, 25]. These components are known as TLR ligands (TLRL). DCs use their PRRs to detect invading pathogens, and attachment to PRRs increases the efficiency of uptake. After internalization of pathogens, activated DCs migrate to the draining lymph nodes where they mature and come in contact with naïve or memory $CD4^+$ or $CD8^+$ T cells or B cells, presenting the antigen via MHC complexes [1, 25]. After recognition of the antigen-MHC class II complex by T cells, additional co-stimulatory signals are produced by DCs, leading to activation of the B and T cells [1, 25]. TLRs are also able to stimulate DC activation and antigen presentation to naïve T cells through MHC class I complex, triggering T cell maturation into Th1 through the secretion of pro-inflammatory cytokine release and expression of co-stimulatory molecules, stimulating CTL responses [8].

A number of strategies have been developed to efficiently deliver antigen to DCs, such as emulsions, viral vectors, liposomes, and biodegradable polymeric particles [27]. Different formulations may be processed via different pathways, affecting their overall immunogenicity. It has been suggested that the antigen uptake mechanism is of importance in determining how antigen is processed and presented on MHC class I and II molecules [28]. Though the exact mechanism of cross-presentation pathways is poorly understood, they have been arranged into two distinct routes: the phagosomal/cytosolic pathway and the endosomal/vacuolar pathway [29]. Similarly to the classical endogenous antigen processing pathway, in the phagosomal/cytosolic route the internalized antigen is processed in the cytosol, after escape from the endosome, where it is transferred to the proteasome and degraded into smaller peptides, which are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), before being loaded on MHC class I molecules [25]. In contrast, the endosomal/vacuolar pathway is TAP independent, and the antigen is processed into smaller peptides in the endosome in a pH-dependent manner by active endolysosomal proteases. Some of these peptides bind to MHC class I molecules inside the endosome and migrate to the cell surface [25]. Soluble antigens appear to be mainly processed through the cytosolic route, whereas particulate antigens are processed predominantly via the endosome [30].

One requirement for effective T cell activation is the appropriate delivery of antigens to DCs, which is often poorly attained when using soluble antigens. Encapsulation of antigens in particulate systems facilitate the delivery process and uptake by DCs,

promoting long-lasting stimulation by sustained antigen release, which is critical to induce an adequate T-cell response [31, 32]. Though immunogenicity is noticeably enhanced by specifically targeting antigens to DCs, additional stimuli are required to properly activate DCs. By co-encapsulating TLR agonists together with the antigen within delivery systems, DCs can be targeted through their TLRs, improving antigen delivery and modulating the immune response in order to achieve a strong cellular response [33]. So, the design of a potent vaccine obviously is not only dependent on the nature of the antigens but also on the way they are formulated.

1.3. Immunotherapy of cancer

Cancer is one of the leading causes of death worldwide. The conventional treatments for cancer consist of surgery, radiotherapy and/or chemotherapy, which are unspecific and poorly effective against metastasized tumors, usually causing severe side effects and decreasing the patient's quality of life. For this reason, there has been a growing interest in new treatment strategies.

It is well known that the immune system is able to respond against tumors, allowing the development of novel therapeutic strategies aiming to activate the immune system against tumor cells [34]. The use of the specificity of the immune system to treat cancer is referred to as cancer immunotherapy. Several studies have been conducted in the area of immunotherapy of cancer, leading to the development of several cancer vaccine strategies that are now extensively studied in multiple clinical trials or have already been approved for standard therapy. Current approaches comprise cell-based, antibody and cytokine therapies [25, 28]. These include adoptive $CD8^+$ T cell transfer [28] and DC-based vaccines [35, 36], such as Provenge® (Dendreon) against prostate cancer [37, 38]; monoclonal antibodies (mAbs) such as rituximab (Rituxan®, Genentech) against B-type leukemias and lymphomas, and trastuzumab (Herceptin®, Genentech) against breast cancer; and the administration of interleukin 2 (IL-2) and interferon alpha (INF-α) used in the treatment of various cancer types [39]. A therapeutic cancer vaccine called Oncophage® against renal cell carcinoma has been approved by the FDA, consisting of an autologous heat shock protein (HSP)–peptide complex produced from each patient's own tumor [25].

One of the most promising strategies in cancer immunotherapy is the use of immune checkpoints blockers [40]. These approaches use mAbs that target immune checkpoints in T cells rather than cancer cells, and have strongly contributed to making cancer immunotherapy the breakthrough of the year in 2013 according to Science magazine [41]. Ipilimumab (YERVOY®, Bristol Myers Squibb), is a mAb that works as an immune checkpoint blocker by targeting cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a protein receptor on the surface of T cells that downregulates CTL responses when bound to an inhibitory signal [39-42]. By blocking this inhibitory mechanism, it activates cancer-specific CTLs. Ipilimumab has been approved by the FDA for the treatment of melanoma, significantly extending patients' lives. In addition to melanoma, ipilimumab is undergoing clinical trials for the treatment of prostate and lung cancer [40, 41].

Another target for immunotherapy of cancer is programmed cell death protein 1 (PD-1), a cell surface receptor expressed on T cells and pro-B cells, which can bind to ligands PD-L1 and PD-L2 [43, 44]. Similarly to CTLA-4, PD-1 downregulates the immune system, promoting self-tolerance and reducing autoimmunity, by inducing apoptosis in antigen-specific T cells while simultaneously reducing apoptosis in regulatory T cells [43, 45]. PD-1/PD-L1 treatment strategies use mAbs that block PD-1 and PD-L1 interaction, thereby enhancing T-cell antitumor activity [41, 45]. Several mAbs targeting PD-1 receptors have shown promising results in clinical trials and are being developed as cancer treatments, such as nivolumab (Opdivo, Bristol Myers Squibb) for lung cancer, melanoma, and renal-cell cancer [46]. Pembrolizumab/lambrolizumab (Keytruda, Merck) has been approved by the FDA as a treatment against metastatic melanoma following treatment with ipilimumab [40, 41, 47]. Treatments combining PD-1 and CTLA-4 blockers have shown greater impact than when used alone, with very promising results [46]. Whereas ipilimumab treatment has been associated with severe immunological adverse effects due to T cell activation and proliferation, resulting in autoimmunity, PD-1 treatments seem to lead to milder side effects [41, 45]. These adverse effects should be taken into account during risk assessment when considering the treatment.

These early clinical successes with checkpoint-blocking antibodies unequivocally demonstrate that immunity to cancer exists but is however in a silenced state in a tumor environment. Therefore, the immune system can be successfully recruited towards tumors but requires adequate activation of the tumor-specific T cells, e.g., by specific vaccination.

1.3.1. Cancer vaccination

In recent years, in addition to the traditional prophylactic vaccines against infectious diseases, there has been an increased interest in broader application of vaccination for protection against other diseases, such as cancer [8, 27].

Currently only few prophylactic cancer vaccines have been approved: two that protect against the high-risk serotypes of HPV (Gardasil, Merck; Cervarix, GSK) and other two against the hepatitis B virus (HBV) (Recombivax HB, Merck; Engerix-B, GSK), which are the leading causes for cervical and liver cancer, respectively. However, prophylactic vaccines do not have therapeutic effects against pre-existing infections and do not prevent their development, as these vaccination strategies rely on antibody protection against the virus upon infection, whereas therapeutic vaccines must have the capability of inducing CTL responses to kill the infected cells of (pre-)malignant lesions [27].

Two main approaches have been used to target DCs in cancer immunotherapy: administration of *ex vivo* tumor antigen loaded DCs and *in vivo* delivery of tumor antigens. Though *ex vivo* loaded DC-based cancer vaccines have been extensively studied with promising therapeutic results, this approach requires each vaccine to be tailor made for each patient, involving extensive and time-consuming lab work as well

as logistics, thus making such vaccines extremely expensive [25]. These limitations may be overcome by delivering tumor associated antigens (TAAs) and adjuvant together to DCs *in vivo*.

Identification of TAAs for a variety of cancers has led to development of vaccines that target these antigens [25, 28]. Most prophylactic vaccines work by inducing an antibody response against specific viral or bacterial antigens. In contrast, therapeutic cancer vaccines require the induction of potent T cell responses against TAA-bearing cells [27, 48]. Cancer vaccines rely on the administration of TAA in order to induce the killing of tumor cells by cytotoxic T lymphocytes (CTLs) orchestrated by activated DCs, resulting in tumor regression. Quite a few cancer vaccines have been tested in the clinic, however, most of these trials have resulted in disappointing clinical results, due to insufficient generation of cell-mediated immunity [27, 28]. Several factors must be considered when developing a cancer vaccine, which in order to be effective must comprise the following three essential elements: an antigen, an immune potentiator, and a delivery system.

1.3.2. Peptide-based cancer vaccines

Several vaccination strategies against cancer have been able to the elicit tumor-specific T cell responses *in vivo*, including whole tumor cells or cell lysates, viral vectors or DNA encoding TAAs, as well as recombinant proteins or synthetic peptides, both of which have been the most popular form of therapeutic vaccines [49, 50].

The first step for developing subunit cancer vaccines is to select the target TAA(s), which can be (a) protein(s) or peptide(s) specific for tumor cells. In order to achieve a successful vaccine, these antigens must include 8-10 amino acid peptide sequences that can be processed and bind to the MHC class I molecules of DCs and will be recognized by T cells to elicit a strong tumor-specific CTL response [27, 28]. The cloning of the first gene reported to encode a CTL-defined human tumor antigen (MAGE-A1) paved the way to the discovery of more TAAs, allowing the production of vaccines for these targets and the study of vaccines against different types of cancer, such as melanoma and cervical cancer [28, 50-55].

Vaccination with TAA-derived synthetic short peptides (SSPs) covering exact MHC class I epitopes initially appeared to be a promising approach for immunotherapy of cancer, as T cells recognize target antigens as peptide fragments presented by MHC class I molecules at the cell surface [27]. However, this approach showed major drawbacks [51, 56], such as peptide-specific immunological tolerance, associated with enhanced tumor growth instead of protection [57, 58], as well as short-lived and suboptimal CTL responses, caused by the lack of proper co-activation of $CD4^+$ T cells and subsequent induction of memory $CD8^+$ T cells [59, 60].

Tolerance was mainly induced because of the exogenous binding of these short peptides to MHC class I molecules that are present not only on professional APCs

like DCs, but also on all somatic cells which generally lack the ability of expressing the surface co-stimulatory molecules that are required to the generation of effector T cells [27, 56-63]. In a direct comparison, vaccination with a SLP bearing both CTL and Th epitopes in Montanide ISA 51 elicited much stronger CTL responses after vaccination than vaccination with a SSP covering an exact HPV16E7 MHC I binding epitope. The former approach resulted in effective eradication of established tumors in a mouse model of HPV16-induced tumors, owing to the activation of both $CD4^+$ and $CD8^+$ T cells [64]. Therefore, the use of longer peptides, such as SLPs, covering both CTL and Th epitopes has been launched as a strategy to overcome the problem of suboptimal CTL responses [27, 65]. These peptides cannot bind to MHC I molecules directly, but have to be taken up and processed by DCs before being presented to T-cells, inducing a more effective immune response [57, 63, 64]. It has been suggested that such SLPs considerably facilitate MHC class I presentation and induce stronger CTL responses in comparison to proteins because SLPs are more efficiently internalized, processed, and cross-presented by DCs [33, 64].

Another disadvantage about the use of SSPs is that the polymorphic nature of MHC molecules (human leukocyte antigen, HLA) in genetically diverse populations restricts their broad use as there is a risk of missing important epitopes [27]. Due to the multiple HLA class I and II molecules, it is unlikely to identify immunogenic CTL and Th epitopes that would cover all individuals, requiring patient selection according to their HLA type. The use of vaccines consisting of overlapping SLPs covering the whole sequence of highly immunogenic regions of TAA proteins may overcome the HLA-dependency problem, allowing the *in vivo* epitope selection according to each HLA-profile after uptake by DCs [27]. Following this concept, numerous SLP vaccines have been studied in clinical trials against different types of cancer and other diseases, one of the most prominent example being HPV-induced cancers [27, 42].

HPV-induced cancers often have viral sequences integrated into the cellular DNA. Some of the HPV “early” genes, such as E6 and E7, are known to act as oncogenes that promote tumor growth and malignant transformation. Because the majority of HPV-16 derived cancers express E6 and E7 oncoproteins, they are attractive candidates as target antigens for immunotherapy of HPV-induced cancer [66-72].

Several protein and peptide vaccines against HPV E6 and/or E7 have been successfully tested in preclinical and clinical models. A cancer vaccine consisting of 13 overlapping SLPs covering the entire sequence of the E6/E7 oncogenic proteins of high-risk HPV16 has been investigated in several (pre-)clinical trials [65]. Therapeutic vaccination with a HPV16 SLP vaccine emulsified in the mineral oil adjuvant Montanide ISA 51 has shown to be able to increase both HPV16-specific $CD4^+$ and $CD8^+$ T cells in murine models of cervical cancer [33, 66, 73]. In clinical trials, this vaccine has shown robust immunogenicity in end-stage cervical cancer patients, and caused complete regression of premalignant HPV16-induced I lesions and eradication of virus in 9 of 20 women with high-grade vulvar intraepithelial neoplasia [52, 65].

Despite these promising results, there is still room for improvement, especially regarding the SLP vaccine formulation. Montanide has been used as the “gold-standard” adjuvant

for protein- and SLP-based cancer vaccines [65, 74, 75]. Montanide is a clinical-grade version of incomplete Freund's adjuvant (IFA) with similar properties. The w/o emulsion is composed of a mineral oil, a surfactant and an aqueous phase, slowly releasing the dispersed antigen when injected subcutaneously. These emulsions are known to elicit long-lasting IgG responses as well as stimulate the activation of CTL and Th cells [53, 76-80], its adjuvant activity being attributed to depot formation and the ability to trigger inflammation at the site of injection [81], while emulsion droplets can promote antigen uptake by DCs [82, 83].

However, the use of Montanide w/o emulsions is far from ideal, not only since they have poorly defined immunogenic properties, but also because their non-biodegradability causes significant local side effects, such as tenderness, swelling, granuloma, local pain, erythema and discomfort, reason why they have not been approved for routine human immunotherapy but are only being used in investigational clinical trials [17, 54, 81, 84]. Furthermore, their use as an adjuvant has some other important limitations: they show poor control of the peptide release rate and lack specific DC-activating capacity. Moreover, the pharmaceutical development and scalability of w/o emulsions is hampered by limited long-term stability and the complexity of the manufacturing process. In addition, formulation of peptides is hampered by the need for organic solvents (like dimethylsulfoxide) to dissolve them prior to parental administration, whereas distribution of peptides in the emulsion is dependent on peptide polarity, making the release of a multipeptide vaccine difficult to predict and control. Thus, there is an urgent medical need for the development of better adjuvants with improved safety and efficacy profiles that can be formulated into vaccine products that are stable during preparation, storage and administration. This can be achieved via different formulation strategies which allow co-delivery of antigens and adjuvants, such as encapsulation in biodegradable particles [79, 85], use of antibody-mediated targeting [86, 87] and/or SLP-TLRL conjugates [78, 88].

1.4. Adjuvants

Adjuvants can be categorized into two groups: immunostimulatory molecules and delivery systems. Immunostimulatory molecules play a major role in directing immunity towards either a bacterial or viral/tumoral defense pathway [1]. Though vaccine delivery systems are often sufficient to induce a long-lasting protective immunity, poorly immunogenic antigens, such as synthetic peptides, are often unable to induce strong responses when incorporated alone, requiring the inclusion of immune-potentiating molecules [89]

1.4.1. Immunostimulatory molecules

DCs are critically involved in the generation and maintenance of an effective CTL response. Therefore, vaccines need to mimic the most successful natural triggers of

DC activation. Immunostimulatory molecules play a major role in directing immunity towards either a bacterial or viral/tumoral defense pathway [1]. Therefore, selecting 'the right' immunostimulatory molecule(s) is crucial in the design of a successful subunit vaccine.

TLRs are the most commonly used target as TLR stimulation is critically involved in the uptake and processing of antigens by DCs, and are able to trigger cross-presentation. TLR activation induces the secretion of proinflammatory cytokines and type I interferon, and leads to upregulation of CD40, CD80 and CD86 costimulatory molecules on the surface of APCs, as well as release of Th1 cytokines such as IL-1, IL-2, IL-6, and TNF, leading to T cell activation [90]. There are at least 10 types of TLRs in humans, which can either be surface bound or expressed intracellularly, recognizing different extracellular or intracellular pathogenic components (Table 1) [1, 8].

Recent research is aimed at activating DCs by targeting TLRs [6, 53, 91-94]. TLRs can be located either on the cell surface or inside intracellular compartments, ultimately indicating their roles at detecting extracellular or intracellular pathogens [8]. TLR1, 2 and 6 are mainly surface TLRs that detect surface bacterial lipoproteins and lipopeptides, which are usually recognized by TLR2 dimerized with TLR1 when triacylated, and TLR2 dimerized with TLR6 when diacylated [8]. A well-known TLR1/2 agonist is Pam3CSK4, a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins, whereas Pam2CSK4, being a diacylated lipopeptide, induces signaling through TLR2/6. Lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria, is a potent activator of TLR4 with the subsequent induction of NF-κB and the production of pro-inflammatory cytokines. TLR5 recognizes flagellin, the protein monomer that constitutes the filament of bacterial flagellae, found on nearly all motile bacteria. TLR3, and 7 to 9 are expressed intracellularly, within the endosomal compartments, and are able to detect bacterial and viral nucleic acids. TLR3 recognizes viral double-stranded RNA (dsRNA), a molecular pattern associated with viral infection, for which polyinosinic-polycytidylic acid (poly(I:C)) is a synthetic analog. TLR7 and TLR8 recognize single-stranded RNA (ssRNA) and small synthetic molecules like imidazoquinolines and nucleoside analogs, such as the imidazoquinoline drug compounds resiquimod (R848) and imiquimod. Finally, TLR9 recognizes unmethylated CpG (cytosine–phosphate–guanine) oligonucleotide (ODN) motifs in DNA molecules, common elements in viruses and bacteria, but not in humans.

Several synthetic TLRLs have been shown to enhance antigen-specific CTL responses in mice when mixed or conjugated with antigen (see Table 1 for examples) [6, 8]. The co-delivery of TLR agonists and antigens through co-encapulation in delivery systems can be an efficient strategy to increase the strength and modulate the quality of the immune response against antigens. Furthermore, as most pathogens present multiple TLR agonists to APCs, simultaneous stimulation of multiple TLRs can result in a synergistic upregulation of inflammatory cytokine production. Hence, the combination of multiple TLRLs may be a promising strategy to induce strong immune responses [6].

In this thesis, either TLR2L Pam3CSK4 or TLR3L Poly(I:C), or a combination of both, have been co-encapsulated with antigen into delivery systems in order to enhance the

Table 1: Human toll-like receptors and their natural and synthetic ligands.

TLR	Location	PAMP recognized	Pathogens	Synthetic agonists	Immune response
TLR 1+2	cell membrane	triacylated lipoproteins	bacteria	Pam3Cys	Induce production of inflammatory cytokines
TLR 2+6	cell membrane	diacylated lipoproteins peptidoglycans LTA fungal β –glucans	mycoplasma bacteria Gram-positive bactéria Fungi	Pam2Cys MALP-2	Induce production of inflammatory cytokines
TLR 3	endosome	double stranded RNA	viruses	Poly(I:C) Poly(A:U)	Synthesis of type 1 interferons
TLR 4	cell membrane	LPS heat shock proteins hyaluronic acid heparan sulfate fibrinogen	Gram-negative bacteria	MPLA LPS analogs	Synthesis of type 1 interferons
TLR 5	cell membrane	flagellin	bacteria	-	Induces production of TNF- α
TLR 7 & 8	endosome	single stranded RNA	viruses	Resiquimod (R848) Imiquimod (R837) Gardiquimod Loxoribine (Guanosine analogs)	Anti-viral response
TLR 9	endosome	unmethylated CpG motifs	Bacteria Viruses protozoa	CPG ODN	Dependent on type of CpG: Type A/D induces IFN- α Type B/K induces IL-12 and TNF- α production
TLR 10	endosome	profillin-like proteins	unknown	unknown	unknown

CpG ODN: Cytosine-guanine rich oligonucleotide; LPS: Lipopolysaccharide; LTA: Lipoteichoic acid; MALP-2: Macrophage-activating lipoprotein-2; MPLA: Monophosphoryl lipid A; Pam2Cys: Dipalmitoyl-S-glyceryl cysteine; Pam3Cys: Tripalmitoyl-S-glyceryl cysteine; poly(I:C): Polyribo(inosinic-cytidylic) acid; TLR: Toll-like receptor

potency of anti-cancer vaccines.

1.4.2. Particulate delivery systems

Most peptide-based cancer vaccine strategies have used Montanide as an adjuvant to increase specific T cell immunity. Over the recent years, because of its drawbacks, alternatives from various types of biomaterials have been used to formulate particulate vaccine carriers, such as liposomes (vesicles formed from phospholipid bilayers) and micro- (MPs) or nanoparticles (NPs) made from polymers like PLGA, poly-lactic acid (PLA) or (derivatives of) chitosan, a polysaccharide obtained by deacetylation of chitin [74, 81, 95]. These particulate systems are biocompatible and biodegradable and can be manufactured reproducibly according to Good Manufacturing Practice (GMP). Furthermore, studies have shown that DC targeted vaccination strategies benefit from encapsulation of antigens in biodegradable particulate systems, as they (i) are able to protect the antigen from premature enzymatic degradation and clearance by the kidneys, increasing their residence time; (ii) serve as depot for sustained antigen release; (iii) are able to co-encapsulate multiple components, delivering antigen and adjuvant together to DCs; and (iv) can be efficiently targeted to specific tissues or immune receptors through targeting moieties coupled to the particle surface [1, 87]. Moreover, particulate antigens enhance antigen-specific humoral and cellular responses more efficiently than soluble ones, as particulate formulations mimic the size and structure of a pathogen facilitating uptake by DCs, and prolong antigen cross-presentation owing to sustained intracellular release of the antigen following uptake [2, 32, 96-99]. These features make biodegradable particles attractive candidates as vaccine delivery systems for peptide-based cancer immunotherapy.

1.4.2.1. Liposomes

Liposomes are artificially-prepared spherical vesicles, with particle sizes ranging between 30 nm to several microns, composed of a lipid bilayer. This bilayer usually consists of synthetic and/or naturally occurring, biodegradable phospholipids and cholesterol, which can be manipulated in order to influence the liposome's physicochemical characteristics, such as size, surface charge and rigidity [32, 98-102]. Liposomes have been extensively studied because of their ability to carry both hydrophilic and hydrophobic compounds, as hydrophilic molecules can be encapsulated in the inner aqueous phase, whereas hydrophobic compounds can be dissolved into the lipidic membrane, and amphiphilic ones can partition between both phases [103, 104]. This ability has rendered interest in liposomal vaccines, as they are suitable to carry a variety of molecules with different characteristics, such as antigens and immunomodulatory compounds; moreover, targeting moieties can be embedded in or covalently attached to the liposomal bilayer to facilitate targeted delivery to DCs [104].

The adjuvant effect of liposomes is highly dependent on their physicochemical properties,

such as vesicle size, surface charge, composition of phospolipid head groups, length and saturation of the lipid tail, and rigidity of the bilayer, which can be customized by adjusting their composition and method of preparation. The surface charge is of particular importance, as cationic liposomes have shown superior adjuvant capacity compared to neutral or negatively charged ones, probably because of electrostatic interactions with the negatively charged surface of DCs [99, 105]. Furthermore, cationic liposomes facilitate adsorption of negatively charged antigens to their surface, such as synthetic peptides, proteins and nucleic acids.

Numerous other liposomal vaccines have been in clinical trials, with shown efficacy, including cancer vaccine candidates, most prominent being Stimuvax® (or L-BLP25, Oncothyreon) against non-small cell lung cancer (NSCLC); and MAGE-A3 (GlaxoSmithKline) against metastatic MAGE-A3-positive melanoma and NSCLC [105-107].

1.4.2.2. PLGA nanoparticles

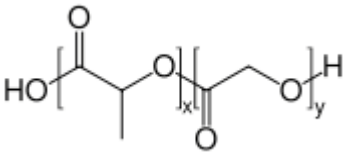
Polymeric particulate delivery systems can consist of bio-degradable or non-biodegradable materials, and can derive from either natural or synthetic sources. Natural polymers include starch, gelatin, alginate, or chitosan, and offer the benefits of being biocompatible, hydrophilic and inexpensive, but their production often results in batch-to-batch inconsistency and incidence of unwanted impurities or contaminants [13]. In contrast, synthetic polymers can be manufactured with high purity and reproducibility and can be tailored to achieve the desired molecular weight, co-polymer composition and rate of degradation [108].

Synthetic particles made from synthetic polymers have been widely explored as peptide antigen delivery vehicles as they offer many advantages over other systems. They are biodegradable and biocompatible and can act as antigen depots, slowly releasing peptides for a pre-determined period of time that can range from a few days to several months, depending on the customized particle degradation rate, potentially eliminating the need for booster doses [6].

PLGA (**Figure 1**) is a copolymer which is Food and Drug Administration (FDA) approved for therapeutic devices and drug delivery systems, owing to its biodegradability and biocompatibility, with several slow-release formulations currently on the market [109-111]. These properties have made PLGA a common choice in the production of a variety of biomedical devices, such as sutures, implants and prosthetic devices, as well as delivery systems, such as micro- and nanoparticles [112, 113].

PLGA undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid, which, under normal physiological conditions, are by-products of various metabolic pathways in the body. Since the body effectively deals with the two monomers, there is minimal systemic toxicity associated with using PLGA for medical applications.

Several forms of PLGA with different properties can be obtained, either depending on the ratio of lactide to glycolide used for the polymerization, or the type of end group (ester-terminated (capped) or carboxylic acid terminated (uncapped)), offering the possibility to tailor the polymer degradation time and regulate drug release [114-117]. Degradation rate of PLGA is related to the lactide/glycolide ratio: the higher the content of glycolic acid, the more hydrophilic it becomes and, consequently, the faster its hydrolysis rate. Also, unlike the homopolymers of lactic and glycolic acid (polylactide and polyglycolide, respectively) which show poor solubilities, PLGA can be dissolved in a wide range of common organic solvents.



PLGA
(x: lactic; y: glycolic)

Figure 1: Chemical structure of poly-(lactic-co-glycolic) acid, with x standing for the number of lactide monomers and y for the number of glycolide monomers.

Consequently, biodegradable particles composed of PLGA have many desirable features for antigen delivery. These particle systems are biocompatible and can be manufactured reproducibly according to Good Manufacturing Practice in a scalable, affordable and reproducible way. Importantly, PLGA is very well suited for making particles of different size, resulting in either MPs or NPs, with controllable release properties [108]. For these reasons, PLGA particles have been studied extensively for the delivery of a wide variety of macromolecules, from DNA to proteins and peptides [53, 81, 112, 118]. Moreover, antigen loaded PLGA particles have been shown to induce cellular immune responses comparable to those elicited with Montanide 51 or IFA [119-121].

PLGA particles can be prepared by a variety of different methods, most commonly used for protein and peptide antigens being the double emulsion with solvent evaporation method [108]. In brief, the polymer is dissolved in an organic volatile solvent (e.g. dichloromethane, ethyl acetate) to which the antigen is added in aqueous solution (the inner water phase, w1) followed by emulsification by homogenization or sonication; this first water in oil (w1/o) emulsion is added to an external aqueous phase (w2) containing a surfactant (e.g., polyvinyl alcohol, polysorbate 20), followed by a second emulsification step, forming the double emulsion (w1/o/w2); the second emulsion is then transferred to an aqueous phase under magnetic stirring at room or reduced temperature to allow for solvent evaporation, leading to precipitation of the polymer and solidification of the particles, which are finally recovered by centrifugation and then freeze-dried [122].

The use of PLGA particles for vaccine delivery offers several advantages from both pharmaceutical and immunological perspectives as compared to soluble antigens or w/o emulsions. Several immunotherapeutic approaches have successfully used both

PLGA MPs and NPs to target DCs [10, 81, 97]. Particles allow for the concomitant delivery of antigen and adjuvant [76, 79], and vaccination with PLGA NPs containing TAA and TLRL has been demonstrated to be as effective as vaccination with IFA [66, 81, 123]. Particles also facilitate endosomal escape, which is a known mechanism leading to antigen cross-presentation by DCs [64, 124]. Furthermore, they allow the coupling of targeting molecules to specific receptors such as CD40 or DEC-205, which can efficiently direct particles to DCs, increasing specific uptake and cellular immune responses [42, 87, 114].

Several factors affect the efficiency of PLGA particles as vaccine delivery vehicles. Particle size is a critical factor, as it can influence bio-distribution and uptake efficiency by APCs. Particles can be defined as NPs or MPs according to their size ranges: NPs for particles within the nano range (10 nm to 1 mm); and MPs for particles in the micron range (1 mm to 1000 mm). It is generally accepted that NPs are advantageous over MPs for drug targeting, as NPs can more easily permeate biological barriers and efficiently reach target tissues [125-127]. However, the ideal size for vaccine delivery is still not consensual [116, 128, 129]. Particle size is known to influence the efficiency and mechanism of uptake, depot formation, and release kinetics [129-132]. The way a cell takes up an antigen-loaded particle can determine how it processes the antigen: MPs are mainly phagocytosed, whereas NPs are generally taken up by endocytosis or pinocytosis. Sustained release of antigen and adjuvants is essential to properly stimulate DCs, whereas a low burst release eliminates potential loss of antigen before the particle is taken up by DCs, increasing antigen presentation and $CD8^+$ T cell activation [31, 32].

However, there are also disadvantages of the use of PLGA, as encapsulation in PLGA particles may result in loss of antigen integrity during preparation and storage, but also after administration, especially regarding protein antigens, where denaturation or degradation can occur due to local acidification of the inner core due to the hydrolysis of the polymer [133]. Though peptides do not require a tertiary structure and may be less susceptible to acidic degradation, their encapsulation in PLGA particles can lead to accelerated deamidation or formation of peptide adducts due to acylation of lysine residues with lactic and glycolic units [134]. These issues are more predominant for MPs than NPs, as their inner core takes longer to degrade the polymer and release the antigen, allowing higher accumulation of acidic molecules [133]. Furthermore, PLGA particles must be freeze-dried to ensure long-term stability during storage and must be re-hydrated prior to administration. Still, formulation of SLPs in PLGA NPs may be advantageous, provided that the formulation is carefully designed and optimized. Nevertheless, despite encouraging results obtained in clinical trials, there are still no PLGA-based particulate vaccines on the market [1, 135].

Incorporating multi-SLP antigens together with TLRL in PLGA particles may result in a ‘pathogen-mimicking’ particle that displays slow antigen release and may be actively phagocytosed by DC, representing a promising approach for generating specific T cell immunity. In the present thesis, PLGA particles were studied to gain better insight into the critical factors influencing delivery and immunogenicity in order to formulate a potent particulate delivery system to improve the safety and efficacy of SLP-based cancer vaccines.

2. Aim and outline of this thesis

The aim of this thesis is to gain a better insight into the role of the formulation of particulate delivery systems in the effectiveness of cancer vaccines through the preparation, physicochemical characterization, and immunological evaluation of a biodegradable, PLGA-based particulate delivery system, incorporating protein or SLP antigens as well as TLRL. The main objectives of this research include:

- Determination of the best size range of particles for vaccine delivery
- Development of SLP loaded PLGA NP formulations
- Development of formulations based on PLGA NPs co-encapsulating SLPs and TLRLs

The overall goal is gaining fundamental insight into how to improve the immunogenicity, clinical efficacy and safety of SLP-based vaccines for cancer immunotherapy, to be used as a safer and more effective alternative to Montanide ISA 51.

In **Chapter 2** the literature regarding PLGA-based particulate systems for protein- and peptide-based vaccine delivery is reviewed, with a strong focus on particle properties affecting immunogenicity.

In **Chapter 3 and 4** of this thesis we use ovalbumin (OVA), a 45-kDa protein purified from chicken egg, as a model protein antigen to conduct initial studies regarding the formulation of PLGA particles as vaccine delivery vehicles. The following chapters (Chapters 5-7) focus on the development of formulations for SLP delivery, using SLPs derived from OVA covering CTL and/or Th epitopes as our model antigens.

The optimal size range of PLGA-based particulate vaccines in order to achieve the most efficient cellular immune responses is studied in **Chapter 3**, where MPs and NPs encapsulating OVA and a TLR3L are compared head-to-head for their capacity to activate B and T cell responses. Having determined that NPs are more efficiently delivered to DCs than MPs, resulting in more effective immune responses *in vivo*, in Chapter 4 we describe the application of PLGA-OVA NPs as a delivery vehicle for *ex vivo* loading of DCs, in order to enhance DC-mediated stimulation of antigen-specific T cells to be used for adoptive T cell immunotherapy.

In **Chapter 5** the development of a new method for SLP encapsulation in PLGA NPs is studied, focusing on formulation conditions required to successfully encapsulate an SLP in PLGA NPs, and optimization of the formulation to improve the efficacy of cross-presentation by DCs of PLGA-SLP NPs in comparison to soluble SLP *in vitro*. Subsequently, in **Chapter 6** we study the co-encapsulation of SLP and TLR2L in PLGA NPs, which are used to study the intracellular mechanisms via which DC process PLGA-SLP NPs and to determine the *in vivo* vaccine potency of PLGA-SLP-TLRL NPs in comparison to soluble SLP and TLRL.

In **Chapter 7** we study the co-encapsulation of two SLPs covering the CTL and Th epitopes of model antigen OVA and two TLRLs in PLGA NPs and liposomes, which were used to ascertain the *in vivo* vaccine efficacy in a direct comparison to Montanide and MF59 analogs, with properties that are very similar to adjuvants currently used in the clinic.

Finally, in **Chapter 8** we discuss the achievements described in this thesis and the perspectives for further research on nanoparticulate delivery systems for SLP-based cancer vaccines.

3. References

1. Leleux, J. and K. Roy, Micro and nanoparticle-based delivery systems for vaccine immunotherapy: an immunological and materials perspective. Advanced healthcare materials, 2013. 2(1): p. 72-94.
2. Elamanchili, P., C.M. Lutsiak, S. Hamdy, M. Diwan, and J. Samuel, "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. Journal of immunotherapy, 2007. 30(4): p. 378-95.
3. Carstens, M.G., Opportunities and challenges in vaccine delivery. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2009. 36(4-5): p. 605-8.
4. O'Hagan, D.T. and R. Rappuoli, Novel approaches to vaccine delivery. Pharm Res, 2004. 21(9): p. 1519-30.
5. Kotton, C.N., Vaccination and immunization against travel-related diseases in immunocompromised hosts. Expert review of vaccines, 2008. 7(5): p. 663-72.
6. Black, M., A. Trent, M. Tirrell, and C. Olive, Advances in the design and delivery of peptide subunit vaccines with a focus on toll-like receptor agonists. Expert review of vaccines, 2010. 9(2): p. 157-73.
7. Reyes-Sandoval, A. and H.C. Ertl, DNA vaccines. Curr Mol Med, 2001. 1(2): p. 217-43.
8. Demento, S.L., A.L. Siefert, A. Bandyopadhyay, F.A. Sharp, and T.M. Fahmy, Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends in biotechnology, 2011. 29(6): p. 294-306.
9. Jensen, P.E., Recent advances in antigen processing and presentation. Nature immunology, 2007. 8(10): p. 1041-8.
10. Park, K., Nano is better than micro for targeted vaccine delivery. Journal of controlled release : official journal of the Controlled Release Society, 2010. 144(2): p. 117.
11. Steinman, R. and K. Inaba, Immunogenicity: role of dendritic cells. BioEssays : news and reviews in molecular, cellular and developmental biology, 1989. 10(5): p. 145-52.
12. Steinman, R.M., The dendritic cell system and its role in immunogenicity. Annual review of immunology, 1991. 9: p. 271-96.
13. Vandana, P. and P. Priyanka, Potential of Nanocarriers in Antigen Delivery: The Path to Successful Vaccine Delivery. Nanocarriers, 2014. 1(1): p. 10-45.
14. Marrack, P., A.S. McKee, and M.W. Munks, Towards an understanding of the adjuvant action of aluminium. Nature reviews. Immunology, 2009. 9(4): p. 287-93.
15. Mannhalter, J.W., H.O. Neychev, G.J. Zlabinger, R. Ahmad, and M.M. Eibl, Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. Clinical and experimental immunology, 1985. 61(1): p. 143-51.
16. Kool, M., V. Petrilli, T. De Smedt, A. Rolaz, H. Hammad, M. van Nimwegen, I.M. Bergen, R. Castillo, B.N. Lambrecht, and J. Tschopp, Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol, 2008. 181(6): p. 3755-9.

17. Peek, L.J., C.R. Middaugh, and C. Berkland, Nanotechnology in vaccine delivery. Advanced drug delivery reviews, 2008. 60(8): p. 915-28.
18. Brunner, R., E. Jensen-Jarolim, and I. Pali-Scholl, The ABC of clinical and experimental adjuvants--a brief overview. Immunology letters, 2010. 128(1): p. 29-35.
19. Guy, B., The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol, 2007. 5(7): p. 505-17.
20. Mbow, M.L., E. De Gregorio, N.M. Valiante, and R. Rappuoli, New adjuvants for human vaccines. Current opinion in immunology, 2010. 22(3): p. 411-6.
21. Bovier, P.A., Epaxal: a virosomal vaccine to prevent hepatitis A infection. Expert review of vaccines, 2008. 7(8): p. 1141-50.
22. Herzog, C., K. Hartmann, V. Kunzi, O. Kursteiner, R. Mischler, H. Lazar, and R. Gluck, Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine. Vaccine, 2009. 27(33): p. 4381-7.
23. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena, Antigen presentation and T cell stimulation by dendritic cells. Annual review of immunology, 2002. 20: p. 621-67.
24. Janeway, C., Immunobiology : the immune system in health and disease. 6th ed. 2005, New York: Garland Science. xxiii, 823 p.
25. Hamdy, S., A. Haddadi, R.W. Hung, and A. Lavasanifar, Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. Advanced drug delivery reviews, 2011. 63(10-11): p. 943-55.
26. van Montfoort, N., M.G. Camps, S. Khan, D.V. Filippov, J.J. Weterings, J.M. Griffith, H.J. Geuze, T. van Hall, J.S. Verbeek, C.J. Melief, and F. Ossendorp, Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc Natl Acad Sci U S A, 2009. 106(16): p. 6730-5.
27. Melief, C.J. and S.H. van der Burg, Immunotherapy of established (pre) malignant disease by synthetic long peptide vaccines. Nat Rev Cancer, 2008. 8(5): p. 351-60.
28. Silva, J.M., M. Videira, R. Gaspar, V. Preat, and H.F. Florindo, Immune system targeting by biodegradable nanoparticles for cancer vaccines. Journal of controlled release : official journal of the Controlled Release Society, 2013. 168(2): p. 179-99.
29. Burgdorf, S. and C. Kurts, Endocytosis mechanisms and the cell biology of antigen presentation. Current opinion in immunology, 2008. 20(1): p. 89-95.
30. Rosalia, R.A., Co-encapsulation of synthetic long peptide antigen and Toll like receptor 2 ligand in poly-(lactic-co-glycolic-acid) particles results in sustained MHC class I cross-presentation by dendritic cells, in Particulate based vaccines for cancer immunotherapy, F.o.M.L.U.M.C.L. Department of Immunohematology, Leiden University, Editor. 2014, Leiden University. p. 95-121.
31. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient CD8(+) T cell activation. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik

e.V, 2013. 83(3): p. 338-45.
32. Demento, S.L., W. Cui, J.M. Criscione, E. Stern, J. Tulipan, S.M. Kaech, and T.M. Fahmy, Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials, 2012. 33(19): p. 4957-64.
33. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, A.L. Silva, W. Jiskoot, T. van Hall, P.A. van Veelen, G. Janssen, K. Franken, L.J. Cruz, A. Tromp, J. Oostendorp, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. European journal of immunology, 2013. 43(10): p. 2554-65.
34. Perrie, Y., A.R. Mohammed, D.J. Kirby, S.E. McNeil, and V.W. Bramwell, Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. International Journal of Pharmaceutics, 2008. 364(2): p. 272-80.
35. Park, T.S., S.A. Rosenberg, and R.A. Morgan, Treating cancer with genetically engineered T cells. Trends in biotechnology, 2011. 29(11): p. 550-7.
36. Rosenberg, S.A. and M.E. Dudley, Adoptive cell therapy for the treatment of patients with metastatic melanoma. Current opinion in immunology, 2009. 21(2): p. 233-40.
37. Grupp, S.A. and C.H. June, Adoptive cellular therapy. Current topics in microbiology and immunology, 2011. 344: p. 149-72.
38. Palucka, K., H. Ueno, L. Roberts, J. Fay, and J. Banchereau, Dendritic cell subsets as vectors and targets for improved cancer therapy. Current topics in microbiology and immunology, 2011. 344: p. 173-92.
39. Cancer Immunology and Immunotherapy. Current topics in microbiology and immunology, ed. G. Dranoff. Vol. 344. 2011.
40. Pardoll, D.M., The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer, 2012. 12(4): p. 252-64.
41. Couzin-Frankel, J., Breakthrough of the year 2013. Cancer immunotherapy. Science, 2013. 342(6165): p. 1432-3.
42. van den Boorn, J.G. and G. Hartmann, Turning tumors into vaccines: co-opting the innate immune system. Immunity, 2013. 39(1): p. 27-37.
43. Iwai, Y., M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato, Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci U S A, 2002. 99(19): p. 12293-7.
44. Ribas, A., Tumor immunotherapy directed at PD-1. The New England journal of medicine, 2012. 366(26): p. 2517-9.
45. Topalian, S.L., F.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, J.D. Powderly, R.D. Carvajal, J.A. Sosman, M.B. Atkins, P.D. Leming, D.R. Spigel, S.J. Antonia, L. Horn, C.G. Drake, D.M. Pardoll, L. Chen, W.H. Sharfman, R.A. Anders, J.M. Taube, T.L. McMiller, H. Xu, A.J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G.D. Kollia, A. Gupta, J.M. Wigginton, and M. Sznol, Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. The New England journal of medicine, 2012. 366(26): p. 2443-54.
46. Wolchok, J.D., H. Kluger, M.K. Callahan, M.A. Postow, N.A. Rizvi, A.M. Lesokhin, N.H. Segal, C.E. Ariyan, R.A. Gordon, K. Reed, M.M. Burke, A. Caldwell, S.A. Kronenberg, B.U. Agunwamba, X. Zhang, I. Lowy, H.D. Inzunza,

W. Feely, C.E. Horak, Q. Hong, A.J. Korman, J.M. Wigginton, A. Gupta, and M. Sznol, Nivolumab plus ipilimumab in advanced melanoma. The New England journal of medicine, 2013. 369(2): p. 122-33.

47. Hamid, O., C. Robert, A. Daud, F.S. Hodi, W.J. Hwu, R. Kefford, J.D. Wolchok, P. Hersey, R.W. Joseph, J.S. Weber, R. Dronca, T.C. Gangadhar, A. Patnaik, H. Zarour, A.M. Joshua, K. Gergich, J. Elassaiss-Schaap, A. Algazi, C. Mateus, P. Boasberg, P.C. Tumeh, B. Chmielowski, S.W. Ebbinghaus, X.N. Li, S.P. Kang, and A. Ribas, Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. The New England journal of medicine, 2013. 369(2): p. 134-44.
48. Ma, W., T. Smith, V. Bogin, Y. Zhang, C. Ozkan, M. Ozkan, M. Hayden, S. Schroter, E. Carrier, D. Messmer, V. Kumar, and B. Minev, Enhanced presentation of MHC class Ia, Ib and class II-restricted peptides encapsulated in biodegradable nanoparticles: a promising strategy for tumor immunotherapy. Journal of translational medicine, 2011. 9: p. 34.
49. Welters, M.J., G.G. Kenter, S.J. Piersma, A.P. Vloon, M.J. Lowik, D.M. Berends-van der Meer, J.W. Drijfhout, A.R. Valentijn, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, R. Offringa, C.J. Melief, and S.H. van der Burg, Induction of tumor-specific $CD4^+$ and $CD8^+$ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 178-87.
50. Solbrig, C.M., J.K. Saucier-Sawyer, V. Cody, W.M. Saltzman, and D.J. Hanlon, Polymer nanoparticles for immunotherapy from encapsulated tumor-associated antigens and whole tumor cells. Molecular pharmaceutics, 2007. 4(1): p. 47-57.
51. Adams, S., D.W. O'Neill, D. Nonaka, E. Hardin, L. Chiriboga, K. Siu, C.M. Cruz, A. Angiulli, F. Angiulli, E. Ritter, R.M. Holman, R.L. Shapiro, R.S. Berman, N. Berner, Y. Shao, O. Manches, L. Pan, R.R. Venhaus, E.W. Hoffman, A. Jungbluth, S. Gnjatic, L. Old, A.C. Pavlick, and N. Bhardwaj, Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. J Immunol, 2008. 181(1): p. 776-84.
52. Welters, M.J., D.V. Filippov, S.J. van den Eeden, K.L. Franken, J. Nouta, A.R. Valentijn, G.A. van der Marel, H.S. Overkleeft, G. Lipford, R. Offringa, C.J. Melief, J.H. van Boom, S.H. van der Burg, and J.W. Drijfhout, Chemically synthesized protein as tumour-specific vaccine: immunogenicity and efficacy of synthetic HPV16 E7 in the TC-1 mouse tumour model. Vaccine, 2004. 23(3): p. 305-11.
53. Hamdy, S., O. Molavi, Z. Ma, A. Haddadi, A. Alshamsan, Z. Gobti, S. Elhasi, J. Samuel, and A. Lavasanifar, Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent $CD8^+$ T cell-mediated anti-tumor immunity. Vaccine, 2008. 26(39): p. 5046-57.
54. Feyerabend, S., S. Stevanovic, C. Gouttefangeas, D. Wernet, J. Hennenlotter, J. Bedke, K. Dietz, S. Pascolo, M. Kuczyk, H.G. Rammensee, and A. Stenzl, Novel multi-peptide vaccination in $Hla\text{-}A2^+$ hormone sensitive patients with biochemical relapse of prostate cancer. The Prostate, 2009. 69(9): p. 917-27.
55. van Poelgeest, M.I., M.J. Welters, E.M. van Esch, L.F. Stynenbosch, G. Kerpershoek, E.L. van Persijn van Meerten, M. van den Hende, M.J. Lowik,

D.M. Berends-van der Meer, L.M. Fathers, A.R. Valentijn, J. Oostendorp, G.J. Fleuren, C.J. Melief, G.G. Kenter, and S.H. van der Burg, HPV16 synthetic long peptide (HPV16-SLP) vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. Journal of translational medicine, 2013. 11: p. 88.

56. Melief, C.J., R.E. Toes, J.P. Medema, S.H. van der Burg, F. Ossendorp, and R. Offringa, Strategies for immunotherapy of cancer. Adv Immunol, 2000. 75: p. 235-82.
57. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, R. Offringa, and S.H. van der Burg, $CD8^+$ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. J Immunol, 2007. 179(8): p. 5033-40.
58. Rosenberg, S.A., J.C. Yang, and N.P. Restifo, Cancer immunotherapy: moving beyond current vaccines. Nature medicine, 2004. 10(9): p. 909-15.
59. Toes, R.E., R. Offringa, R.J. Blom, C.J. Melief, and W.M. Kast, Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. Proc Natl Acad Sci U S A, 1996. 93(15): p. 7855-60.
60. Toes, R.E., R.J. Blom, R. Offringa, W.M. Kast, and C.J. Melief, Enhanced tumor outgrowth after peptide vaccination. Functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors. J Immunol, 1996. 156(10): p. 3911-8.
61. van den Broeke, L.T., E. Daschbach, E.K. Thomas, G. Andringa, and J.A. Berzofsky, Dendritic cell-induced activation of adaptive and innate antitumor immunity. J Immunol, 2003. 171(11): p. 5842-52.
62. Matloubian, M., R.J. Concepcion, and R. Ahmed, $CD4^+$ T cells are required to sustain $CD8^+$ cytotoxic T-cell responses during chronic viral infection. Journal of virology, 1994. 68(12): p. 8056-63.
63. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C.J. Melief, Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. The Journal of experimental medicine, 1998. 187(5): p. 693-702.
64. Zwaveling, S., S.C. Ferreira Mota, J. Nouta, M. Johnson, G.B. Lipford, R. Offringa, S.H. van der Burg, and C.J. Melief, Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol, 2002. 169(1): p. 350-8.
65. Melief, C.J., Synthetic vaccine for the treatment of lesions caused by high risk human papilloma virus. Cancer journal, 2011. 17(5): p. 300-1.
66. Zhang, H., H. Hong, D. Li, S. Ma, Y. Di, A. Stoten, N. Haig, K. Di Gleria, Z. Yu, X.N. Xu, A. McMichael, and S. Jiang, Comparing pooled peptides with intact protein for accessing cross-presentation pathways for protective $CD8^+$ and $CD4^+$ T cells. The Journal of biological chemistry, 2009. 284(14): p. 9184-91.
67. Sabbatini, P., T. Tsuji, L. Ferran, E. Ritter, C. Sedrak, K. Tuballes, A.A. Jungbluth, G. Ritter, C. Aghajanian, K. Bell-McGuinn, M.L. Hensley, J. Konner, W. Tew, D.R. Spriggs, E.W. Hoffman, R. Venhaus, L. Pan, A.M. Salazar, C.M. Diefenbach, L.J. Old, and S. Gnjatic, Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clinical cancer research : an

official journal of the American Association for Cancer Research, 2012. 18(23): p. 6497-508.
68. Speetjens, F.M., P.J. Kuppen, M.J. Welters, F. Essahsah, A.M. Voet van den Brink, M.G. Lantrua, A.R. Valentijn, J. Oostendorp, L.M. Fathers, H.W. Nijman, J.W. Drijfhout, C.J. van de Velde, C.J. Melief, and S.H. van der Burg, Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clinical cancer research : an official journal of the American Association for Cancer Research, 2009. 15(3): p. 1086-95.
69. Quakkelaar, E.D., M.F. Fransen, W.W. van Maren, J. Vaneman, N.M. Loof, S.H. van Heiningen, J.S. Verbeek, F. Ossendorp, and C.J. Melief, IgG-mediated anaphylaxis to a synthetic long peptide vaccine containing a B cell epitope can be avoided by slow-release formulation. J Immunol, 2014. 192(12): p. 5813-20.
70. Audran, R., M. Cachat, F. Lurati, S. Soe, O. Leroy, G. Corradin, P. Druilhe, and F. Spertini, Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. Infection and immunity, 2005. 73(12): p. 8017-26.
71. Sirima, S.B., A.B. Tiono, A. Ouedraogo, A. Diarra, A.L. Ouedraogo, J.B. Yaro, E. Ouedraogo, A. Gansane, E.C. Bougouma, A.T. Konate, Y. Kabore, A. Traore, R. Chilengi, I. Soulama, A.J. Luty, P. Druilhe, S. Cousens, and I. Nebie, Safety and immunogenicity of the malaria vaccine candidate MSP3 long synthetic peptide in 12-24 months-old Burkinabe children. PloS one, 2009. 4(10): p. e7549.
72. Spitzer, N., A. Jardim, D. Lippert, and R.W. Olafson, Long-term protection of mice against Leishmania major with a synthetic peptide vaccine. Vaccine, 1999. 17(11-12): p. 1298-300.
73. van der Burg, S.H. and C.J. Melief, Therapeutic vaccination against human papilloma virus induced malignancies. Current opinion in immunology, 2011. 23(2): p. 252-7.
74. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, F. Essahsah, L.M. Fathers, R. Offringa, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, S.H. van der Burg, and C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
75. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, R. Offringa, S.H. van der Burg, and C.J. Melief, Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 169-77.
76. Wischke, C., J. Zimmermann, B. Wessinger, A. Schendler, H.H. Borchert, J.H. Peters, T. Nesselhut, and D.R. Lorenzen, Poly(I:C) coated PLGA microparticles induce dendritic cell maturation. International journal of pharmaceutics, 2009. 365(1-2): p. 61-8.
77. Zom, G.G., S. Khan, C.M. Britten, V. Sommandas, M.G. Camps, N.M. Loof,

C.F. Budden, N.J. Meeuwenoord, D.V. Filippov, G.A. van der Marel, H.S. Overkleeft, C.J. Melief, and F. Ossendorp, Efficient induction of antitumor immunity by synthetic toll-like receptor ligand-peptide conjugates. Cancer immunology research, 2014. 2(8): p. 756-64.

78. Zom, G.G., S. Khan, D.V. Filippov, and F. Ossendorp, TLR ligand-peptide conjugate vaccines: toward clinical application. Adv Immunol, 2012. 114: p. 177-201.
79. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander, Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. Journal of drug targeting, 2009. 17(8): p. 652-61.
80. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner, Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. European journal of immunology, 2007. 37(8): p. 2063-74.
81. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. International journal of cancer. Journal international du cancer, 2011. 129(2): p. 407-16.
82. Vitoriano-Souza, J., N. Moreira, A. Teixeira-Carvalho, C.M. Carneiro, F.A. Siqueira, P.M. Vieira, R.C. Giunchetti, S.A. Moura, R.T. Fujiwara, M.N. Melo, and A.B. Reis, Cell recruitment and cytokines in skin mice sensitized with the vaccine adjuvants: saponin, incomplete Freund's adjuvant, and monophosphoryl lipid A. PloS one, 2012. 7(7): p. e40745.
83. Harris, R.C., K.A. Chianese-Bullock, G.R. Petroni, J.T. Schaefer, L.B. Brill, 2nd, K.R. Molhoek, D.H. Deacon, J.W. Patterson, and C.L. Slingluff, Jr., The vaccine-site microenvironment induced by injection of incomplete Freund's adjuvant, with or without melanoma peptides. Journal of immunotherapy, 2012. 35(1): p. 78-88.
84. Diefenbach, C.S., S. Gnjatic, P. Sabbatini, C. Aghajanian, M.L. Hensley, D.R. Spriggs, A. Iasonos, H. Lee, B. Dupont, S. Pezzulli, A.A. Jungbluth, L.J. Old, and J. Dupont, Safety and immunogenicity study of NY-ESO-1b peptide and montanide ISA-51 vaccination of patients with epithelial ovarian cancer in high-risk first remission. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(9): p. 2740-8.
85. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup, TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, 2008. 26(13): p. 1626-37.
86. Rosalia, R.A., L.J. Cruz, S. van Duikeren, A.T. Tromp, A.L. Silva, W. Jiskoot, T. de Gruijl, C. Lowik, J. Oostendorp, S.H. van der Burg, and F. Ossendorp, CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses. Biomaterials, 2015. 40: p. 88-97.
87. Cruz, L.J., R.A. Rosalia, J.W. Kleinovink, F. Rueda, C.W. Lowik, and F. Ossendorp, Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8(+) T cell response: A comparative study. Journal of controlled release : official journal of the Controlled Release Society, 2014. 192: p. 209-18.

88. Khan, S., J.J. Weterings, C.M. Britten, A.R. de Jong, D. Graafland, C.J. Melief, S.H. van der Burg, G. van der Marel, H.S. Overkleeft, D.V. Filippov, and F. Ossendorp, Chirality of TLR-2 ligand Pam3CysSK4 in fully synthetic peptide conjugates critically influences the induction of specific $CD8^+$ T-cells. Molecular immunology, 2009. 46(6): p. 1084-91.
89. Salvador, A., M. Igartua, R.M. Hernandez, and J.L. Pedraz, An overview on the field of micro- and nanotechnologies for synthetic Peptide-based vaccines. J Drug Deliv, 2011. 2011: p. 181646.
90. Van Gool, S.W., P. Vandenberghe, M. de Boer, and J.L. Ceuppens, CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. Immunological reviews, 1996. 153: p. 47-83.
91. Khan, S., M.S. Bijker, J.J. Weterings, H.J. Tanke, G.J. Adema, T. van Hall, J.W. Drijfhout, C.J. Melief, H.S. Overkleeft, G.A. van der Marel, D.V. Filippov, S.H. van der Burg, and F. Ossendorp, Distinct uptake mechanisms but similar intracellular processing of two different toll-like receptor ligand-peptide conjugates in dendritic cells. The Journal of biological chemistry, 2007. 282(29): p. 21145-59.
92. Bonifaz, L.C., D.P. Bonnyay, A. Charalambous, D.I. Darguste, S. Fujii, H. Soares, M.K. Brimnes, B. Moltedo, T.M. Moran, and R.M. Steinman, *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. The Journal of experimental medicine, 2004. 199(6): p. 815-24.
93. Akira, S. and K. Takeda, Toll-like receptor signalling. Nature reviews. Immunology, 2004. 4(7): p. 499-511.
94. Gorden, K.K., X. Qiu, J.J. Battiste, P.P. Wightman, J.P. Vasilakos, and S.S. Alkan, Oligodeoxynucleotides differentially modulate activation of TLR7 and TLR8 by imidazoquinolines. J Immunol, 2006. 177(11): p. 8164-70.
95. van den Hende, M., M.I. van Poelgeest, J.M. van der Hulst, J. de Jong, J.W. Drijfhout, G.J. Fleuren, A.R. Valentijn, A.R. Wafelman, G.M. Slappendel, C.J. Melief, R. Offringa, S.H. van der Burg, and G.G. Kenter, Skin reactions to human papillomavirus (HPV) 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia. International journal of cancer. Journal international du cancer, 2008. 123(1): p. 146-52.
96. Wieber, A., T. Selzer, and J. Kreuter, Characterisation and stability studies of a hydrophilic decapeptide in different adjuvant drug delivery systems: a comparative study of PLGA nanoparticles versus chitosan-dextran sulphate microparticles versus DOTAP-liposomes. International journal of pharmaceutics, 2011. 421(1): p. 151-9.
97. Joshi, M.D., W.J. Unger, G. Storm, Y. van Kooyk, and E. Mastrobattista, Targeting tumor antigens to dendritic cells using particulate carriers. Journal of controlled release : official journal of the Controlled Release Society, 2012. 161(1): p. 25-37.
98. Kwon, Y.J., S.M. Standley, S.L. Goh, and J.M. Frechet, Enhanced antigen presentation and immunostimulation of dendritic cells using acid-degradable cationic nanoparticles. Journal of controlled release : official journal of the Controlled Release Society, 2005. 105(3): p. 199-212.
99. Reddy, S.T., M.A. Swartz, and J.A. Hubbell, Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends in immunology,

2006. 27(12): p. 573-9.
100. Tacken, P.J., R. Torensma, and C.G. Figdor, Targeting antigens to dendritic cells *in vivo*. Immunobiology, 2006. 211(6-8): p. 599-608.
101. Han, H.D., L.S. Mangala, J.W. Lee, M.M. Shahzad, H.S. Kim, D. Shen, E.J. Nam, E.M. Mora, R.L. Stone, C. Lu, S.J. Lee, J.W. Roh, A.M. Nick, G. Lopez-Berestein, and A.K. Sood, Targeted gene silencing using RGD-labeled chitosan nanoparticles. Clinical cancer research : an official journal of the American Association for Cancer Research, 2010. 16(15): p. 3910-22.
102. Moingeon, P., C. de Taisne, and J. Almond, Delivery technologies for human vaccines. British medical bulletin, 2002. 62: p. 29-44.
103. Watson, D.S., A.N. Endsley, and L. Huang, Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine, 2012. 30(13): p. 2256-72.
104. Barnier Quer, C., Adjuvanted nanoparticulate seasonal influenza vaccines, in Division of Drug Delivery Technology of the Leiden/Amsterdam Center for Drug Research (LACDR), Faculty of Science, Leiden University2013.
105. Nordly, P., H.B. Madsen, H.M. Nielsen, and C. Foged, Status and future prospects of lipid-based particulate delivery systems as vaccine adjuvants and their combination with immunostimulators. Expert opinion on drug delivery, 2009. 6(7): p. 657-72.
106. Foged, C., C. Arigita, A. Sundblad, W. Jiskoot, G. Storm, and S. Frokjaer, Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. Vaccine, 2004. 22(15-16): p. 1903-13.
107. Vangasseri, D.P., Z. Cui, W. Chen, D.A. Hokey, L.D. Falo, Jr., and L. Huang, Immunostimulation of dendritic cells by cationic liposomes. Molecular membrane biology, 2006. 23(5): p. 385-95.
108. Astete, C.E. and C.M. Sabliov, Synthesis and characterization of PLGA nanoparticles. J Biomater Sci Polym Ed, 2006. 17(3): p. 247-89.
109. Butts, C., A. Maksymiuk, G. Goss, D. Soulieres, E. Marshall, Y. Cormier, P.M. Ellis, A. Price, R. Sawhney, F. Beier, M. Falk, and N. Murray, Updated survival analysis in patients with stage IIIB or IV non-small-cell lung cancer receiving BLP25 liposome vaccine (L-BLP25): phase IIB randomized, multicenter, open-label trial. Journal of cancer research and clinical oncology, 2011. 137(9): p. 1337-42.
110. Jyoti Menaria, S.K., Varuna Verma, Cancer Vaccine: An Overview. Scholars Journal of Applied Medical Sciences 2013. 1(3): p. 161-171.
111. Yuchen Fan, Q.Z., Development of liposomal formulations: From concept to clinical investigations. Asian Journal of Pharmaceutical Sciences, 2013. 8(2): p. 81-87.
112. Jiang, W., R.K. Gupta, M.C. Deshpande, and S.P. Schwendeman, Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Advanced drug delivery reviews, 2005. 57(3): p. 391-410.
113. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug delivery, 2010. 17(7): p. 490-9.

114. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, R. Torensma, and C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. Journal of controlled release : official journal of the Controlled Release Society, 2010. 144(2): p. 118-26.
115. Fahmy, T.M., S.L. Demento, M.J. Caplan, I. Mellman, and W.M. Saltzman, Design opportunities for actively targeted nanoparticle vaccines. Nanomedicine, 2008. 3(3): p. 343-55.
116. Jain, S., D.T. O'Hagan, and M. Singh, The long-term potential of biodegradable poly(lactide-co-glycolide) microparticles as the next-generation vaccine adjuvant. Expert review of vaccines, 2011. 10(12): p. 1731-42.
117. Samadi, N., A. Abbadessa, A. Di Stefano, C.F. van Nostrum, T. Vermonden, S. Rahimian, E.A. Teunissen, M.J. van Steenbergen, M. Amidi, and W.E. Hennink, The effect of lauryl capping group on protein release and degradation of poly(D,L-lactic-co-glycolic acid) particles. Journal of controlled release : official journal of the Controlled Release Society, 2013. 172(2): p. 436-43.
118. Walter, E., D. Dreher, M. Kok, L. Thiele, S.G. Kiama, P. Gehr, and H.P. Merkle, Hydrophilic poly(DL-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells. Journal of controlled release : official journal of the Controlled Release Society, 2001. 76(1-2): p. 149-68.
119. Slutter, B., L. Plapied, V. Fievez, M.A. Sande, A. des Rieux, Y.J. Schneider, E. Van Riet, W. Jiskoot, and V. Preat, Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination. Journal of controlled release : official journal of the Controlled Release Society, 2009. 138(2): p. 113-21.
120. Yang, A., L. Yang, W. Liu, Z. Li, H. Xu, and X. Yang, Tumor necrosis factor alpha blocking peptide loaded PEG-PLGA nanoparticles: preparation and *in vitro* evaluation. International journal of pharmaceutics, 2007. 331(1): p. 123-32.
121. Wischke, C. and S.P. Schwendeman, Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. International journal of pharmaceutics, 2008. 364(2): p. 298-327.
122. Mohammadi-Samani, S. and B. Taghipour, PLGA micro and nanoparticles in delivery of peptides and proteins; problems and approaches. Pharm Dev Technol, 2014.
123. Silva, A.L., R.A. Rosalia, E. Varypataki, S. Sibuea, F. Ossendorp, and W. Jiskoot, Poly-(lactic-co-glycolic-acid)-based particulate vaccines: Particle uptake by dendritic cells is a key parameter for immune activation. Vaccine, (0).
124. Shen, H., A.L. Ackerman, V. Cody, A. Giodini, E.R. Hinson, P. Cresswell, R.L. Edelson, W.M. Saltzman, and D.J. Hanlon, Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology, 2006. 117(1): p. 78-88.
125. Link, A., F. Zabel, Y. Schnetzler, A. Titz, F. Brombacher, and M.F. Bachmann, Innate immunity mediates follicular transport of particulate but not soluble protein antigen. J Immunol, 2012. 188(8): p. 3724-33.
126. Manolova, V., A. Flace, M. Bauer, K. Schwarz, P. Saudan, and M.F. Bachmann, Nanoparticles target distinct dendritic cell populations according to their size.

European journal of immunology, 2008. 38(5): p. 1404-13.
127. Simon, L.C. and C.M. Sabliov, The effect of nanoparticle properties, detection method, delivery route and animal model on poly(lactic-co-glycolic) acid nanoparticles biodistribution in mice and rats. Drug metabolism reviews, 2013.
128. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert review of vaccines, 2010. 9(9): p. 1095-107.
129. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. The AAPS journal, 2013. 15(1): p. 85-94.
130. Johansen, P., T. Storni, L. Rettig, Z. Qiu, A. Der-Sarkissian, K.A. Smith, V. Manolova, K.S. Lang, G. Senti, B. Mullhaupt, T. Gerlach, R.F. Speck, A. Bot, and T.M. Kundig, Antigen kinetics determines immune reactivity. Proc Natl Acad Sci U S A, 2008. 105(13): p. 5189-94.
131. Fifis, T., A. Gamvrellis, B. Crimeen-Irwin, G.A. Pietersz, J. Li, P.L. Mottram, I.F. McKenzie, and M. Plebanski, Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol, 2004. 173(5): p. 3148-54.
132. Gutierro, I., R.M. Hernandez, M. Igartua, A.R. Gascon, and J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine, 2002. 21(1-2): p. 67-77.
133. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
134. Ghassemi, A.H., M.J. van Steenbergen, A. Barendregt, H. Talsma, R.J. Kok, C.F. van Nostrum, D.J. Crommelin, and W.E. Hennink, Controlled release of octreotide and assessment of peptide acylation from poly(D,L-lactide-co-hydroxymethyl glycolide) compared to PLGA microspheres. Pharm Res, 2012. 29(1): p. 110-20.
135. Klencke, B., M. Matijevic, R.G. Urban, J.L. Lathey, M.L. Hedley, M. Berry, J. Thatcher, V. Weinberg, J. Wilson, T. Darragh, N. Jay, M. Da Costa, and J.M. Palefsky, Encapsulated plasmid DNA treatment for human papillomavirus 16-associated anal dysplasia: a Phase I study of ZYC101. Clinical cancer research : an official journal of the American Association for Cancer Research, 2002. 8(5): p. 1028-37.

Chapter 2

PLGA particulate delivery systems for protein- and peptide-based vaccines: linking particle properties to immunogenicity

Ana Luísa Silva, Peter Soema, Bram A. Slütter, Ferry Ossendorp, Wim Jiskoot

Accepted for publication at
Human Vaccines & Immunotherapeutics

Abstract

Vaccination has had a major impact in global health, and continuous efforts in this field have led to the development of newer and safer vaccines based on subunit antigens rather than whole inactivated or live attenuated vaccines. Among the emerging subunit vaccines are recombinant protein- and synthetic peptide-based vaccine formulations. However, proteins and peptides have a low intrinsic immunogenicity. A common strategy to overcome this is to co-deliver (an) antigen(s) with (an) immune modulator(s) by co-encapsulating them in a particulate delivery system, such as poly(lactic-co-glycolic acid) (PLGA) particles. Particulate PLGA formulations offer many advantages for antigen delivery as they are biocompatible and biodegradable; can protect the antigens from degradation and clearance; allow for co-encapsulation of antigens and immune modulators; can be targeted to antigen presenting cells; and their particulate nature can increase uptake and cross-presentation by mimicking the size and shape of an invading pathogen. This review discusses the use of PLGA particulate formulations for subunit vaccine delivery and provides an overview of the formulation parameters influencing their adjuvanticity, such as size, charge, antigen localization, release profile, and the co-delivery of immune modulators and/or specific targeting molecules, and further outlines how these characteristics affect uptake, processing and antigen presentation by dendritic cells and the ensuing immune response. Finally, we address the use of PLGA delivery systems for peptide-based vaccines.

Keywords: Vaccine, delivery systems, antigen, synthetic peptide, adjuvant, dendritic cells, PLGA, nanoparticles, microparticles.

1. Introduction

Vaccination consists of the administration of antigens in order to elicit an adaptive antigen-specific immune response and confer long-term protection against subsequent exposure to the antigen [1]. Traditional vaccine formulations, consisting of either live attenuated or killed pathogens, have been very successful in the last century to prevent widespread infectious diseases [2, 3]. Still, despite their success [4, 5], there are serious safety concerns associated with these vaccines, which include the possibility of reactivation of the attenuated pathogens [6, 7] and the inability of immune-compromised patients to clear the vaccine leading to disease symptoms [8]. These issues have led to the demand for safer alternatives and vaccine development shifted from using whole inactivated pathogens to subunits of the pathogen. These subunits may be antigenic proteins, peptides, capsular polysaccharides or any specific part of the pathogen which has been demonstrated to stimulate a protective immune response. Examples of subunit vaccines include hepatitis B, tetanus, diphtheria, and human papillomavirus (HPV) vaccines. However, the need for eliciting both humoral and cellular immune responses has limited the efficacy of subunit vaccines against certain diseases for which effective vaccines are still unavailable, such as AIDS, malaria, tuberculosis, or cancer. While subunits are safer than whole pathogens, they

generally are less immunogenic, demanding the use of adjuvants [5]. Adjuvants are immunostimulatory molecules and/or delivery systems [9] used in vaccine formulations to enhance the magnitude of antigen-specific immune responses.

Immunostimulatory molecules activate the immune system through their interaction with specific receptors of APCs, which can recognize certain evolutionary conserved molecular motifs associated with groups of pathogens, the pathogen-associated molecular patterns (PAMPs). The identification process is regulated by membrane-bound pattern recognition receptors (PRRs) on the DC surface or internal compartments. PRRs present on DC include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). These receptors recognize molecules such as bacterial lipopolysaccharides, viral nucleic acids, bacterial peptides, peptidoglycans or lipoproteins, acting as communicators between innate and adaptive immunity and has been intensively studied over the last few decades [10, 11]. TLRLs have been shown to enhance and modulate the immune response when mixed, conjugated, or co-delivered together with antigen [7, 11]. This knowledge opens the door to the rational design of vaccine formulations that co-deliver TLRLs to increase the immunogenicity of the antigen.

Next to immunostimulatory molecules, subunit vaccines may benefit from encapsulation in particulate delivery systems, which include microparticles (MP) (> 1 μm) and nanoparticles (NP) (< 1000 nm). Particles may promote immunogenicity through the following mechanism:

(1) Stability improvement of the antigen: particulate delivery systems can protect encapsulated or associated antigen from chemical and enzymatic degradation and rapid clearance via the kidneys, resulting in increased residence time [1, 9];

(2) Controlled antigen release: particulate formulations can be tailored to serve as intra- and/or extracellular depot for sustained release of the antigen, increasing antigen exposure to DCs and prolonged antigen presentation [12];

(3) Facilitated DC uptake: particulate delivery systems can mimic the size and shape of an invading pathogen, which facilitates uptake by DCs [11, 13];

(4) Targeted delivery: particles *per se are passively directed to APCs because of their particulate form, but can also be specifically targeted to specific tissues or subsets of immune cells (like DCs) via targeting moieties, such as TLR ligands or DC-specific antibodies* [14-17];

(5) Enhanced cross-presentation: particles may facilitate endosomal escape, which is a known mechanism leading to antigen cross-presentation by DCs and induction of a CTL response [18, 19];

(6) Concomitant delivery of multiple components: particulate formulations can co-deliver a combination of molecules, such as (multiple) antigens and/or immu-

nostimulatory molecules and/or targeting ligands, mimicking pathogens and facilitating uptake by antigen APCs and stimulating immune activation [12, 13];

(7) Regulation of the type of immune response: immunological properties of particles can be tailored by changing their size, surface charge, or hydrophobicity, amongst others [1, 9].

(8) Dose reduction: owing to the potential synergistic effect of all the above-mentioned effects, particles can serve to decrease the dose of antigen required to elicit an immune response [11].

A large number of particulate systems has been reported, such polymeric particles, liposomes, virus like particles and virosomes, immune stimulatory complexes (ISCOMs), emulsions, or inorganic nanobeads, but particularly, poly(D,L-lactide-co-glycolide) (PLGA)-based delivery systems are well studied and are promising candidates for antigen delivery [20]. Since the initial description of PLGA particle as potential adjuvants by O'Hagan et al [21], PLGA particles have been formulated in a wide variety of ways resulting in various size, charge, antigen stability, loading capacity and release profiles. These key formulation aspects can greatly affect the end product characteristics and consequently the potency of the vaccine. These factors will be discussed in detail in this review, as well as the latest advances in peptide-based vaccines using PLGA-based particulate systems.

2. Poly(D,L-lactide-co-glycolide) particulate systems for subunit vaccine delivery

Poly(lactic-co-glycolic acid) (PLGA) and its derivatives are aliphatic polyesters that are available in different ratios of lactic acid and glycolic acid, various molecular weights, and type of end groups (ester-terminated (capped) or carboxylic acid terminated (uncapped)). PLGA polymers have been widely studied over the past few decades for several biomedical applications due to their excellent safety records, varying from sutures to bone reconstruction, as well as in implants for sustained drug delivery, and it has long been approved for parenteral human use by the FDA [22-24]. After their administration, PLGA particles undergo degradation by bulk erosion, during which water diffuses into the polymeric matrix, hydrolyzing the ester bonds throughout the polymer and reducing its molecular weight until degradation products are formed that can be dissolved [9]. This process increases porosity in the matrix, allowing the sustained release of the entrapped material as degradation continues. Finally, PLGA is hydrolyzed into the original monomers, lactic acid and glycolic acid, which are by-products of various metabolic pathways and therefore are not associated with significant toxicity [25]. Degradation rate of PLGA is related to molecular weight, hydrophilicity and crystallinity, but also other factors such as pH of the medium, water uptake rate, process of ester hydrolysis, swelling ratio and degradation by-products [9, 26]. Lower molecular

weight molecules degrade faster, as shorter molecules can be more easily hydrolyzed and dissolved, leaving the polymeric matrix. Higher hydrophilicity can also lead to faster degradation: the hydophilicity is mainly influenced by the monomers' ratio, with glycolic acid being more hydrophilic than lactic acid, so the higher the content of glycolic acid, the more hydrophilic, increasing hydrolysis rate [25]. An exception to this rule is the co-polymer with 50:50 lactide:glycolide ratio, which has the fastest degradation rate, even among polymer compositions with higher glycolic acid content. This is due to the influence of crystallinity: the higher the crystallinity, the slower the degradation, and at a 50:50 ratio the polymer is the least crystalline, resulting in the fastest degradation rate [9, 27]. Uptake of PLGA particles by APCs may further expedite the degradation of PLGA, as the acidic environment of the endosomal compartment (pH ~4.5 – 6.5) [28] accelerates degradation compared to physiological pH (pH 7.4) since low pH catalyzes breakage of the ester linkage of the polymer backbone enhancing polymer erosion [29, 30].Thus, depending on the type of PLGA polymer used, PLGA particles can be made with distinct release kinetics [15, 31-33]. Next to release characteristics various other physical trades of PLGA particle can manipulated including particle size and size distribution, zeta potential, polydispersity index, encapsulation efficiency and drug loading [26]. All these characteristics can be controlled during the synthesis of the particles which can be manufactured according to good manufacturing practice in a scalable, affordable and reproducible way [25]..

While many properties are favorable and controllable, there are also drawbacks in using PLGA particles as a delivery system, especially concerning the stability of encapsulated protein antigens, which will be discussed in more detail later on. Therefore, antigen stability after encapsulation and storage should be evaluated, and each formulation should be specifically customized for each antigen, accordingly to its properties [5].

3. PLGA particle characteristics affecting adjuvanticity

Depending on the preparation method and conditions, PLGA particles can be made with diameters ranging from 80 nm to 250 μm [7]. Moreover, various experimental conditions can be chosen and varied, such as type of solvents and polymer, polymer molecular weight, polymer concentration, type and concentration of surfactants, homogenization mechanism, duration and intensity, or volume ratio of phases. Each of these different factors can affect the particle size, size distribution, zeta potential, encapsulation efficiency, drug loading and release profile [26], which in turn affect the immunogenicity of the formulation. In following section we will systemically review these effects.

3.1. Particle size

Particle size is one of the most critical factors affecting interaction with APCs as well as their biodistribution. Particle size is strongly dependent on the type and concentration of surfactants, polymer concentration, phase volume ratios and homogenization speed [26]. Higher polymer concentration leads to bigger particles, due to higher viscosity of the oil phase, making it harder to break the droplets, as well as and higher (w1/o)/w2 ratios; while higher surfactant concentrations lead to more stable emulsions and can produce smaller particles [26]. The method of homogenization and its speed are also among the most important factors: for instance, microparticles are usually produced using an homogenizer and/or magnetic stirring, whereas nanoparticles are produced by sonication, since the higher the homogenization speed, the smaller the particles.

Particle size is also known to influence the loading capacity, depot formation and release kinetics [34-36]. The particle size and size distribution are determinant for antigen release rate, as the total surface area for protein delivery depends on the particle size [26]. The smaller the particle, the faster the antigen release, as smaller particles have a larger surface area, and therefore a greater proportion of antigen located near its surface, which can lead to a higher burst release [37, 38]. On the other hand, microparticles have larger cores from which the encapsulated antigen slowly diffuses out, and require more time to be degraded, usually showing very low antigen release rates and overall antigen release when comparing to nanoparticles, affecting the total amount of antigen actually being delivered to DCs [38].

Smaller particles are generally regarded as more effective delivery vehicles, since their size would allow easier travel through membranes and other biological barriers and efficiently reach target tissues [39-41]. However, there is still no definitive answer to which size PLGA particles are the most effective for vaccine delivery, and results of different studies comparing nanoparticles and microparticles are contradictory [32, 35, 36]. A strong correlation between particle size and the mechanism of antigen uptake, processing and presentation by APCs has been reported in different studies [34-36, 42-44]. APCs are known to take up and process particles with dimensions comparable to viruses and bacteria [45]. The way APCs take up the vaccine can determine how it processes the antigen. Soluble antigens which are preferentially presented by the MHC class II pathway and are only poorly cross-presented. Particles in the range of 20-200 nm are efficiently taken up by DCs via endocytosis or pinocytosis and facilitate the induction of cellular immune responses, whereas microparticles of 0.5-5 μm are taken up via phagocytosis or macropinocytosis mainly generating humoral responses [35, 36, 46]. Particles larger than 10 μm are hardly taken up, leading to defective immune activation [47-49]. It has also been postulated that large microparticles (> 10 μm) preferentially attach to the surface of macrophages thus serving as an extracellular depot system for continuous antigen release [36]. Comparative studies about the effect of PLGA particle size on the observed immune response have been summarized on **Table 1**. These studies suggest that the efficiency of internalization significantly affects the resulting immune response. However, one should bear in mind that particle properties other than size may also affect their fate and biological effects (see following

sections).

The size of MPs should not be too large, as Thomas et al. showed that hepatitis B surface antigen (HBsAg) in PLGA MPs with a size of 5 µm elicited a significantly higher serum antibody response than 12 µm MPs upon pulmonary administration in rats [50]. Confocal imaging also showed that smaller particles were taken up more efficiently by alveolar macrophages, which might explain the increased immunogenicity.

A study investigating the immunogenicity of differently sized PLGA particles (200, 500 and 1 µm) encapsulating bovine serum albumin (BSA) reported that 1000 nm-sized particles were capable of inducing stronger IgG responses *in vivo* than 200 and 500 nm NPs, which induced similar IgG titers to soluble BSA and alum, following immunization via intranasal, oral and s.c. routes in mice [51]. Similar studies were conducted also with PLA MPs encapsulating HBsAg, showing that MPs of 2-8 µm induced stronger anti-HBsAg antibody responses than NPs of 200-600 nm after intramuscular (i.m.) immunization of rats [52]. However, NPs were efficiently taken up by macrophages, whereas PLA MPs were not taken up but found attached to the surface of the macrophages. Immunization with PLA MPs (2–8 µm) promoted IL-4 secretion, upregulated MHC class II molecules and favored a Th2 response, whereas immunization with PLA NPs (200–600 nm) was associated with higher levels of IFN-γ production, upregulation of MHC class I molecules along with antibody isotypes related to a Th1 response [52]. Comparable results were obtained with i.m. vaccination of rats with tetanus toxoid (TT) in PLA particles: PLA MPs sized 2 to 8 µm were more effective in generating antibody titers than particles smaller than 2 µm, potentiating humoral immune response [49]. The choice of particle size may be dependent on the type of immune response desired: NPs favored a Th1 bias, whereas MPs promote Th2 based responses.

After comparing the immunogenicity of TT loaded PLGA NPs (500-600 nm) to MPs (4 µm), both types of particles were mixed together into a formulation that included both NPs and MPs loaded with TT [53]. After i.m. immunization of rats, this mixture elicited higher antibody responses compared to the NPs or MPs alone, which elicited similar responses. A mixture of both size classes could also be considered to stimulate both Th1 and Th2 type responses.

Joshi et al. compared 17 µm, 7 µm, 1 µm, and 300 nm sized PLGA particles co-encapsulating OVA and CpG , by selectively recovering different sized particles with different centrifugation cycles, showing a size-dependent burst release over 48 h followed by a plateau, with total OVA and CpG release ranging from 100% for 300 nm NPs to circa 10% for 17 µm MPs [35]. In a direct comparison, they observed that the efficiency of particle uptake and upregulation of MHC class I and CD86 expression on BMDC was correlated with smaller particle size [35]. The same trend was observed following intraperitoneal (i.p.) vaccination, with the 300 nm NP generating the highest antigen-specific cytotoxic T cell responses, and the highest IgG2a:IgG1 ratio of OVA-specific antibodies, in proportion to DC uptake. These results concur with our own observations, since we have recently compared PLGA NP circa 300 nm with MP > 20 µm, co-encapsulating OVA and poly(I:C), with similar compositions and release

Table 1: Comparative studies about the effect of PLGA particle size on the observed immune response.

Formulation	Particle size	Antigen/TLRL	*In vitro* / *in vivo*	Adminstration route	Response	References
PLGA MPs	5 µm, 12 µm	HBsAg protein	*In vitro* & *in vivo*	Pulmonary	5 µm > 12 µm MPs uptaken by rat alveolar macrophages; Ab responses: 5 µm > 12 µm MPs	[50]
PLGA NPs & MPs	200 nm, 500 nm, 1 µm	BSA protein	*In vitro* & *in vivo*	s.c.	Ab responses: 200 nm ~ 500 nm < 1 µm particles.	[43]
PLA NPs & MPs	200-600 nm, 2-8 µm	HBsAg protein	*In vitro* & *in vivo*	i.m.	NPs >> MPs uptaken by macrophages; MPs ↑ anti-HBsAg Ab responses & ↑ IL-4 secretion related to a Th2 response; NPs ↑ IFN-γ production & ↑ Ab isotype related to a Th1 response.	[52]
PLA MPs	< 2 µm, 2-8 µm, 10-70 µm, 50-150 µm	TT	*In vivo*	i.m.	Ab responses ↑ by 2-8 µm MPs > <2 µm MPs >> 10-70 µm ~ 50-150 µm.	[49]
PLGA NPs & MPs	500-600 nm, 3.5 µm	TT	*In vivo*	i.m.	NPs and MPs mixed together ↑ Ab responses > NPs ~ MPs alone	[53]
PLGA NPs & MPs	17 µm, 7 µm, 1 µm, 300 nm	OVA / Cpg ODN	*In vitro* & *in vivo*	i.p.	particle uptake & upregulation of MHC class I and CD86 expression & ↑ OVA-specific $CD8^+$ T cells & ↑ IgG2a:IgG1 following the same size trend: : 17 µm << 7 µm < 1 µm < 300 nm	[35]
PLGA NPs & MPs	300 nm,> 20 µm	OVA / poly(I:C)	*In vitro* & *in vivo*	s.c.	NPs >> MPs internalized by DCs & ↑ $CD8^+$ T cell activation *in vitro*; vaccination with NPs ↑ OVA-specific $CD8^+$ T cells & Ab production, MPs did not	[48]
PLGA NPs & MPs	600 nm, 1 – 1.5 µm	OVA	*In vitro*	n/a	MPs > NPs induced *in vitro* MHC class I Ag cross-presentation	[54]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ~: similar; ↑: increased/high: ↓: decreased/low

properties, for their capacity to induce MHC class I cross-presentation *in vitro* and improve immune responses *in vivo* [48]. NPs were efficiently internalized by DCs *in vitro*, whereas MP were not. Subcutaneous vaccination of C57BL/6 mice with NPs resulted in significantly better priming of Ag-specific $CD8^+$ T cells compared to MP. NP also induced a balanced TH1/TH2-type antibody response, whereas MP failed to increase antibody titers [48]. NP also outperformed IFA as an adjuvant, by more efficiently boosting $CD8^+$ T cell activation and (IgG2a) antibody production [48]. Conversely, in a study by Lee et al., MPs in the range of 1.11~1.44 μm more efficiently induced *in vitro* MHC class I cross-presentation of OVA peptides via than 0.56 μm NPs [54]. However, since only 1.11 μm MPs were fully characterized and evaluated *in vivo*, we cannot exclude that differences in (burst) release may be responsible for the differences observed *in vitro*, neither if the results would be translated *in vivo*.

The impact of antigen delivery system size on the resultant immune response also depends on the route of administration employed. Particles in the size range of 20-50 nm are suitable for transport through lymphatic vessels to reach lymph nodes, where they can increase the probability of immune cell interaction [9]. In contrast, large particles (500–2000 nm) depended on cellular transport by skin DCs [40]. These studies suggest that particulate vaccines should be formulated in the nano-size range to achieve efficient uptake, significant MHC class I cross-presentation and effective CTL responses.

3.2. Controlled antigen/adjuvant release

In addition to their ability to protect antigens, favor uptake by APCs and enhance the immune response, controlled release systems can extend antigen release for prolonged periods of time [55, 56]. Antigen/adjuvant release from PLGA particles is dependent on a variety of factors, such as size, polymer composition, porosity of the matrix, antigen loading or the way it is associated with PLGA particles, i.e. encapsulated/entrapped or adsorbed onto the surface. In the first case, antigen release depends on the degradation, erosion or dissolution of the polymer; whereas in the second case it is dependent on the interactions between the polymer and the antigen [57]. Entrapment of the antigen within the particle matrix protects antigen from external environment but may lead to incomplete release, which could lead to a weak immune response; in contrast, adsorption may lead to high burst release, prematurely releasing the antigen from the particulate carrier before uptake by DCs, which can lead to deficient immune responses [37]L. Frequently, a combination of adsorbed and encapsulated antigen occurs, resulting in a characteristic triphasic release profile with an initial burst release followed by a lag phase and a final sustained release phase of the encapsulated antigen dictated by polymer erosion [57, 58]. Initial burst release of antigen can be generally explained by two mechanisms: either it occurs mainly due to the release of antigens that are adsorbed or located in the surface layer, or the morphology of NP/MP causes the initial antigen escape through pores and cracks that may form during the NP/MP fabrication process [59-61]. Several factors affect burst release:

higher hydrophilicity, lower molecular weight and lower polymer concentration can lead to higher burst release [26]. The higher glycolide content makes the polymer more hydrophilic, facilitating water uptake from the release medium which results in a higher initial burst release, whereas the higher lactide content makes it more hydrophobic, thus resulting in much slower release [62]. Other parameters may be altered that impact release, such as switching the molecular weight or to ester end-capped version of the respective polymer [33]. By adding salts to the inner water phase (w1), the porosity of the resulting particles can be controlled by increasing the osmotic gradient and the flux of water from w2 into the w1/polymer phase, increasing antigen release rate [48]. Suspensions of sugars [63] or salts in the oil phase are expected to act in a similar way, resulting in a major increase in water-uptake, e.g., by incorporation of suspended NaCl, which has been shown with PLGA films [64]. A larger inner surface, induced by a higher porosity of the particles, can potentially increase the uptake of the release medium into the particles and accelerate the drug pore-diffusion and release [65]. After burst, the release of encapsulated material from such systems is dependent on diffusivity through the polymer barrier (a more hydrophobic polymer will create a higher barrier), porosity, size of antigen molecule and distribution throughout the matrix, leading to prolonged antigen release, thereby enhancing the duration of antigen exposure to APCs and thus the potency of the resultant response [66].

Antigen release kinetics regulates the antigen exposure to the immune system. If most of the cargo is burst released immediately after immunization and before uptake, antigen will be delivered to APCs in soluble form, losing the benefit of particulate delivery [37]. In contrast, if the release profile is too slow or incomplete, there will not be enough antigen available for presentation by APCs. Moreover, Hailemichael et al. showed that Montanide-based persisting vaccine depots can induce specific T cell sequestration, dysfunction and deletion at vaccination sites; whereas short-lived formulations may overcome these limitations and result in greater therapeutic efficacy of peptide-based cancer vaccines [67]. Still, sustained release of antigen/adjuvant seems crucial to properly activate DCs, whereas a low burst eliminates potential antigen loss before uptake, increasing antigen presentation and $CD8^+$ T cell activation [12, 37]. Kanchan et al. reported that slow and continuous release of antigen/adjuvant may prolong MHC antigen presentation, which play a key role in T cell stimulation and activation, and played a critical role in eliciting memory antibody responses [68]. It has been reported that extended antigen release may enhance not only the level, but also the quality of immune responses [36]. Johansen et al. demonstrated that antigenic delivery increasing exponentially over time induced more potent $CD8^+$ T cell responses and antiviral immunity than a single dose or multiple equivalent doses (zero order) [34]. Shen et al. showed that OVA-loaded PLGA MPs enhanced exogenous antigen MHC class I cross-presentation at 1000-fold lower concentration than soluble antigen, and served as an intracellular antigen reservoir, leading to sustained MHC class I presentation of OVA for 72 h [19]. Likewise, Waeckerle-Men et al. showed that MHC classes I and II-restricted presentation of encapsulated proteins and peptides by DCs was markedly prolonged and presented 50-fold more efficiently on class I molecules than soluble antigens [69]. A difference in performance between PLGA NPs connected to the kinetics of antigen delivery was showed by Demento et al., with “slow” releasing NPs eliciting prolonged antibody titers comparing to “fast” releasing ones, and favored long-term effector-

memory cellular responses [12]. Finally, Zhang et al. formulated OVA-loaded PLGA NPs by encapsulating antigen within NPs or by simply mixing soluble antigen with the NPs, observing that the combined formulations induced more powerful antigen-specific immune responses than each single-component formulation. The enhanced immune responses elicited by the combined vaccine formulation may be ascribed to the combination of a depot effect at the injecton site, adequate initial antigen exposure and long-term antigen persistence leading to prolonged antigen presentation [70].

3.3. Surface characteristics

Surface characteristics such as shape, hydrophobicity, and zeta potential are reported to influence phagocytic uptake by APCs. Because cells are negatively charged, cationic particles induce more efficient phagocytic uptake than anionic particles owing to electrostatic attraction to the negatively charged APC membranes [71, 72]. Strategies aimed at improving the efficacy of PLGA particles as antigen delivery vehicles involve coating them with ionic surfactants or polymers such as poly(ethylene glycol) (PEG), sodium dodecyl sulphate (SDS), aminodextran, chitosan, poly(ethylene imine) (PEI), poly(L-lysine), protamine or cetyltrimethylammonium bromide (CTAB) [57, 73, 74]. Coating can be achieved either by incorporating these agents in the particle matrix either together with the polymer or in the external aqueous phase during the emulsification process, or by adsorption to the surface of pre-formed particles by resuspending them in a solution containing the coating and incubating for a determined amount of time. Besides changing surface charge, some of these molecules have bioadhesive properties, such as chitosan [1], which has been employed to develop formulations for mucosal delivery. Polycations can also aid in phagosomal/endosomal escape after being internalized by APCs [1], potentially influencing the antigen presentation pathway and type of immune response.

Wishke et al. studied the impact of the surface properties of MPs (5 – 10 μm) on phagocytosis and the phenotype of DCs, using bovine serum albumin bearing fluorescein isothiocyanate groups (FITC-BSA) as model antigen [74]. Anionic particles were obtained by using polyvinyl alcohol (PVA) as stabilizing agent; whereas for cationic surfaces CTAB and chitosan/PVA or diethylaminoethyl cellulose (DEAE)-dextran/PVA blends were evaluated. Whereas CTAB modified MPs lost their positive charge and aggregated due to CTAB desorption, the modification with chitosan and DEAE-dextran resulted in stable MPs. DEAE-dextran modified MPs increased the cellular uptake of the protein loaded MPs.

Positively charged PLGA microspheres (1 – 5 μm) containing hepatitis B surface antigen (HBsAg) were prepared with cationic agents stearylamine and PEI in the external aqueous phase [71]. Compared to unmodified formulations, positive surface charge enhanced both the systemic and mucosal immune response upon immunization of rats via the intranasal route, showing increased levels of IgG in serum and sIgA in salivary, vaginal and bronchoalveolar lavages. PLGA microspheres coated with chitosan

were developed for nasal immunization using recombinant HBsAg [75]. The modified PLGA microspheres showed the lowest nasal clearance rate and a 30-fold increase of serum IgG levels in comparison with unmodified PLGA microspheres upon nasal administration. OVA-loaded PLGA nanoparticles coated with N-trimethyl chitosan (TMC) were more efficiently taken up by DCs and showed a longer nasal residence time than uncoated particles [76].

Protamine, a cationic polypeptide, has been used as a surface coating material because of its ability of increasing cell penetration [77]. Protamine coating of PLGA microparticles (~7 μm) encapsulating the purified phospholipase A2 (PLA2) from bee venom or ovalbumin (OVA) as model antigens injected s.c. in mice led to increased antibody and T-cell responses as compared to uncoated particles (~3 μm). This was most likely mediated by an increased uptake, as protamine-coated particles (~3 μm) carrying green fluorescent protein plasmid were efficiently internalized in vitro by non-phagocytic cells and impressively increased transfection [77]. In another study from the same group, PLGA microparticles containing PLA2 allergen in combination with adsorbed protamine and CpG (~8 μm) resulted in strong PLA2-specific antibody responses and the induction of the Th1-associated isotype IgG2a [78].

The CTL-restricted OVA peptide SIINFEKL was microencapsulated into bare PLGA MPs (~2.6 μm), chitosan-coated PLGA MPs with CpG either covalently coupled or physically adsorbed onto the MP surface (~3.1 μm), and protamine-coated PLGA MPs with adsorbed CpG (~2.2 μm) [79]. For the covalent coupling, chitosan coated MPs were functionalized with sulfo-MBS (m-maleimidobenzoyl-N- hydroxysuccinimide ester), a water-soluble heterobifunctional cross-linker which forms amide bonds with primary amino groups via the N-hydroxysuccinimide ester while the maleimido residue reacts with sulfhydryl groups to form stable thioether bonds, and then incubated with 5′-thiol-modified CpG. However, only the uncoated PLGA MP with adsorbed CpG mediated a prominent CTL response in mice after s.c. immunization, eliciting the production of 1.2% of IFN-γ secreting and SIINFEKL-specific $CD8^+$ T cells, with failure of the other formulations being ascribed to the low release of antigen and CpG.

During the encapsulation process antigens are exposed to the organic solvents required to dissolve the polymer (e.g. dichloromethane, ethyl acetate), emulsification steps (which may include interfaces, temperature excursions, sonication, and vigorous shaking) and drying (usually lyophilization), all of which may compromise antigen stability [9]. Moreover, after administration the degradation of the polymer matrix creates an acidic and potentially harmful microenvironment which can result in denaturation, chemical degradation or aggregation of the antigen, which may endanger the integrity and immunogenic potential of the vaccine [80, 81]. In particular, protein antigens tend to aggregate or degrade upon entrapment into PLGA or during release from the matrix [81]. Though peptide antigens may be less susceptible to the above-mentioned stress factors than proteins, as they do not possess a defined tertiary structure, that can also lead to deamidation or formation of peptide adducts due to acylation of lysine residues with lactic and glycolic acid units [82]. This may be partially solved by optimized manufacturing methods or addition of stabilizing agents, such as basic inorganic salts (e.g. magnesium hydroxide, calcium carbonate, sodium bicarbonate, zinc carbonate),

other proteins, surfactants or sugars [81, 83-85].

Attachment of antigens via covalent chemistry or adsorption through electrostatic interactions onto the surface particles might be an alternative to encapsulation to prevent antigen instability. Adsorption procedures can be performed under milder condition than encapsulation, the antigen is released faster and is immediately available from the particle surface to be processed following uptake, not being dependent on polymer degradation to release entrapped antigen, and antigen loading is often more efficient [21, 86]. However, adsorption may also result in burst or premature release, therefore release kinetics should be taken into account when considering adsorption or encapsulation of antigens into PLGA particles.

A single s.c. injection of HBsAg adsorbed to PLGA-CTAB microspheres (~5-7 μm) gave similar humoral and cellular responses a two injections of HBsAg in alum [87]. Anionic MPs with a mean size of 1 μm were prepared by adding SDS to the external water phase in the preparation process in order to adsorb p55 Gag protein [88]. SDS-coated PLGA particles with adsorbed p55 gag were able to induce high specific CTL responses after i.m. administration in mice. IgG titers were significantly increased compared to soluble p55 Gag protein [89]. The same group also investigated particles consisting of PLGA coated with the anionic surfactant dioctylsulfosuccinate (DSS). 1 μm sized PLGA-DSS particles with Neisseria meningitidis B antigen adsorbed elicited high antigen-specific IgG titers in mice after i.p. administration compared to an antigen-alum formulation [90]. Similar particles were used to adsorb gp120dV2, a recombinant HIV glycoprotein with an isoelectric point of 8.5 [91]. In contrast with encapsulated antigen, surface adsorption of this protein did not affect its binding capability to CD4 [91, 92].

In conclusion, modifying the surface charge may help increase particle uptake efficiency and result in a stronger immune response, especially when considering mucosal delivery. Furthermore, modification of the particle surface using either polycations or polyanions has been used to create cationic or anionic particles to which charged antigens/adjuvants can be adsorbed. Most protein antigens and/or adjuvants are negatively charged, so positive charge would allow such antigens to be adsorbed to the surface instead of being encapsulated into particles, which may be beneficial to improve antigen stability.

3.4. Targeted delivery to DCs

3.4.1. TLRL co-delivery in PLGA systems

One of the greatest benefits of particulate antigen delivery systems is their ability to co-deliver antigens and immunomodulators simultaneously to the same APCs [93]. The concomitant delivery of TLRLs and antigens in PLGA particles has been proven.

successful to enhance antigen-specific CTL responses [79, 94]. The appropriate selection of the TLRL for co-delivery will determine the bias towards Th1 or Th2 responses [93]. Furthermore, as most pathogens simultaneously present multiple TLR agonists to APCs, the combination of multiple TLRLs may result in a synergistic effect and a promising strategy to induce strong protective immune responses [7]. Over the last decades, some of these ligands have been used in several vaccine formulations to target and activate TLRs. A summary of PLGA vaccine formulations containing TLRLs can be found in **Table 2**.

CpG is a ligand to TLR9 which is known to induce a MHC I class driven antigen presentation, resulting in a cellular immune response. Separate groups of C57BL/6 mice were immunized s.c twice with TT and CpG in PLGA NPs (test group), TT-loaded NPs, TT-loaded NPs mixed with soluble CpG, TT and CpG both in solution (reference group), TT alone in solution, and alum adsorbed TT [95]. While CpG increased the immunogenicity of soluble TT antigens when co-delivered s.c. as a soluble adjuvant in mice, its effect was significantly higher when administered in a particulate PLGA formulation. The TT/CpG NPs group showed strong antigen-specific T cell proliferation ex vivo significantly higher than that observed for T cells isolated from the reference group, and was associated with higher levels of interferon γ secretion. IgG titers that were also 16 times higher than the reference group, and differences of 8-fold for IgG1 and IgG3, and 5-fold for IgG2b titers were observed. Overall, the results show that co-delivery of CpG and TT resulted in induction of both Th1 and Th2 immune responses with a bias towards Th1 type. Characterization studies showed that co-encapsulation of CpG and TT in PLGA particles resulted in CpG localization on the outer side of the particles [95]. The importance of co-encapsulation was studied by co-encapsulating ovalbumin (OVA) and CpG-chitosan complexes in PLGA MPs, showing that the co-encapsulation of CpG with OVA in PLGA MPs significantly improved the antibody response and isotype shifting in comparison with mice immunized i.d. with OVA-loaded MPs [96]. Heit et al. compared the *in vivo* immunogenicity of MPs (<10 μm) co-entrapping OVA and CpG with that of CpG-OVA conjugates, or a mixture CpG and OVA [97, 98]. *In vivo*, MP-based vaccination triggered clonal expansion of Ag-specific MHC class I-restricted $CD8^+$ T cells comparable to CpG-Ag conjugates. Conjugation of antigens to adjuvants is however a difficult process that has to be performed and optimized for each individual antigen, whereas particulate formulations offer a more generic approach. Using infection- and tumor-model system, PLGA MP-based single step s.c. vaccination at the tail base of mice conferred protective and even therapeutic immunity against OVA-expressing B16 melanoma tumor cells [98].

TLR3 ligand poly(I:C) is also known to enhance cross-priming of $CD8^+$ cytotoxic T lymphocytes [99]. Poly(I:C) adsorbed to (DEAE)–dextran-coated PLGA MPs containing FITC-BSA were effectively phagocytized by DCs ex vivo and induced a maturation similar to that achieved with a cytokine cocktail or higher concentrations of soluble poly(I:C) [100]. Schlosser et al. demonstrated that MHC I cross-priming in mice after s.c. vaccination with PLGA MPs was enhanced when OVA was co-encapsulated together with either a CpG or poly(I:C) as compared to co-inoculation of OVA-loaded MPs with soluble or separately encapsulated adjuvants [94]. Using four different read-out systems: (i) SIINFEKL/H-2Kb tetramer positive CTLs, (ii) IFN-γ production,

Table 2: Examples of reports of PLGA formulations using Toll-like receptor ligands and their immunological effects.

Receptor	Ligand	Formulation	Antigen	*In vitro / in vivo*	Adminstration route	Response	References
TLR 1/2	Pam3CSK4	PLGA NPs (~350 nm)	OVA24 peptide	*In vitro & in vivo*	s.c.	TLR 2 stimulation ↑ MHC class I presentation of OVA24-NPs by DCs *in vitro* & ↑ prolonged Ag presentation and $CD8^+$ T cell activation *in vivo* after adoptive transfer of NP-loaded DCs	[111]
	Pam3CSK4	PLGA NPs (~500) and MPs (~2 µm; <7 µm)	$CS_{252-260}$ coupled to Pam3CSK4 (Pam-$CS_{252-260}$)	*In vivo*	i.p	Pam-$CS_{252-260}$ particles ↑ cytolytic activity > $CS_{252-260}$-MPs or sPam-$CS_{252-260}$; 500 nm NPs > 2 µm ~ <7 µm MPs inducing CTL responses	[110]
TLR 3	Poly(I:C)	(DEAE)–dextran-PLGA MPs (~3 µm)	FITC-BSA	*In vitro*	n/a	poly(I:C) coated-MPs ↑ expression of CD80, CD86, and CD83 at the DC surface ~ cytokine cocktail or ↑ concentrations of sPoly(I:C).	[100]
TLR 4	MPLA	PLGA MPs (1 – 10 µm)	OVA_{323-39} peptide; MUC1 mucin peptide	*In vivo*	s.c.	Ag/MPLA-MPs ↑ T cell proliferative response & production of IFN-g by T cells, eliciting a specific Th1 immune response > Ag-MPs or Ag mixed with alum	[102, 103]
	MPLA	PLGA NPs (350 – 450 nm)	OVA protein	*In vitro & in vivo*	i.p. or s.c.	OVA/MPLA-NPs ↑ $CD8^+$ T cell proliferative responses & IFN-g *in vitro* & >13-folds increase in clonal expanded $CD4^+$ T cells *in vivo* > OVA-NPs	[104]
	MPLA	PLGA NPs (~300 nm)	HBcAg protein	*In vivo*	s.c.	HBcAg/MPLA-NPs ↑ Th1 cellular response with predominant IFN-g profile > sHBcAg, sHBcAg/sMPLA, or HBcAg-NPs	[105]
	MPLA	PLGA NPs (~500 nm)	$HBcAg_{129–140}$	*In vivo*	s.c.	$HBcAg_{129–140}$/MPLA-NPs ↑ Th1-type response > control formulation of $HBcAg_{129–140}$ in CFA	[132]

TLR 4	MPLA	PLGA NPs (350 – 450 nm)	OVA; MUC1 lipopeptide (BLP25)	*In vitro & in vivo*	n/a	OVA/MPLA-NPs ↑ *in vitro* and *in vivo* antigen-specific primary Th1 immune responses > OVA-NPs or sOVA/sMPLA after adoptive transfer of antigen-pulsed DCs; MUC1/MPLA-NPs delivery to DCs ↑ MUC1 reactive T cells *in vitro* > MUC1-NPs, MPLA-NPs, sMUC1, or sMUC1 with MPLA-NPs	[13]
	7-acyl lipid A	PLGA NPs (350 – 410 nm)	$TRP2_{180-188}$ peptide	*In vivo*	s.c.	$TRP2_{180-188}$/7-acyl lipid A-NPs ↑ $CD8^+$ T cell-mediated anti-tumor immunity & therapeutic anti-tumor effect & levels of IFN-g and pro-inflammatory Th1-related cytokines > $TRP2_{180-188}$-NPs	[107]
	MPLA	PLGA NPs (~80 nm)	$TRP2_{180-188}$ peptide	*In vitro & in vivo*	i.d.	NP ↑ uptake *in vitro* & *in vivo*; $TRP2_{180-188}$/MPLA-NPs ↓ growth of s.c. inoculated B16 melanoma cells in a prophylactic setting > $TRP2_{180-188}$-NPs, $sTRP2_{180-188}$/sMPLA	[108]
	MPLA or RC529	PLGA MPs (3 – 5 μm)	gp120 protein; Men B	*In vivo*	i.p.	Ag adsorbed on TLRL-MPs ↑ IgG serum titers > Ag adsorbed-MPs with sTLRL.	[109]
TLR 9	CpG ODN	PLGA NPs (~300 nm)	Tetanus toxoid (TT)	*In vitro & in vivo*	s.c.	TT/CpG-NPs ↑ antigen-specific T cell proliferation ex vivo & IFN-γ secretion & 16-fold IgG titers > sTT/sCPG; co-encapsulation ↑ Th1 and Th2 immune responses towards Th1 type bias.	[95]
	CpG ODN	PLGA MPs (<10 μm)	OVA protein; CpG-OVA conjugate	*In vitro & in vivo*	s.c.	OVA/CpG-MPs were uptaken by DCs *in vitro*; OVA/CpG-MPs ↑ Ag-specific $CD4^+$ and $CD8^+$ T cells ~ CPG-OVA conjugates *in vivo*. In a tumor challenge, MPs caused complete tumor regression in four out of five mice.	[98]
	CpG ODN	PLGA MPs (<25 μm)	PLA2 protein	*In vivo*	s.c.	PLA2/CPG-MPs ↑ PLA2-specific Ab responses & ↑ Th1-associated isotype IgG2a. The effect of CpG ↑ when protamine was co-encapsulated for complexation of CpG.	[78]

TLR 9	CpG ODN	bare, chitosan-coated, and protamine-coated PLGA MPs (<10 µm)	SIINFEKL peptide	*In vivo*	s.c.	Only uncoated SIINFEKL-MPs with adsorbed CpG ↑ IFN-g secreting and SIINFEKL-specific $CD8^+$ T cells.	[79]
	CpG ODN	PLGA MPs (~1 – 1.5 µm) coated with CTAB or DSS	p55 gag or gp120 env proteins	*In vitro & in vivo*	i.m.	CpG adsorbed to PLGA-CTAB MPs co-administered with gp120 env or p55 gag proteins adsorbed to PLGA-DSS MPs ↑ Ag-specific serum IgG titers, as well as CTL responses against p55 gag > sCpG/sAg,	[88-91]
	CpG ODN-chitosan complexes	PLGA 502 and 752 MPs (~1 – 2 µm)	OVA protein	*In vivo*	i.d.	OVA/CpG-MPs ↑ Ab response and isotype shifting to Th1 > OVA- MPs.	[96]
	CpG ODN or Poly(I:C)	PLGA MPs (<5 µm)	OVA protein	*In vivo*	s.c.	CpG/OVA- or poly(I:C)/OVA-MPs ↑ (i) SIINFEKL/H-2Kb tetramer positive CTLs, (ii) IFN-g production, (iii) *in vivo* cytotoxicity & (iv) protection from vaccinia virus > to OVA-MPs with sTLRL or OVA-MPs with TLRL-MPs.	[94]
	CpG ODN & Poly(I:C)	PLGA MPs (~0.5 - 5 µm)	OVA protein	*In vivo*	s.c.	OVA/CpG-MPs with MP-poly(I:C) ≥ IFA in eradication of preexisting tumors and suppression of lung metastases	[101]
	CpG ODN or/and Poly(I:C)	PLGA NPs (~1 µm)	OVA protein	*In vitro*		poly(I:C)/OVA- or CpG/OVA-NPs ↑ prolonged MHC class I- & II-restricted presentation & ↑ OVA-specific $CD8^+$ and $CD4^+$ T cells; combination of both TLRLs synergistically ↑ MHC class I-restricted, but not class II, Ag presentation.	[99]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ≥: equal or higher than; ~: similar; ↑: increased/high: ↓: decreased/low; CFA: complete Freund's adjuvant; sX: soluble X.

(iii) *in vivo* cytotoxicity against SIINFEKL-charged target cells, and (iv) the protection from infection with vaccinia virus encoding OVA, they found that co-encapsulated TLR ligand and antigen consistently yielded stronger CTL responses. For instance, a single immunization with MPs containing co-encapsulated OVA and CpG yielded 9% SIINFEKL/H-2Kb tetramer positive CTLs, whereas approximately half of the response was detected when a mixture of the two MP preparations was administered. This effect was observed for both adjuvants examined, though the responses to poly(I:C) were generally lower. Mueller et al. compared the immune response to IFA and a mixture of PLGA MPs combining both OVA and CpG (MP-OVA/CpG) or a mixture of MP-OVA/CpG and MP-poly(I:C), observing that the PLGA MP mixture was as efficient as or superior to IFA in eradication of pre-existing tumors and suppression of lung metastases [101]. PLGA MPs opsonized with mouse IgG, either loaded with OVA and either poly(I:C) or CpG, were incubated with DCs and significantly increased and prolonged both MHC class I- and class II-restricted OVA presentation, with each TLRL showing similar potency. A combination of the two TLR agonists synergistically increased the MHC class I-restricted, but not the class II-restricted, presentation of exogenous antigen [99].

Monophosphoryl lipid A (MPLA) is a detoxified form of lipid A derived from LPS which is a potent TLR4 agonist [7]. Immunization of mice s.c. with PLGA MPs incorporating MPLA with an OVA peptide, consisting of residues 323–339 containing Th and B epitopes of OVA, resulted in an increase in the production of IFN-γ, eliciting a specific Th1 immune response [102] Similar results were obtained with cancer-associated 24mer human MUC1 mucin peptide [103]. Delivery of MUC1 mucin peptide by Poly(d,l-lactic-co-glycolic acid. Hamdy et al. studied the co-delivery of MPLA and OVA in PLGA NPs (OVA/MPLA NPs). The primary $CD4^+$ T cell responses to OVA/MPLA NPs were investigated using OVA-specific T cells from DO11.10 transgenic mice. Following adoptive transfer of these cells, mice were immunized s.c. by NP formulations. For assessing the $CD8^+$ T cell responses, bone marrow derived DCs were pulsed with different OVA formulations and co-cultured with $CD8^+$ T cells from OT-1 mice. Co-delivery of MPLA and OVA in PLGA NPs induced a higher $CD8^+$ T cell proliferative responses and IFN-γ *in vitro* and >13-folds increase in clonal expanded $CD4^+$ T cells *in vivo* following either i.p. or s.c. route of immunization, compared to OVA-PLGA particles without MPLA [104]. The expanded T cells were capable of cytokine secretion and expressed an activation and memory surface phenotype. Similar studies also showed a significantly stronger cell-mediated response in mice after s.c. vaccination with hepatitis B core antigen (HBcAg) protein or peptide ($HBcAg_{129–140}$) and MPLA in PLGA nanoparticles than when using the free antigen, the free antigen with MPLA, or particles loaded with antigen alone [105, 106]. Elamanchili et al. demonstrated that delivery of OVA and MPLA in PLGA NPs to DCs induced potent *in vitro* and *in vivo* antigen-specific primary Th1 immune responses in mice compared to OVA encapsulated alone or soluble OVA and MPLA, after adoptive transfer of antigen-pulsed DCs [13]. Similarly, co-delivery of MUC1 lipopeptide (BLP25, a cancer vaccine candidate) and MPLA loaded in PLGA NPs to human DCs significantly enhanced proliferation of antigen-specific T cells *in vitro* comparing to NP-MUC1, NP-MPLA, soluble MUC1, or soluble MUC1 mixed with NP-MPLA [13]. This combination of MUC1 and MPLA in PLGA NPs was shown to break tolerance and elicit strong T-cell responses against self-antigens *in vivo* after

adoptive transfer of DCs loaded with the vaccine formulations. Hamdy et al. showed that s.c. vaccination of mice bearing melanoma B16 tumors with PLGA NPs co-encapsulating the poorly immunogenic melanoma antigen, tyrosinase-related protein 2 (TRP2), along with TLR4 ligand (7-acyl lipid A, an analog for MPLA) was able to induce potent $CD8^+$ T cell-mediated anti-tumor immunity and therapeutic anti-tumor effect in comparison to antigen encapsulated alone [107]. Zhang et al. observed that i.d. vaccination of mice with PLGA NPs carrying melanoma antigenic peptide $TRP2_{180-188}$ and MPLA significantly delayed growth of subcutaneously inoculated B16 melanoma cells in a prophylactic setting compared to NPs with $TRP2_{180-188}$ alone, or soluble $TRP2_{180-188}$ mixed with MPLA [108]. In another approach, enhanced immunogenicity in mice was obtained with i.p. vaccination with HIV-1 gp120 protein or antigen from Neisseria meningitidis serotype B (Men B) adsorbed to the anionic surface of PLGA MPs treated with ionic surfactant DSS that contained either encapsulated MPLA or the synthetic LPS analog RC529 [109]. Delivery of MPLA or RC529 encapsulated in PLGA MPs with antigen adsorbed onto the MPs resulted in even further enhancement of IgG serum titers over those obtained with soluble TLRLs. This effect was observed for both antigens regardless of whether or not the TLRL and the antigen were used with the same or with separate particles.

In addition, PLGA NPs (~450 - 600 nm) and MPs (~1 – 3 μm or ~6 – 32 μm) have been used to deliver a lipid modified peptide (lipopeptide) from berghei circumsporozoite protein ($CS_{252-260}$, SYIPSAEKI), coupled to TLR2 agonist Pam3Cys with a Ser-Lys-Lys-Lys-Lys spacer (here designated Pam-$CS_{252-260}$). Particles of mean size < 500 nm were better inducers of CTL than larger microparticles (> 2 μm). Pam-$CS_{252-260}$ loaded PLGA particles administrated i.p. to mice elicited higher levels of cytolytic activity than $CS_{252-260}$-MPs or soluble Pam-$CS_{252-260}$ [110]. Recent work by our group using a 24 residue synthetic long peptide (SLP) from OVA SLP-OVA24 as vaccine antigen and TLR2 ligand Pam3CSK4 co-encapsulated in PLGA NPs showed that TLR2 stimulation enhanced MHC class I presentation by DCs and significantly enhanced prolonged antigen presentation and $CD8^+$ T cell activation *in vivo* after adoptive transfer of antigen-loaded DCs [111].

The best way to deliver adjuvants in PLGA particles, either to entrap or to adsorb them onto their surface, is yet to be resolved. The better choice likely depends on the cellular location of their target receptors: if they act on the cell surface, it might be desirable to have the adjuvant readily available on uptake; but if they need to be internalized to interact with endosomal receptors, encapsulation within the particle might be preferable [112].

3.4.2. Targeted delivery to other DC receptors

Aside from TLR ligands, there are many more targeting ligands that have been used with PLGA particles to increase the immunogenicity of subunit vaccines (see **Table 3**). This can be achieved by modifying the particle surface with ligands that can target specific surface receptors of APCs, by either physical association or conjugation reactions

Table 3: Examples of studies of PLGA particles targeted to DCs.

Receptor	Formulation	Antigen / adjuvant	*In vitro / in vivo*	Administration route	Response compared to untargeted particles	References
Integrin, lectin & mannose	PLGA MPs (~2.5 µm) c.c. to RGD peptide; WGA; mannose-PEG3-NH2	-	*In vitro*	n/a	↑ uptake of targeted MPs	[117]
Integrin	PLGA NPs (~200 nm) c.c. to RGD peptide	OVA	*In vitro & in vivo*	oral	↑uptake by M cells & ↑ IgG responses *in vivo*	[116]
	PLGA MPs (~1 µm) with alginate or c.c. RGD-alginate	SPf66; S3	*In vivo*	i.d.	↑ Ab and cellular responses and more balanced Th1/Th2 responses; ↑ IFN-γ secretion and splenocyte proliferation	[118]
Mannose	Mannan c.c. to PLGA NPs (~400 nm)	OVA	*In vitro & in vivo*	s.c.	↑ antigen-specific $CD4^+$ and $CD8^+$ T cell responses *in vitro* and & vivo	[123]
	Mannan-coated on or c.c. to PLGA NPs (~400-500 nm)	-	*In vitro*	n/a	↑ DC uptake & cell surface markers (CD40, CD86) & secretion of inflammatory cytokines (IL-12, IL-6 and TNF-α)	[122, 120]
DC-SIGN	PLGA MPs (2 µm) and NPs (200 nm) c.c. to humanized hD1 anti-DC-SIGN antibody	BSA; TT	*In vitro*	n/a	MPs were taken up nonspecifically; NPs effectively targeted DCs: ↑ uptake & Ag-specific T cell responses at 10–100 fold lower concentrations	[15]
DEC-205	PLGA NPs (~200 nm) c.c. to bfFp containing anti-DEC-205 antibody fragment	OVA	*In vitro & in vivo*	s.c.	2-fold ↑ receptor-mediated uptake of bfFp functionalized NPs *in vitro*; ↑ OVA-specific IgG responses *in vivo*	[127]
DEC-205	PLGA NPs (~200-250) c.c. to anti-DEC-205 mAb	OVA / KRN	*In vitro & in vivo*	footpads	↑ antigen-specific humoral & CTL responses & promoted potent antitumor responses	[129]

DEC-205; CD40; CD11	PLGA NPs (200 nm) c.c. either with anti-DEC-205, -aCD40 or -CD11 mAbs	OVA / poly(I:C) & R848	*In vitro & in vivo*	s.c.	↑ uptake of targeted NPs & IL-12 production & expression of IFN-g *in vitro*; ↑ OVA-specific $CD8^+$ T cell responses *in vivo*	[14]
CD40	PLGA NPs (200 nm) c.c. with anti-aCD40 mAb	OVA; HPV-E7 / poly(I:C) & Pam3CSK4	*In vitro & in vivo*		↑ selective delivery to DCs & ↑ $CD8^+$ T cell priming *in vitro*; ↑ tumor control & prolonged survival of tumor-bearing mice *in vivo*	[17]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ≥: equal or higher than; ~: similar; ↑: increased/high: ↓: decreased/low; CFA: complete Freund's adjuvant; sX: soluble X; c.c.: chemically conjugated; bfFp: bifunctional fusion protein of strepatividin

[1, 5]. Physical association is driven by electrostatic and hydrophobic interactions, whereas preformed PLGA nanoparticles with carboxyl end groups can be chemically conjugated with molecules with terminal amine groups via amide coupling reactions using carbodiimide reagents [113]. To do that, the surface of PLGA is first derivatized by PEG-NH2 with functional end groups that can react with different ligands [113]. For instance, surface modification with biotin-PEG-NH2: as avidin and its homologues show very high affinity to biotin, biotinylated PEG-PLGA particles allow noncovalent binding with avidin-ligand conjugates or vice versa, allowing targeting ligands such as antibodies to be attached to PLGA particles using these methods [113]. Interaction between PLGA particles functionalized with specific ligands and/or antibodies against DC receptors may improve targeting to DCs, increase particle uptake by DCs through receptor-mediated endocytosis and modulate DC maturation, and thereby enhance the effectiveness of the vaccine formulation [10].

M-cell targeting can be considered if the vaccine is administered at a mucosal tissue [114, 115]. Integrins are heterodimeric transmembrane subunits that have specific affinities toward peptides with an arginine-glycine-aspartate (RGD) sequence [113]. Grafting of the integrin binding targeting peptide RGD can be used to promote the uptake of NPs via interaction with β1 integrins on M cells. Garinot et al. improved the efficiency of orally delivered vaccines in mice, using PEGylated OVA-loaded PLGA NPs displaying RGD molecules at their surface, which were able to elicit IgG responses *in vivo* [116]. Brandhonneur et al. studied the influence of target ligand-grafted PLGA MPs (~2.5 µm) on the rate of uptake by alveolar macrophages using three different ligands: WGA (lectin weat germ agglutinin, which interacts with lectin receptors), a RGD (arginine-glycine-aspartate) containing peptide (interacting on integrins), and mannose-PEG_3-NH_2 (interacting with mannose receptor) covalently coupled the particle surface using the carbodiimide method, showing that a much higher uptake was observed for targeted MPs because of the specific mechanism of phagocytosis. [117]. Mata et al. developed PLGA MPs (1 µm) containing a small percentage of alginate (PLGA-alg MP) or RGD-modified alginate (PLGA-alg-RGD MP). Two malaria synthetic peptides, SPf66 and S3, were microencapsulated into PLGA, PLGA-alg and PLGA-alg-RGD MP. Intradermal (i.d.) vaccination of mice demonstrated that incorporation of alginate elicited higher humoral and cellular immune responses leading to more balanced Th1/Th2 responses, and that the addition of RGD increased cell targeting, enhancing cellular responses such as IFN-γ secretion and slenocyte proliferation [118].

C-type lectin receptors (CLRs) are endocytic receptors that recognize exogenous and endogenous carbohydrates which are present on the surface of DCs and macrophages [113]. Antigens associated with specific sugar residues can target to these receptors on DCs, including the mannose receptor, DEC-205 (also known as CD205), and DC-specific intracellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN) [119]. Two main strategies can be used to target CLRs, either by grafting particles with specific sugar residues which are natural ligands to these endocytic receptors (e.g. sugars with terminal mannose, fucose or N-acetylglucosamine) or by coupling mAbs against them [120, 121]. Many CLRs expressed by DCs are directly implicated in immunoregulatory processes, such as antigen uptake, intracellular trafficking and antigen presentation [119].

PLGA particles decorated with mannan, a natural polymannose isolated from the cell wall of Saccharomyces cerevisiae, have been designed for targeted DC delivery via mannose receptors. Incorporation of mannan in NPs (~400 – 500 nm) made from capped and uncapped PLGA was achieved either through addition in first or second aqueous phases during NP preparation or by attachment onto the surface of freeze dried NPs by physical adsorption or chemical conjugation to COOH terminated polymer. PLGA NPs containing chemically conjugated or physically adsorbed mannan significantly enhanced DC uptake [122] and induced DC maturation, as evidenced by the up-regulation of cell surface markers (CD40, CD86) and secretion of inflammatory cytokines (IL-12, IL-6 and TNF-α) [120]. PLGA NPs (~400 nm) with chemically conjugated mannan showed increased $CD4^+$ and $CD8^+$ T cell immune response against encapsulated OVA in comparison to their untargeted counterparts [123]. In addition to being a targeting ligand, mannan is also reported to have immunostimulatory properties, possibly due to TLR agonism [123]. The type of sugar can determine which receptor is targeted and the immunological outcome. The nature of mannan has been shown to guide the type of immune response, with antigens conjugated to oxidized mannan inducing mostly cellular responses, whereas antigens coupled to reduced mannan induced humoral responses [124]. Oxidized mannan facilitates endosomal escape of the mannosylated antigen into the cytoplasm, resulting in a $CD8^+$ T cell response, whereas formulations with reduced mannan remain in the endosome, being degraded by the lysosomal enzymes, and resulting in a $CD4^+$ T cell response [125]. A drawback of targeting mannose receptors is the fact that they are also expressed by other cell types, such as monocytes, macrophages, and other subsets of other cells, which might result in unspecific uptake [47].

DEC-205 is a CLR that has been used to target DCs *in vivo*, that is mainly expressed on mature DCs, and also by B cells, T cells, monocytes, macrophages and natural killer (NK) cells at lower levels [121]. DEC-205 is a membrane protein which binds to carbohydrates and mediates endocytosis and antigen presentation and [47]. However, delivery of OVA conjugated to mAb targeting DEC-205 without additional maturation stimuli results in the induction of regulatory T cells and immune tolerance, whereas concomitant delivery of agonistic α-CD40 mAb with targeted OVA results in strong induction of OVA-specific $CD4^+$ and $CD8^+$ T-cell responses [126]. Raghuwanshi et al. prepared OVA-loaded PLGA NPs (~200 nm) for targeted delivery to dendritic cells [127]. First, NPs were prepared by coupling of biotin-PEG2000-amine to the carboxylic group of PLGA. Then, NPs were mixed with a recombinant bifunctional fusion protein (bfFp) of strepatividin and a single chain antibody fragment that recognizes mouse DEC-205 receptors. *In vitro* studies showed a two-fold increase in uptake of targeted NPs, and s.c. immunization of mice using the targeted NPs in combination with a DC maturation agent (anti-CD40 mAb) showed enhanced IgG titers against OVA. However, co-administration of anti-CD40 mAb was shown to be required as DEC-205 targeting in its absence leads to antigen-specific tolerance, as shown in previous studies [126, 127].

Invariant Natural Killer T (iNKT) can enhance cross-priming in a CD1d-dependent manner as they recognize (glyco)lipid antigens [128]. The most extensively studied

CD1d antigen is high-affinity α-galactosylceramide (α-GalCer), an immunostimulatory ligand that can elicit the secretion of a wide array of cytokines by iNKT cells upon recognition, enhancing DC activation and acting as a strong adjuvant for Th1 and CTL responses [27]. Targeted delivery of immunostimulant α-GalCer analogue KRN7000 and OVA by anti-DEC-205 conjugated NPs (~200 - 250 nm) triggered optimal antigen-specific humoral and CTL responses and promoted potent antitumor responses mediated by iNKT cells [129].

DC-SIGN is the most DC-specific CLR and is mainly expressed on immature DCs [121]. A recent study by Cruz et al. using BSA- or TT-loaded MPs (2 μm) and NPs (200 nm) consisting of a PLGA core coated with a PEG-lipid layer conjugated to the humanized anti-DC-SIGN targeting antibody hD1 demonstrated that NPs were targeted more efficiently than MPs to DCs, leading to improved antigen presentation by human DCs and activation of antigen-specific T-cell responses at 10–100 fold lower concentrations of antigen compared to the non-targeted NPs [15]. In another study, similar PLGA NPs (~200 - 250 nm) co-encapsulating OVA and TLRLs poly(I:C) and R848, were coupled to different mAbs against distinct DC surface molecules: DEC-205; TNF family receptor with known DC activating properties αCD40; and CD11c, an integrin receptor [14]. Targeted NPs were more efficiently internalized, and increased IL-12 production and expression of IFN-γ *in vitro* than their non-targeted counterparts, with CD40-targeted NPs performing slightly better than DEC-205 or CD11c targeted NPs. Upon s.c. vaccination, all targeted NP consistently showed higher efficacy than non-targeted NP to stimulate $CD8^+$ T cell responses, though no significant differences were observed between NPs targeted to different receptors [14]. In a subsequent study, s.c. injection of αCD40-targeted PLGA NPs (~200 - 250 nm) loaded with OVA or HPV-E7 protein and TLRLs poly(I:C) and Pam3CSK4 led to very efficient and selective delivery to DCs and efficient $CD8^+$ T cell priming. CD40-targeted NPs encapsulating HPV-E7 protein significantly enhanced antigen-specific $CD8^+$ T cells compared to non-targeted NPs for a mixture of soluble HPV-E7-protein and adjuvants. Therapeutic application of CD40-targeted NPs improved priming of $CD8^+$ T cells against the two independent tumor associated antigens and enhanced tumor control and prolonged survival of tumor-bearing mice, showing that targeting to specific DC receptors is an effective way to increase the efficacy of particulate vaccines [17].

4. Recent advances in PLGA particles for peptide-based vaccine delivery

Peptide-based vaccine formulations offer several advantages, as peptides can be easily synthesized and characterized in a highly reproducible manner in large scale and are generally more stable than whole proteins [7]. Furthermore, they may be especially valuable as anticancer vaccines, where the use of the whole protein is inadequate due to its similarity to endogenous human protein or carcinogenic properties; and the lack of redundant components significantly reduces the risk of allergic or autoimmune

responses [72]. 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 [7]. Currently available experimental adjuvants such as Montanide are associated with side effects, while commercially available adjuvants are ineffective at inducing cellular immunity [72]. Encapsulation of synthetic peptides in PLGA particles has been shown to promote cross-presentation, resulting in both humoral and cell-mediated immunity [10]. Different synthetic antigenic peptides have been successfully entrapped into PLGA particles resulting in increased immunogenicity of the peptide vaccines. A summary of peptide-based formulations is presented in **Table 4**.

Partidos et al. injected mice i.p. with the CTL epitope (LDRLVRLIG) representing the 51-59 residues from measles virus nucleoprotein (MVNP51-59) encapsulated in PLGA MPs, which elicited a higher specific cytotoxicity compared with results obtained with IFA [130]. However, simply mixing empty PLGA MPs with the peptide resulted in the induction of CTL responses comparable to those induced by the encapsulated peptide formulation, likely due to surface adsorption of the peptide to the particles [131]. PLGA NPs (~500 nm) containing HBcAg129–140 peptide and MPLA induced a strong Th1-type response with a predominant IFN-γ profile after a single s.c. immunization, which was enhanced after a booster immunization, while mice immunized with a CFA-peptide formulation showed a Th2 bias. Furthermore, mice primed with the CFA-peptide formulation still developed a strong Th1-type response when boosted with the NP formulation [132].

Peptides derived from malarial antigens have also been studied. In a study by Men et al., two synthetic peptides, P30B2 (composed of one universal T helper epitope from tetanus toxin P30 and a B cell epitope B2 derived from the repeat sequence of Plasmodium berghei) and (NANP)6P2P30 (composed of P30 and another T helper epitope from tetanus toxin P2 and a B cell epitope B2 derived from the repeat sequence of Plasmodium falciparum), were incorporated into MPs (1-20 μm) of different compositions eliciting strong and sustained proliferative and antibody responses comparable to those obtained with IFA-peptide preparations after s.c. immunization at the tail base [133]. Malarial peptide SPf66, the first chemically synthesized vaccine to elicit a partial protective immune response against malaria, when encapsulated in PLGA MPs (~1 μm) induced a superior immune response in comparison to alum-adjuvanted SPf66, and a similar response compared with the peptide formulated with Freund's complete adjuvant (FCA) [134]; leading to high antibody levels and protection against P. falciparum challenge when administered to Aoutus monkeys [135]. Another study by Mata et al. compared the immune response against the S3 malarial synthetic peptide injected s.c. using PLGA MPs (~3 μm), Montanide, and alum, with Montanide and MPs behaving comparably and resulting in mixed Th1/Th2 immune responses [136]. Prolonged antibody responses were also obtained by Mata et al. with two malarial peptides derived from the constant region of 3D7 and FC27 Plasmodium falciparum MSP2 dimorphic proteins encapsulated in PLGA MP formulations after s.c. or i.d. injections [137]. More recently, Mata et al. developed PLGA MPs (1 μm) containing a small percentage of alginate (PLGA-alg MP) or RGD-modified alginate (PLGA-alg-RGD MP) [118]. Malaria peptides SPf66

Table 4: Examples of PLGA formulations for peptide-based vaccine delivery.

Formulation	Antigen	Adjuvant	*In vitro* / *in vivo*	Administration route	Response	References
PLGA MPs (~1.6 µm)	$MVNP_{51-59}$	n/a	*In vivo*	i.p.	$MVNP_{51-59}$-MPs ↑ specific CTL responses against $MVNP_{51-59}$ & MV for 120 days > $sMVNP_{51-59}$ and in saline or in IFA; mixing empty PLGA MPs with $sMVNP_{51-59}$ ↑ CTL responses ~ $MVNP_{51-59}$-MPs	[130, 131]
PLGA NPs (~500 nm)	$HBcAg_{129–140}$	MPLA	*In vivo*	s.c.	$HBcAg_{129–140}$-NPs ↑ Th1-type response > $HBcAg_{129–140}$ in CFA ↑ Th2-type; mice primed with CFA- $HBcAg_{129–140}$ ↑ Th1-type response when boosted with $HBcAg_{129–140}$-NPs.	[132]
PLA and PLGA MPs (1-20 µm)	P30B2; (NANP)6P2P30	n/a	*In vivo*	s.c.	MPs ↑ Ab responses & specific isotype subclass Ab production ~ IFA-peptide preparations	[133]
PLGA MPs (~1 µm)	SPf66	n/a	*In vivo*	s.c.	SPf66-MPs ↑ immune response ~ SPf66-CFA > alum-SPf66, leading to ↑ Ab levels and protection against P. falciparum challenge in Aoutus monkeys	[134, 135]
PLGA MPs (~3 µm)	S3	n/a	*In vivo*	i.d.	S3-MPs ~ S3 in Montanide resulting in mixed Th1/Th2 immune responses; but < IFN-γ secretion than Montanide. MPs ↑ Ab levels & ↑ isotype IgG2a & IFN-γ levels.	[136]
PLGA MPs (~1 µm)	MSP2 3D7; MSP2 FC27	n/a	*In vivo*	s.c. or i.d.	MSP2 3D7- & FC27-MPs ↑ potent and long-lasting Ab responses; Montanide was effective against the 3D7 peptide but not FC27.	[137]
PLGA MPs (1 – 10 µm); PLGA NPs (500-900 nm);	OVA_{323-39} peptide; MUC1 mucin peptide	MPLA	*In vitro* & *in vivo*	s.c.	Ag/MPLA-MPs ↑ production of IFN-g, eliciting a specific Th1 immune response > Ag-MPs or Ag mixed with alum	[102, 103]
PLGA NPs (350 – 450 nm)	MUC1 lipopeptide (BLP25)	MPLA	*In vitro* & *in vivo*	n/a	MUC1/MPLA-NPs ↑ proliferation of MUC1 specific T-cells *in vitro* > MUC1-NPs, MPLA-NPs, sMUC1, or sMUC1 with MPLA-NPs; MUC1/MPLA-NPs broke tolerance to self-antigen MUC1 *in vivo* after adoptive transfer of NP-loaded DCs	[13]

PLGA NPs (~330 nm)	OVA24	n/a	*In vitro*	n/a	Low-burst release-NPs ↑ MHC class I Ag presentation *in vitro* > high-burst release-NPs > sOVA24	[37]
PLGA NPs (~300 nm)	OVA24	Pam3CSK4	*In vitro* & *in vivo*	s.c.	NPs ↑ MHC class I Ag presentation *in vitro*; ↑ prolonged Ag presentation & $CD8^+$ T cell activation *in vivo* after adoptive transfer of NP-loaded DCs	[111]
PLGA NPs (~350 nm)	OVA24	Poly(I:C) & pam3csk4	*In vitro* & *in vivo*	s.c.	↑ Ag uptake by DCs and activation of T cells *in vitro*; ↑ OVA-specific $CD8^+$ T cell proliferation *in vivo* ~ MF59 and Montanide	Varypataki et al., in prep.
pLHMGA NPs (~400 – 500 nm)	HPV $E7_{43-69}$	Poly(I:C)	*In vivo*	s.c.	HPV E743–69/Poly(I:C)-NPs or HPV E743–69-NPs with sPoly(I:C) ↑ HPV-specific $CD8^+$ T cells & prolonged the survival of mice in a therapeutic tumor setting ~ to IFA.	[143]
PLGA NPs (180-280 nm)	MART-1_{27-35}; $gp100_{209-217}$; $mSTEAP_{326-335}$	n/a	*In vitro*	n/a	Ag-NPs ↑ Ag presentation by DC pulsed with NPs & ↑ CTL activation > sAg or empty NPs	[144]
PLGA NPs (~80 nm)	$TRP2_{180-188}$	MPLA	*In vitro* & *in vivo*	i.d.	NP ↑ uptake *in vitro* & *in vivo*; TRP2180-188/MPLA-NPs ↑ CTL responses > TRP2180-188-CFA; TRP2180-188/MPLA-NPs ↓ growth of s.c. inoculated B16 melanoma cells in a prophylactic setting compared to TRP2180-188-NPs or sTRP2180-188 / sMPLA	[108]
PLGA NPs (350 – 410 nm)	$TRP2_{180-188}$ peptide	7-acyl lipid A	*In vitro* & *in vivo*	s.c.	TRP2180-188/7-acyl lipid A-NPs ↑ $CD8^+$ T cell-mediated anti-tumor immunity & therapeutic anti-tumor effect & levels of IFN-g and pro-inflammatory Th1-related cytokines > TRP2180-188-NPs	[107]
PLGA-PEG-PCL NPs in a 70:15:15 w/w ratio (~140-190 nm) grafted with mannose	Melan-A:26, gp100:209 or gp100:44	Poly(I:C), CpG, and mannose	*In vitro* & *in vivo*	s.c.	Ag/TLRLs-NPs ↑ IgG2c/IgG1 ratios & ↑ IFN-γ and IL-2; mannose-functionalization of NPs ↑ Th1 immune response; MHC class I- or class II-restricted melanoma Ag/TLRLs-mannose-NPs ↓ the growth rate of murine B16F10 melanoma tumors in therapeutic and prophylatic settings	[145]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ≥: equal or higher than; ~: similar/comparable; ↑: increased/high: ↓: decreased/low; CFA: complete Freund's adjuvant; sX: soluble X.

and S3 were microencapsulated into PLGA, PLGA-alg and PLGA-alg-RGD MP. Intradermal vaccination of mice demonstrated that incorporation of alginate elicited higher humoral and cellular immune responses leading to more balanced Th1/Th2 responses, and that the addition of RGD increased cell targeting, enhancing cellular responses such as IFN-γ secretion and splenocyte proliferation.

An extensively studied application for peptide-based vaccines is for immunotherapy of cancer by encapsulation of tumor-derived peptides in PLGA particles. Vaccination with synthetic long peptides (SLPs), containing the CTL and/or Th epitopes of a TAA, has shown superior efficacy to protein antigen [138] or minimal MHC class I restricted epitopes in mouse models [18, 139]. 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 the activation of both $CD4^+$ and $CD8^+$ T cells and eliciting a stronger cellular immune response [18, 140, 141].

Immunization of mice s.c. with PLGA MPs (1-10 μm) incorporating MPLA with an OVA peptide, consisting of residues 323–339 containing Th and B epitopes of OVA, resulted in an increase in the production of IFN-g, eliciting a specific Th1 immune response [102]. Ovalbumin peptide encapsulated in poly(d,l lactic-co-glycolic acid. Similar results were obtained with PLGA NPs (500-900 nm) loaded with cancer-associated 24mer human MUC1 mucin peptide [103]. Delivery of MUC1 mucin peptide by Poly(d,l-lactic-co-glycolic acid. Elamanchili et al. showed that co-delivery of MUC1 lipopeptide (BLP25, a cancer vaccine candidate) and MPLA loaded in PLGA NPs (340-450 nm) to human DCs significantly enhanced proliferation of antigen-specific T cells *in vitro* comparing to NP-MUC1, NP-MPLA, soluble MUC1, or soluble MUC1 mixed with NP-MPLA [13]. This combination of MUC1 and MPLA in PLGA NPs was shown to break tolerance and elicit strong T-cell responses against self-antigens *in vivo* after adoptive transfer of DCs loaded with the vaccine formulations [13, 142].

Our group has demonstrated that encapsulation of OVA24, a model 24-residue synthetic long peptide (SLP) antigen covering a CTL epitope of ovalbumin (SIINFEKL) in PLGA NPs of circa 330 nm, engineered to diplay a low burst release, showed enhanced MHC class I restricted T cell activation *in vitro* when compared to high-burst releasing NPs and soluble OVA24 [37]. Co-encapsulation of OVA24 with TLR2 ligand Pam3CSK4 in PLGA NPs enhanced MHC class I presentation by DCs *in vitro* and significantly enhanced prolonged antigen presentation and sustained $CD8^+$ T cell proliferation *in vivo* after adoptive transfer of PLGA NP-loaded DCs [111]. Moreover, recent studies comparing the co-delivery of two SLPs containing the CTL and Th epitopes of OVA (OVA24 and OVA17, respectively), and the TLRLs poly(I:C) and Pam3CSK4 in different delivery systems, showed that subcutaneous vaccination in mice with PLGA NPs resulted in enhanced OVA-specific $CD8^+$ T cell proliferation *in vivo* at least comparable to the formulations that are currently used in clinic, MF59 and Montanide (Varypataki et al., manuscript in preparation). NPs (~400 – 500 nm) based on hydrophilic polyester poly(D,L lactic-co-hydroxymethyl glycolic acid) (pLHMGA) were loaded with a 27-mer SLP containing the CTL epitope RAHYNIVTF of HPV E7 oncoprotein (E743–69) together with poly(I:C) and compared to E743–69 and poly(I:C) formulated in IFA [143]. Encapsulation of antigen substantially enhanced the population of HPV-specific $CD8^+$

T cells when combined with poly(I:C) either co-encapsulated or in its soluble form, and vaccination with NPs substantially prolonged the survival of mice (three weeks) in a therapeutic tumor setting, with a therapeutic efficacy in tumor eradication equivalent to that of the IFA formulation. Moreover, as opposed to IFA, subcutaneous administration of pLHMGA NPs was not associated with local adverse effects.

Some melanoma specific antigens that have been studied for cancer vaccines include MART-1, gp100, TRP1, and TRP2. Encapsulation of MHC class Ia, Ib, and class II-restricted peptide epitopes MART-1_{27-35}, $gp100_{209-217}$ and $mSTEAP_{326-335}$ in PLGA NPs (180-280 nm), resulted in a significantly enhanced antigen presentation of the encapsulated peptides upon internalization of the NPs by DCs *in vitro*, and confirmed that the improved peptide presentation is actually associated with more efficient generation of peptide-specific CTL and T helper cell responses [144].

Zhang et al. loaded PLGA NPs (~80 nm) with TAA peptides from melanoma ($hgp100_{25–33}$ or $TRP2_{180–188}$) observing that the NPs were efficiently uptaken by murine DCs *in vitro* and *in vivo* and induced stronger cellular immune responses than the peptides mixed with CFA [108]. Moreover, i.d. vaccination with PLGA-NP carrying both $TRP2_{180-188}$ and MPLA significantly delayed growth of s.c. inoculated B16-F10 melanoma tumor cells in a prophylactic setting compared to NPs with TRP2 alone, TRP2 peptide mixed with MPLA. Additionally, i.p. administration of IFN-γ from day 1 after the tumor inoculation was highly effective for controlling tumor growth in challenged mice.

Similarly, subcutaneous administration of co-encapsulated $TRP2_{180–188}$ and 7-acyl lipid A into PLGA NPs (350-410 nm) to B16-F10 tumor-bearing mice resulted in anti-tumor activity related with increased levels of IFN-γ and pro-inflammatory Th1-related cytokines compared to control groups [107].

Silva et al. studied the co-entrapment of melanoma-associated antigens and the TLRLs poly(I:C) and CpG, in mannose-functionalized aliphatic polyester-based NPs (constituted by a blend of PLGA, poly(D,L-lactic-co-glycolide-b-ethylene glycol) (PEG-b-PLGA) and poly(ε- caprolactone-b-ethylene glycol) (PEG-b-PCL) in a 70:15:15 w/w ratio) to be targeted to mannose receptors on antigen-presenting cells and induce anti-tumor immune responses [145]. The s.c. co-delivery of the model antigen OVA and the TLR ligands was crucial to induce high IgG2c/IgG1 ratios and high levels of IFN-γ and IL-2, while mannose-functionalization of PLGA-PEG-PCL NPs potentiated the Th1 immune response. The nanoparticulate vaccines decreased the growth rate of murine B16F10 melanoma tumors in therapeutic and prophylatic settings. The combination of mannose-functionalized NPs containing MHC class I- and class II-restricted melanoma antigens (Melan-A:26 and gp100:209) co-entrapped in the same mannose-NPs demonstrated to have a detrimental effect on the anti-tumor immune response, whereas the combination of mannose-NPs containing either a MHC class I- or class II-restricted antigens along with both TLRLs demonstrated the most potent anti-tumor immune response, suggesting the importance of the activation of both $CD4^+$ and $CD8^+$ T-cells.

Although there are still no PLGA-based particulate vaccines on the market [1], one has

reached clinical trial. A phase I clinical trial evaluated the safety and immunogenicity of a synthetic HIV peptide (HIV-1 MN V3) administered i.m. with alum or administered orally encapsulated into PLGA microspheres, unfortunately with poor results [5, 146]. Still, antigenic peptides incorporated into PLGA particulate carriers have demonstrated a considerable potential as prophylactic and therapeutic vaccines, and it is expected that more PLGA-based vaccines enter clinical trials in the near future.

5. Conclusions

Vaccination with subunit antigens is not always successful due to their limited bioavailability and poor immunogenicity. Moreover, soluble antigens are often inefficiently cross-presented. Delivery systems can be used in order to overcome these problems, by protecting antigens from degradation and increase their biodistribution and ability to reach and be uptaken by APCs.

Depending on their physicochemical characteristics, delivery systems can modulate the immune response, mainly due to direct influence in the following mechanisms: facilitated uptake by APCs, regulation of the internalization pathways and ability to endosomal escape, and interaction with specific receptors that mediate the immune response towards humoral or cellular bias. The main immunogenic properties of viruses that elicit potent immune responses may serve as a base for rational vaccine design [147].

Most studies are clear: size plays a crucial role in vaccine efficacy. Smaller particles tend to be more immunogenic due to their easier uptake by DCs and more efficient transport in the lymphatic system, where they can reach immature DC subsets; still, microparticles can form stable antigen depots and are more suitable for intranasal or inhalable pulmonary vaccination [1]. Recent studies have suggested that smaller particles mostly induce cellular immunity while larger particles tend to induce humoral responses [1, 36]. Other important factors include release kinetics; surface characteristics; concomitant delivery of antigen and immunostimulants, allowing DCs to associate danger signals with the antigen, while co-encapsulation of multiple TLRLs may result in a synergistic effect; coating or coupling of DC-specific targeting moieties, increasing DC uptake and enhancing antigen presentation to T cells. Future developments in vaccine delivery will likely involve the combination of immunostimulants with delivery vehicles modified with DC-specific targeting ligands/antibodies.

In summary, vaccines that mimic the size, charge, release kinetics and PAMPs of pathogens may be the future of peptide-based immunotherapy of cancer and/or other diseases that cannot be treated by conventional vaccines.

6. References

1. Leleux, J. and K. Roy, Micro and nanoparticle-based delivery systems for vaccine immunotherapy: an immunological and materials perspective. Adv Healthc Mater, 2013. 2(1): p. 72-94.
2. Bazin, H., A brief history of the prevention of infectious diseases by immunisations. Comp Immunol Microbiol Infect Dis, 2003. 26(5-6): p. 293-308.
3. Parrino, J. and B.S. Graham, Smallpox vaccines: Past, present, and future. J Allergy Clin Immunol, 2006. 118(6): p. 1320-6.
4. O'Hagan, D.T. and R. Rappuoli, Novel approaches to vaccine delivery. Pharm Res, 2004. 21(9): p. 1519-30.
5. Salvador, A., M. Igartua, R.M. Hernandez, and J.L. Pedraz, An overview on the field of micro- and nanotechnologies for synthetic Peptide-based vaccines. J Drug Deliv, 2011. 2011: p. 181646.
6. Seligman, S.J. and E.A. Gould, Live flavivirus vaccines: reasons for caution. Lancet, 2004. 363(9426): p. 2073-5.
7. Black, M., A. Trent, M. Tirrell, and C. Olive, Advances in the design and delivery of peptide subunit vaccines with a focus on toll-like receptor agonists. Expert Rev Vaccines, 2010. 9(2): p. 157-73.
8. Kotton, C.N., Vaccination and immunization against travel-related diseases in immunocompromised hosts. Expert Rev Vaccines, 2008. 7(5): p. 663-72.
9. Vandana, P. and P. Priyanka, Potential of Nanocarriers in Antigen Delivery: The Path to Successful Vaccine Delivery. Nanocarriers, 2014. 1(1): p. 10-45.
10. Hamdy, S., A. Haddadi, R.W. Hung, and A. Lavasanifar, Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. Adv Drug Deliv Rev, 2011. 63(10-11): p. 943-55.
11. Demento, S.L., A.L. Siefert, A. Bandyopadhyay, F.A. Sharp, and T.M. Fahmy, Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends Biotechnol, 2011. 29(6): p. 294-306.
12. Demento, S.L., W. Cui, J.M. Criscione, E. Stern, J. Tulipan, S.M. Kaech, and T.M. Fahmy, Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials, 2012. 33(19): p. 4957-64.
13. Elamanchili, P., C.M. Lutsiak, S. Hamdy, M. Diwan, and J. Samuel, "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. J Immunother, 2007. 30(4): p. 378-95.
14. Cruz, L.J., R.A. Rosalia, J.W. Kleinovink, F. Rueda, C.W. Lowik, and F. Ossendorp, Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8(+) T cell response: a comparative study. J Control Release, 2014. 192: p. 209-18.
15. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, R. Torensma, and C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN in vitro. J Control Release, 2010. 144(2): p. 118-26.
16. van den Boorn, J.G. and G. Hartmann, Turning tumors into vaccines: co-opting the innate immune system. Immunity, 2013. 39(1): p. 27-37.

17. Rosalia, R.A., L.J. Cruz, S. van Duikeren, A.T. Tromp, A.L. Silva, W. Jiskoot, T. de Gruijl, C. Lowik, J. Oostendorp, S.H. van der Burg, and F. Ossendorp, CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses. Biomaterials, 2015. 40: p. 88-97.
18. Zwaveling, S., S.C. Ferreira Mota, J. Nouta, M. Johnson, G.B. Lipford, R. Offringa, S.H. van der Burg, and C.J. Melief, Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol, 2002. 169(1): p. 350-8.
19. Shen, H., A.L. Ackerman, V. Cody, A. Giodini, E.R. Hinson, P. Cresswell, R.L. Edelson, W.M. Saltzman, and D.J. Hanlon, Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology, 2006. 117(1): p. 78-88.
20. Mundargi, R.C., V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J Control Release, 2008. 125(3): p. 193-209.
21. Singh, M., J. Kazzaz, M. Ugozzoli, J. Chesko, and D.T. O'Hagan, Charged polylactide co-glycolide microparticles as antigen delivery systems. Expert Opin Biol Ther, 2004. 4(4): p. 483-91.
22. Jain, R.A., The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials, 2000. 21(23): p. 2475-90.
23. Ignjatovic, N.L., Z.R. Ajdukovic, V.P. Savic, and D.P. Uskokovic, Size effect of calcium phosphate coated with poly-DL-lactide- co-glycolide on healing processes in bone reconstruction. J Biomed Mater Res Part B Appl Biomater, 2010. 94(1): p. 108-17.
24. Shive, M. and J. Anderson, Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev, 1997. 28(1): p. 5-24.
25. Astete, C.E. and C.M. Sabliov, Synthesis and characterization of PLGA nanoparticles. J Biomater Sci Polym Ed, 2006. 17(3): p. 247-89.
26. Mohammadi-Samani, S. and B. Taghipour, PLGA micro and nanoparticles in delivery of peptides and proteins; problems and approaches. Pharm Dev Technol, 2014.
27. Silva, J.M., M. Videira, R. Gaspar, V. Preat, and H.F. Florindo, Immune system targeting by biodegradable nanoparticles for cancer vaccines. J Control Release, 2013. 168(2): p. 179-99.
28. Sorkin, A. and M. Von Zastrow, Signal transduction and endocytosis: close encounters of many kinds. Nat Rev Mol Cell Biol, 2002. 3(8): p. 600-14.
29. Zolnik, B.S. and D.J. Burgess, Effect of acidic pH on PLGA microsphere degradation and release. J Control Release, 2007. 122(3): p. 338-44.
30. Yoo, J.Y., J.M. Kim, K.S. Seo, Y.K. Jeong, H.B. Lee, and G. Khang, Characterization of degradation behavior for PLGA in various pH condition by simple liquid chromatography method. Biomed Mater Eng, 2005. 15(4): p. 279-88.
31. Fahmy, T.M., S.L. Demento, M.J. Caplan, I. Mellman, and W.M. Saltzman, Design opportunities for actively targeted nanoparticle vaccines. Nanomedicine (Lond), 2008. 3(3): p. 343-55.

32. Jain, S., D.T. O'Hagan, and M. Singh, The long-term potential of biodegradable poly(lactide-co-glycolide) microparticles as the next-generation vaccine adjuvant. Expert Rev Vaccines, 2011. 10(12): p. 1731-42.
33. Samadi, N., A. Abbadessa, A. Di Stefano, C.F. van Nostrum, T. Vermonden, S. Rahimian, E.A. Teunissen, M.J. van Steenbergen, M. Amidi, and W.E. Hennink, The effect of lauryl capping group on protein release and degradation of poly(D,L-lactic-co-glycolic acid) particles. J Control Release, 2013. 172(2): p. 436-43.
34. Johansen, P., T. Storni, L. Rettig, Z. Qiu, A. Der-Sarkissian, K.A. Smith, V. Manolova, K.S. Lang, G. Senti, B. Mullhaupt, T. Gerlach, R.F. Speck, A. Bot, and T.M. Kundig, Antigen kinetics determines immune reactivity. Proc Natl Acad Sci U S A, 2008. 105(13): p. 5189-94.
35. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. AAPS J, 2013. 15(1): p. 85-94.
36. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
37. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient CD8(+) T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45.
38. Wang, G. and H. Uludag, Recent developments in nanoparticle-based drug delivery and targeting systems with emphasis on protein-based nanoparticles. Expert Opin Drug Deliv, 2008. 5(5): p. 499-515.
39. Link, A., F. Zabel, Y. Schnetzler, A. Titz, F. Brombacher, and M.F. Bachmann, Innate immunity mediates follicular transport of particulate but not soluble protein antigen. J Immunol, 2012. 188(8): p. 3724-33.
40. Manolova, V., A. Flace, M. Bauer, K. Schwarz, P. Saudan, and M.F. Bachmann, Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol, 2008. 38(5): p. 1404-13.
41. Simon, L.C. and C.M. Sabliov, The effect of nanoparticle properties, detection method, delivery route and animal model on poly(lactic-co-glycolic) acid nanoparticles biodistribution in mice and rats. Drug Metab Rev, 2013.
42. Fifis, T., A. Gamvrellis, B. Crimeen-Irwin, G.A. Pietersz, J. Li, P.L. Mottram, I.F. McKenzie, and M. Plebanski, Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol, 2004. 173(5): p. 3148-54.
43. Gutierro, I., R.M. Hernandez, M. Igartua, A.R. Gascon, and J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine, 2002. 21(1-2): p. 67-77.
44. Tran, K.K. and H. Shen, The role of phagosomal pH on the size-dependent efficiency of cross-presentation by dendritic cells. Biomaterials, 2009. 30(7): p. 1356-62.
45. Xiang, S.D., A. Scholzen, G. Minigo, C. David, V. Apostolopoulos, P.L. Mottram, and M. Plebanski, Pathogen recognition and development of particulate vaccines: does size matter? Methods, 2006. 40(1): p. 1-9.
46. Sharp, F.A., D. Ruane, B. Claass, E. Creagh, J. Harris, P. Malyala, M. Singh,

D.T. O'Hagan, V. Petrilli, J. Tschopp, L.A. O'Neill, and E.C. Lavelle, Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci U S A, 2009. 106(3): p. 870-5.
47. Joshi, M.D., W.J. Unger, G. Storm, Y. van Kooyk, and E. Mastrobattista, Targeting tumor antigens to dendritic cells using particulate carriers. J Control Release, 2012. 161(1): p. 25-37.
48. Silva, A.L., R.A. Rosalia, E. Varypataki, S. Sibuea, F. Ossendorp, and W. Jiskoot, Poly-(lactic-co-glycolic-acid)-based particulate vaccines: Particle uptake by dendritic cells is a key parameter for immune activation. Vaccine, (0).
49. Katare, Y.K., T. Muthukumaran, and A.K. Panda, Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles. Int J Pharm, 2005. 301(1-2): p. 149-60.
50. Thomas, C., V. Gupta, and F. Ahsan, Particle size influences the immune response produced by hepatitis B vaccine formulated in inhalable particles. Pharm Res, 2010. 27(5): p. 905-19.
51. Gutierro, I., R.M. Hernández, M. Igartua, A.R. Gascón, and J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine, 2002. 21(1-2): p. 67-77.
52. Kanchan, V. and A.K. Panda, Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. Biomaterials, 2007. 28(35): p. 5344-57.
53. Raghuvanshi, R.S., Y.K. Katare, K. Lalwani, M.M. Ali, O. Singh, and A.K. Panda, Improved immune response from biodegradable polymer particles entrapping tetanus toxoid by use of different immunization protocol and adjuvants. Int J Pharm, 2002. 245(1-2): p. 109-21.
54. Lee, Y.R., Y.H. Lee, S.A. Im, K. Kim, and C.K. Lee, Formulation and Characterization of Antigen-loaded PLGA Nanoparticles for Efficient Cross-priming of the Antigen. Immune Netw, 2011. 11(3): p. 163-8.
55. Aguado, M.T., Future approaches to vaccine development: single-dose vaccines using controlled-release delivery systems. Vaccine, 1993. 11(5): p. 596-7.
56. Preis, I. and R.S. Langer, A single-step immunization by sustained antigen release. J Immunol Methods, 1979. 28(1-2): p. 193-7.
57. Correia-Pinto, J.F., N. Csaba, and M.J. Alonso, Vaccine delivery carriers: insights and future perspectives. Int J Pharm, 2013. 440(1): p. 27-38.
58. Dailey…, L., New poly (lactic-co-glycolic acid) derivatives: Modular polymers with tailored properties. Drug Discovery Today: Technologies, 2005.
59. Yeo, Y. and K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res, 2004. 27(1): p. 1-12.
60. Sah, H., R. Toddywala, and Y.W. Chien, Continuous release of proteins from biodegradable microcapsules and in vivo evaluation of their potential as a vaccine adjuvant. Journal of Controlled Release, 1995. 35(2–3): p. 137-144.
61. Sah, H., R. Toddywala, and Y.W. Chien, The influence of biodegradable microcapsule formulations on the controlled release of a protein. Journal of Controlled Release, 1994. 30(3): p. 201-211.
62. Panyam, J., M.M. Dali, S.K. Sahoo, W. Ma, S.S. Chakravarthi, G.L. Amidon, R.J. Levy, and V. Labhasetwar, Polymer degradation and in vitro release of

a model protein from poly(d,l-lactide-co-glycolide) nano- and microparticles. Journal of Controlled Release, 2003. 92(1–2): p. 173-187.
63. Leelarasamee, N., S.A. Howard, C.J. Malanga, L.A. Luzzi, T.F. Hogan, S.J. Kandzari, and J.K. Ma, Kinetics of drug release from polylactic acid-hydrocortisone microcapsules. J Microencapsul, 1986. 3(3): p. 171-9.
64. Zhang, Y., S. Zale, L. Sawyer, and H. Bernstein, Effects of metal salts on poly(DL-lactide-co-glycolide) polymer hydrolysis. J Biomed Mater Res, 1997. 34(4): p. 531-8.
65. Mansour, H.M., M. Sohn, A. Al-Ghananeem, and P.P. Deluca, Materials for pharmaceutical dosage forms: molecular pharmaceutics and controlled release drug delivery aspects. Int J Mol Sci, 2010. 11(9): p. 3298-322.
66. Govender, T., S. Stolnik, M.C. Garnett, L. Illum, and S.S. Davis, PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Control Release, 1999. 57(2): p. 171-85.
67. Hailemichael, Y., Z. Dai, N. Jaffarzad, Y. Ye, M.A. Medina, X.F. Huang, S.M. Dorta-Estremera, N.R. Greeley, G. Nitti, W. Peng, C. Liu, Y. Lou, Z. Wang, W. Ma, B. Rabinovich, R.T. Sowell, K.S. Schluns, R.E. Davis, P. Hwu, and W.W. Overwijk, Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat Med, 2013. 19(4): p. 465-72.
68. Kanchan, V., Y.K. Katare, and A.K. Panda, Memory antibody response from antigen loaded polymer particles and the effect of antigen release kinetics. Biomaterials, 2009. 30(27): p. 4763-76.
69. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, H.P. Merkle, and M. Groettrup, Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine, 2006. 24(11): p. 1847-57.
70. Zhang, W., L. Wang, Y. Liu, X. Chen, Q. Liu, J. Jia, T. Yang, S. Qiu, and G. Ma, Immune responses to vaccines involving a combined antigen–nanoparticle mixture and nanoparticle-encapsulated antigen formulation. Biomaterials, 2014. 35(23): p. 6086-6097.
71. Thomas, C., V. Gupta, and F. Ahsan, Influence of surface charge of PLGA particles of recombinant hepatitis B surface antigen in enhancing systemic and mucosal immune responses. Int J Pharm, 2009. 379(1): p. 41-50.
72. Skwarczynski, M. and I. Toth, Recent advances in peptide-based subunit nanovaccines. Nanomedicine (Lond), 2014. 9(17): p. 2657-69.
73. Fischer, S., E. Uetz-von Allmen, Y. Waeckerle-Men, M. Groettrup, H.P. Merkle, and B. Gander, The preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles. Biomaterials, 2007. 28(6): p. 994-1004.
74. Wischke, C., H.H. Borchert, J. Zimmermann, I. Siebenbrodt, and D.R. Lorenzen, Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. J Control Release, 2006. 114(3): p. 359-68.
75. Jaganathan, K.S. and S.P. Vyas, Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally. Vaccine, 2006. 24(19): p. 4201-

11.
76. Slutter, B., S. Bal, C. Keijzer, R. Mallants, N. Hagenaars, I. Que, E. Kaijzel, W. van Eden, P. Augustijns, C. Lowik, J. Bouwstra, F. Broere, and W. Jiskoot, Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine, 2010. 28(38): p. 6282-91.
77. Martinez Gomez, J.M., N. Csaba, S. Fischer, A. Sichelstiel, T.M. Kundig, B. Gander, and P. Johansen, Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis. J Control Release, 2008. 130(2): p. 161-7.
78. Martinez Gomez, J.M., S. Fischer, N. Csaba, T.M. Kundig, H.P. Merkle, B. Gander, and P. Johansen, A protective allergy vaccine based on CpG- and protamine-containing PLGA microparticles. Pharm Res, 2007. 24(10): p. 1927-35.
79. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander, Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. Journal of drug targeting, 2009. 17(8): p. 652-61.
80. Schwendeman, S.P., Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems. Crit Rev Ther Drug Carrier Syst, 2002. 19(1): p. 73-98.
81. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
82. Ghassemi, A.H., M.J. van Steenbergen, A. Barendregt, H. Talsma, R.J. Kok, C.F. van Nostrum, D.J. Crommelin, and W.E. Hennink, Controlled release of octreotide and assessment of peptide acylation from poly(D,L-lactide-co-hydroxymethyl glycolide) compared to PLGA microspheres. Pharm Res, 2012. 29(1): p. 110-20.
83. Audran, R., Y. Men, P. Johansen, B. Gander, and G. Corradin, Enhanced immunogenicity of microencapsulated tetanus toxoid with stabilizing agents. Pharm Res, 1998. 15(7): p. 1111-6.
84. Zhu, G. and S.P. Schwendeman, Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives. Pharm Res, 2000. 17(3): p. 351-7.
85. Zhu, G., S.R. Mallery, and S.P. Schwendeman, Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide). Nat Biotechnol, 2000. 18(1): p. 52-7.
86. Singh, M., J. Kazzaz, M. Ugozzoli, P. Malyala, J. Chesko, and D.T. O'Hagan, Polylactide-co-glycolide microparticles with surface adsorbed antigens as vaccine delivery systems. Curr Drug Deliv, 2006. 3(1): p. 115-20.
87. Saini, V., V. Jain, M.S. Sudheesh, K.S. Jaganathan, P.K. Murthy, and D.V. Kohli, Comparison of humoral and cell-mediated immune responses to cationic PLGA microspheres containing recombinant hepatitis B antigen. Int J Pharm, 2011. 408(1-2): p. 50-7.
88. Kazzaz, J., J. Neidleman, M. Singh, G. Ott, and D.T. O'Hagan, Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes

against recombinant p55 gag from HIV-1. Journal of controlled release : official journal of the Controlled Release Society, 2000. 67(2-3): p. 347-56.
89. O'Hagan, D.T., M. Singh, J. Kazzaz, M. Ugozzoli, M. Briones, J. Donnelly, and G. Ott, Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen. Vaccine, 2002. 20(27-28): p. 3389-98.
90. Singh, M., J. Kazzaz, J. Chesko, E. Soenawan, M. Ugozzoli, M. Giuliani, M. Pizza, R. Rappouli, and D.T. O'Hagan, Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. J Pharm Sci, 2004. 93(2): p. 273-82.
91. Singh, M., J. Chesko, J. Kazzaz, M. Ugozzoli, E. Kan, I. Srivastava, and D.T. O'Hagan, Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. Pharm Res, 2004. 21(12): p. 2148-52.
92. Chesko, J., J. Kazzaz, M. Ugozzoli, M. Singh, D.T. O'Hagan, C. Madden, M. Perkins, and N. Patel, Characterization of antigens adsorbed to anionic PLG microparticles by XPS and TOF-SIMS. J Pharm Sci, 2008. 97(4): p. 1443-53.
93. De Temmerman, M.L., J. Rejman, J. Demeester, D.J. Irvine, B. Gander, and S.C. De Smedt, Particulate vaccines: on the quest for optimal delivery and immune response. Drug Discov Today, 2011. 16(13-14): p. 569-82.
94. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup, TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, 2008. 26(13): p. 1626-37.
95. Diwan, M., M. Tafaghodi, and J. Samuel, Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres. Journal of controlled release : official journal of the Controlled Release Society, 2002. 85(1-3): p. 247-62.
96. San Román, B., J.M. Irache, S. Gómez, N. Tsapis, C. Gamazo, and M.S. Espuelas, Co-encapsulation of an antigen and CpG oligonucleotides into PLGA microparticles by TROMS technology. Eur J Pharm Biopharm, 2008. 70(1): p. 98-108.
97. Heit, A., F. Schmitz, M. O'Keeffe, C. Staib, D.H. Busch, H. Wagner, and K.M. Huster, Protective CD8 T cell immunity triggered by CpG-protein conjugates competes with the efficacy of live vaccines. J Immunol, 2005. 174(7): p. 4373-80.
98. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner, Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. Eur J Immunol, 2007. 37(8): p. 2063-74.
99. Lee, Y.R., Y.H. Lee, S.A. Im, I.H. Yang, G.W. Ahn, K. Kim, and C.K. Lee, Biodegradable nanoparticles containing TLR3 or TLR9 agonists together with antigen enhance MHC-restricted presentation of the antigen. Arch Pharm Res, 2010. 33(11): p. 1859-66.
100. Wischke, C., J. Zimmermann, B. Wessinger, A. Schendler, H.-H. Borchert, J.H. Peters, T. Nesselhut, and D.R. Lorenzen, Poly(I:C) coated PLGA microparticles induce dendritic cell maturation. Int J Pharm, 2009. 365(1-2): p. 61-8.

101. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. Int J Cancer, 2011. 129(2): p. 407-16.
102. Newman, K.D., J. Samuel, and G. Kwon, Ovalbumin peptide encapsulated in poly(d,l lactic-co-glycolic acid) microspheres is capable of inducing a T helper type 1 immune response. J Control Release, 1998. 54(1): p. 49-59.
103. Newman, K.D., D.L. Sosnowski, G.S. Kwon, and J. Samuel, Delivery of MUC1 mucin peptide by Poly(d,l-lactic-co-glycolic acid) microspheres induces type 1 T helper immune responses. J Pharm Sci, 1998. 87(11): p. 1421-7.
104. Hamdy, S., P. Elamanchili, A. Alshamsan, O. Molavi, T. Satou, and J. Samuel, Enhanced antigen-specific primary $CD4^+$ and $CD8^+$ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles. Journal of biomedical materials research Part A, 2007. 81(3): p. 652-62.
105. Chong, C.S.W., M. Cao, W.W. Wong, K.P. Fischer, W.R. Addison, G.S. Kwon, D.L. Tyrrell, and J. Samuel, Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery. Journal of controlled release : official journal of the Controlled Release Society, 2005. 102(1): p. 85-99.
106. Lutsiak, M.E.C., G.S. Kwon, and J. Samuel, Biodegradable nanoparticle delivery of a Th2-biased peptide for induction of Th1 immune responses. J Pharm Pharmacol, 2006. 58(6): p. 739-47.
107. Hamdy, S., O. Molavi, Z. Ma, A. Haddadi, A. Alshamsan, Z. Gobti, S. Elhasi, J. Samuel, and A. Lavasanifar, Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent $CD8^+$ T cell-mediated anti-tumor immunity. Vaccine, 2008. 26(39): p. 5046-57.
108. Zhang, Z., S. Tongchusak, Y. Mizukami, Y.J. Kang, T. Ioji, M. Touma, B. Reinhold, D.B. Keskin, E.L. Reinherz, and T. Sasada, Induction of anti-tumor cytotoxic T cell responses through PLGA-nanoparticle mediated antigen delivery. Biomaterials, 2011. 32(14): p. 3666-78.
109. Kazzaz, J., M. Singh, M. Ugozzoli, J. Chesko, E. Soenawan, and D.T. O'Hagan, Encapsulation of the immune potentiators MPL and RC529 in PLG microparticles enhances their potency. J Control Release, 2006. 110(3): p. 566-73.
110. Nixon, D.F., C. Hioe, P.D. Chen, Z. Bian, P. Kuebler, M.L. Li, H. Qiu, X.M. Li, M. Singh, J. Richardson, P. McGee, T. Zamb, W. Koff, C.Y. Wang, and D. O'Hagan, Synthetic peptides entrapped in microparticles can elicit cytotoxic T cell activity. Vaccine, 1996. 14(16): p. 1523-30.
111. Rosalia, R.A., Co-encapsulation of synthetic long peptide antigen and Toll like receptor 2 ligand in poly-(lactic-co-glycolic-acid) particles results in sustained MHC class I cross-presentation by dendritic cells, in Particulate based vaccines for cancer immunotherapy, F.o.M.L.U.M.C.L. Department of Immunohematology, Leiden University, Editor. 2014, Leiden University. p. 95-121.
112. Singh, M., J. Kazzaz, M. Ugozzoli, P. Malyala, J. Chesko, and D.T. O'Hagan, Polylactide-co-glycolide microparticles with surface adsorbed antigens as vaccine delivery systems. Current drug delivery, 2006. 3(1): p. 115-20.

113. Sah, H., L.A. Thoma, H.R. Desu, E. Sah, and G.C. Wood, Concepts and practices used to develop functional PLGA-based nanoparticulate systems. Int J Nanomedicine, 2013. 8: p. 747-65.
114. Rajapaksa, T.E., M. Stover-Hamer, X. Fernandez, H.A. Eckelhoefer, and D.D. Lo, Claudin 4-targeted protein incorporated into PLGA nanoparticles can mediate M cell targeted delivery. Journal of controlled release : official journal of the Controlled Release Society, 2010. 142(2): p. 196-205.
115. Gupta, P.N., K. Khatri, A.K. Goyal, N. Mishra, and S.P. Vyas, M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. Journal of drug targeting, 2007. 15(10): p. 701-13.
116. Garinot, M., V. Fievez, V. Pourcelle, F. Stoffelbach, A. des Rieux, L. Plapied, I. Theate, H. Freichels, C. Jerome, J. Marchand-Brynaert, Y.J. Schneider, and V. Preat, PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. J Control Release, 2007. 120(3): p. 195-204.
117. Brandhonneur, N., F. Chevanne, V. Vie, B. Frisch, R. Primault, M.F. Le Potier, and P. Le Corre, Specific and non-specific phagocytosis of ligand-grafted PLGA microspheres by macrophages. Eur J Pharm Sci, 2009. 36(4-5): p. 474-85.
118. Mata, E., M. Igartua, M.E. Patarroyo, J.L. Pedraz, and R.M. Hernandez, Enhancing immunogenicity to PLGA microparticulate systems by incorporation of alginate and RGD-modified alginate. Eur J Pharm Sci, 2011. 44(1-2): p. 32-40.
119. Tacken, P.J., R. Torensma, and C.G. Figdor, Targeting antigens to dendritic cells in vivo. Immunobiology, 2006. 211(6-8): p. 599-608.
120. Haddadi, A., S. Hamdy, Z. Ghotbi, J. Samuel, and A. Lavasanifar, Immunoadjuvant activity of the nanoparticles' surface modified with mannan. Nanotechnology, 2014. 25(35): p. 355101.
121. Tacken, P.J., I.J. de Vries, R. Torensma, and C.G. Figdor, Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. Nat Rev Immunol, 2007. 7(10): p. 790-802.
122. Ghotbi, Z., A. Haddadi, S. Hamdy, R.W. Hung, J. Samuel, and A. Lavasanifar, Active targeting of dendritic cells with mannan-decorated PLGA nanoparticles. Journal of drug targeting, 2010.
123. Hamdy, S., A. Haddadi, A. Shayeganpour, J. Samuel, and A. Lavasanifar, Activation of antigen-specific T cell-responses by mannan-decorated PLGA nanoparticles. Pharm Res, 2011. 28(9): p. 2288-301.
124. Apostolopoulos, V., G.A. Pietersz, B.E. Loveland, M.S. Sandrin, and I.F. McKenzie, Oxidative/reductive conjugation of mannan to antigen selects for T1 or T2 immune responses. Proc Natl Acad Sci U S A, 1995. 92(22): p. 10128-32.
125. Apostolopoulos, V., G.A. Pietersz, S. Gordon, L. Martinez-Pomares, and I.F. McKenzie, Aldehyde-mannan antigen complexes target the MHC class I antigen-presentation pathway. Eur J Immunol, 2000. 30(6): p. 1714-23.
126. Bonifaz, L.C., D.P. Bonnyay, A. Charalambous, D.I. Darguste, S. Fujii, H. Soares, M.K. Brimnes, B. Moltedo, T.M. Moran, and R.M. Steinman, In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med, 2004. 199(6): p. 815-24.

127. Raghuwanshi, D., V. Mishra, M.R. Suresh, and K. Kaur, A simple approach for enhanced immune response using engineered dendritic cell targeted nanoparticles. Vaccine, 2012. 30(50): p. 7292-9.
128. Macho Fernandez, E., J. Chang, J. Fontaine, E. Bialecki, F. Rodriguez, E. Werkmeister, V. Krieger, C. Ehret, B. Heurtault, S. Fournel, B. Frisch, D. Betbeder, C. Faveeuw, and F. Trottein, Activation of invariant Natural Killer T lymphocytes in response to the alpha-galactosylceramide analogue KRN7000 encapsulated in PLGA-based nanoparticles and microparticles. Int J Pharm, 2012. 423(1): p. 45-54.
129. Macho-Fernandez, E., L.J. Cruz, R. Ghinnagow, J. Fontaine, E. Bialecki, B. Frisch, F. Trottein, and C. Faveeuw, Targeted delivery of alpha-galactosylceramide to CD8alpha$^+$ dendritic cells optimizes type I NKT cell-based antitumor responses. J Immunol, 2014. 193(2): p. 961-9.
130. Partidos, C.D., P. Vohra, C. Anagnostopoulou, D.H. Jones, G.H. Farrar, and M.W. Steward, Biodegradable microparticles as a delivery system for measles virus cytotoxic T cell epitopes. Mol Immunol, 1996. 33(6): p. 485-91.
131. Partidos, C.D., P. Vohra, D. Jones, G. Farrar, and M.W. Steward, CTL responses induced by a single immunization with peptide encapsulated in biodegradable microparticles. J Immunol Methods, 1997. 206(1-2): p. 143-51.
132. Lutsiak, M.E., G.S. Kwon, and J. Samuel, Biodegradable nanoparticle delivery of a Th2-biased peptide for induction of Th1 immune responses. J Pharm Pharmacol, 2006. 58(6): p. 739-47.
133. Men, Y., B. Gander, H.P. Merkle, and G. Corradin, Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres. Vaccine, 1996. 14(15): p. 1442-50.
134. Rosas, J.E., R.M. Hernandez, A.R. Gascon, M. Igartua, F. Guzman, M.E. Patarroyo, and J.L. Pedraz, Biodegradable PLGA microspheres as a delivery system for malaria synthetic peptide SPf66. Vaccine, 2001. 19(31): p. 4445-51.
135. Rosas, J.E., J.L. Pedraz, R.M. Hernandez, A.R. Gascon, M. Igartua, F. Guzman, R. Rodriguez, J. Cortes, and M.E. Patarroyo, Remarkably high antibody levels and protection against P. falciparum malaria in Aotus monkeys after a single immunisation of SPf66 encapsulated in PLGA microspheres. Vaccine, 2002. 20(13-14): p. 1707-10.
136. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug Deliv, 2010. 17(7): p. 490-9.
137. Mata, E., A.M. Carcaboso, R.M. Hernandez, M. Igartua, G. Corradin, and J.L. Pedraz, Adjuvant activity of polymer microparticles and Montanide ISA 720 on immune responses to Plasmodium falciparum MSP2 long synthetic peptides in mice. Vaccine, 2007. 25(5): p. 877-85.
138. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, A.L. Silva, W. Jiskoot, T. van Hall, P.A. van Veelen, G. Janssen, K. Franken, L.J. Cruz, A. Tromp, J. Oostendorp, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Dendritic cells process synthetic long peptides better than whole

protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65.
139. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, S.H. van der Burg, and R. Offringa, Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation. Eur J Immunol, 2008. 38(4): p. 1033-42.
140. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C.J. Melief, Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med, 1998. 187(5): p. 693-702.
141. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, R. Offringa, and S.H. van der Burg, $CD8^+$ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. J Immunol, 2007. 179(8): p. 5033-40.
142. Elamanchili, P., M. Diwan, M. Cao, and J. Samuel, Characterization of poly(D,L-lactic-co-glycolic acid) based nanoparticulate system for enhanced delivery of antigens to dendritic cells. Vaccine, 2004. 22(19): p. 2406-12.
143. Rahimian, S., M.F. Fransen, J.W. Kleinovink, J.R. Christensen, M. Amidi, W.E. Hennink, and F. Ossendorp, Polymeric nanoparticles for co-delivery of synthetic long peptide antigen and poly IC as therapeutic cancer vaccine formulation. J Control Release, 2015. 203: p. 16-22.
144. Ma, W., T. Smith, V. Bogin, Y. Zhang, C. Ozkan, M. Ozkan, M. Hayden, S. Schroter, E. Carrier, D. Messmer, V. Kumar, and B. Minev, Enhanced presentation of MHC class Ia, Ib and class II-restricted peptides encapsulated in biodegradable nanoparticles: a promising strategy for tumor immunotherapy. J Transl Med, 2011. 9: p. 34.
145. Silva, J.M., E. Zupancic, G. Vandermeulen, V.G. Oliveira, A. Salgado, M. Videira, M. Gaspar, L. Graca, V. Preat, and H.F. Florindo, In vivo delivery of peptides and Toll-like receptor ligands by mannose-functionalized polymeric nanoparticles induces prophylactic and therapeutic anti-tumor immune responses in a melanoma model. J Control Release, 2015. 198: p. 91-103.
146. Lambert, J.S., M. Keefer, M.J. Mulligan, D. Schwartz, J. Mestecky, K. Weinhold, C. Smith, R. Hsieh, Z. Moldoveanu, P. Fast, B. Forrest, and W. Koff, A Phase I safety and immunogenicity trial of UBI microparticulate monovalent HIV-1 MN oral peptide immunogen with parenteral boost in HIV-1 seronegative human subjects. Vaccine, 2001. 19(23-24): p. 3033-42.
147. Bachmann, M.F. and G.T. Jennings, Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol, 2010. 10(11): p. 787-96.

Chapter 3

Poly-(lactic-co-glycolic-acid)-based particulate vaccines: particle uptake by dendritic cells is a key parameter for immune activation

Ana Luisa Silva*, Rodney A. Rosalia*, Eleni M. Varypataki, Shanti Sibuea, Ferry Ossendorp, Wim Jiskoot

*contributed equally to this study

Vaccine 2015 33(7):847-54

Abstract

Poly(lactic-co-glycolic acid) (PLGA) particles have been extensively studied as biodegradable delivery system to improve the potency and safety of protein-based vaccines. In this study we analyzed how the size of PLGA particles, and hence their ability to be engulfed by dendritic cells (DC), affects the type and magnitude of the immune response in comparison to sustained release from a local depot. PLGA microparticles (MP, volume mean diameter ≈ 112 µm) and nanoparticles (NP, Z-average diameter ≈ 350 nm) co-encapsulating ovalbumin (OVA) and poly(I:C), with comparable antigen (Ag) release characteristics, were prepared and characterized. The immunogenicity of these two distinct particulate vaccines was evaluated *in vitro* and *in vivo*. NP were efficiently taken up by DC and greatly facilitated MHC I Ag presentation *in vitro*, whereas DC cultured in the presence of MP failed to internalize significant amounts of Ag and hardly showed MHC I Ag presentation. Vaccination of mice with NP resulted in significantly better priming of Ag-specific $CD8^+$ T cells compared to MP and OVA emulsified with incomplete Freund's adjuvant (IFA). Moreover, NP induced a balanced TH1/TH2-type antibody response, compared to vaccinations with IFA which stimulated a predominant TH2-type response, whereas MP failed to increase antibody titers. In conclusion, we postulate that particle internalization is of crucial importance and therefore particulate vaccines should be formulated in the nano- but not micro-size range to achieve efficient uptake, significant MHC class I cross-presentation and effective T and B cell responses.

Keywords: Protein antigen, PLGA, microparticles, nanoparticles, adjuvants, immunotherapy, cellular immune response

1. Introduction

In the past few years, extensive efforts in the immunotherapy field have led to the development of several therapeutic vaccine strategies [1-3]. Protein vaccines are popular forms of therapeutic vaccines [3, 4] which have been tested successfully in (pre-) clinical studies against various immunological diseases [5, 6]. The potency of protein vaccines can be significantly amplified via the encapsulation in biodegradable particles. The use of particles facilitates the uptake of the antigen (Ag) by dendritic cells (DC), allows the co-delivery of Ag and Toll-like receptor ligands (TLRL) [7, 8] and improves Ag processing, presentation and T cell priming by DC compared to use of soluble Ag. Generally, particulate Ag is better routed into MHC class I cross-presentation pathways and preserved inside intracellular compartments, resulting in sustained and efficient priming of $CD8^+$ T cell responses [9-13]. DC have superior capacity to cross-present exogenous Ag in MHC I molecules and are considered the major target for vaccines aimed at activating a robust $CD8^+$ T cell mediated immunity [3, 14]

Most clinical trials for cancer immunotherapy have relied on the use of Montanide, a GMP-grade version of incomplete Freund's adjuvant (IFA), which is a water-in-oil (w/o) emulsion for Ag delivery. The immune-activating properties of Montanide are partially

explained through the formation of a local Ag depot and the onset of inflammation, which attracts immune cells towards the site of injection [15] where the Ag is taken up primarily in its soluble form [16]. However, the use of Montanide is associated with significant local adverse effects [5], reason why there is an urgent need for alternatives [17].

Nanoparticles (NP) and microparticles (MP) prepared from biodegradable poly(L-lactic-co-glycolic acid) (PLGA) have been studied extensively for the sustained delivery of proteins and therapeutic agents and as an potential alternative to w/o emulsions [18-20]. Plain PLGA particles have sub-optimal adjuvant properties *in vivo* resulting in poor DC maturation [9, 21], which can be overcome by the inclusion of TLRL, leading to an efficient induction of TH1-mediated T cell responses with the capacity to control tumors or protect against a viral challenge [7, 22-25].

It is generally assumed that NP, compared to MP, are better for targeted drug delivery due to a better biodistribution [26, 27] and ability to cross biological barriers [28]. Still, there is little agreement when it comes to therapeutic vaccines: which size leads to the most efficient MHC class I Ag cross-presentation remains controversial [29-31].

To study the importance of particle uptake for the induction of an immune response, we developed NP and MP containing equivalent amounts of Ag and TLRL with comparable release profiles *in vitro*. Particles co-encapsulating model Ag ovalbumin (OVA) and TLR3L poly(I:C) were formulated to obtain NP that will be efficiently internalized by DC [32], releasing the Ag mostly intracellularly [12], versus MP with a size (> 20 μm) that is too large to be taken up by DC [31], thus functioning exclusively as a local Ag/TLR3L depot under the skin, similarly to Montanide.

2. Materials and Methods

2.1. Reagents

PLGA Resomer RG502H (50:50 MW 5,000–15,000 Da) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany); PLGA Resomer RG752H (75:25 MW 4,000-15,000 Da), Dichloromethane (DCM), dimethyl sulfoxide (DMSO), and Hepes from Sigma–Aldrich (Steinheim, Germany); Ovalbumin (OVA) grade V, 44 kDa from Worthington (New Jersey, USA); Alexa Fluor 488 (AF488) labeled OVA from Invitrogen (Merelbeke, Belgium); Tween 20 from Merck Schuchardt (Hohenbrunn, Germany); polyvinyl alcohol (PVA) 4 – 88 (31 kDa) from Fluka (Steinheim, Germany); Poly(I:C) LMW and rhodamine labeled poly(I:C) from InvivoGen (San Diego, USA); Phosphate-buffered saline (NaCl 8.2 g/L; Na2HPO4.12 H2O 3.1 g/L; NaH2PO4.2H2O 0.3 g/L) (PBS) from B. Braun (Melsungen, Germany); all fluorescently labeled antibodies from BD Pharmingen (San Diego, USA); incomplete Freund's adjuvant (IFA) from Difco Laboratories (Detroit, USA). APC-SIINFEKL/H2-Kb tetramers, SIINFEKL (OVA8) and ASNENMETM (FLU9)

and carboxyfluorescein succinimidyl ester (CFSE)-labeled synthetic short peptides were produced in house. All other chemicals were of analytical grade and aqueous solutions prepared with Milli-Q water.

2.2. Cells

D1 cells, a murine GM-CSF dependent immature dendritic cell line, were cultured as described previously [33]. Bone-marrow derived DC (BMDC) were freshly isolated from femurs from mice and cultured as published previously [34] and yielded cells which were at least 90% positive for murine DC marker CD11c. B3Z $CD8^+$ T-cell hybridoma cell line, specific for the H-2Kb-restricted OVA257–264 CTL epitope SIINFEKL was cultured as described before [35].

2.3. Animals

C57BL/6 (Ly5.2/CD45.2; H-2b) mice were obtained from Charles River Laboratories. Ly5.1/CD45.1 congenic (C57BL/6 background) mice were bred in the specific pathogen-free animal facility of the Leiden University Medical Center. All animal experiments were approved by the animal experimental committee of Leiden University.

2.4. Preparation and characterization of OVA- and poly(I:C)-loaded PLGA particles

2.4.1. Preparation of NP and MP and IFA

PLGA 50:50 and PLGA 75:25 NP were prepared as described [36], using 1 mg OVA, 0.25 mg poly(I:C) and 1 µg poly(I:C)-rhodamine dissolved in 85 µl of 25 mM Hepes, pH 7.4, as inner aqueous phase. For NP and MP used in the release and uptake studies, 1% (w/w total OVA) of OVA-AF488 was added to the inner phase during preparation for detection purposes.

PLGA 50:50 MP were prepared by adding 1 mg OVA, 0.25 mg poly(I:C), and 1 µg poly(I:C)-rhodamine dissolved in 500 µl of 25 mM Hepes pH 7.4 to 1 ml DCM containing 125 mg PLGA 50:50. The mixture was homogenized for 30 s at 25,000 rpm (Heidolph Ultrax 900, Sigma, Germany) and transferred to 10 ml of 2% (w/v) PVA under magnetic stirring for 10 min at 750 rpm at room temperature, followed by 1 hour at 500 rpm at 40ºC to allow evaporation of DCM. MP were harvested and washed twice by centrifugation (2000 g, 2 min). To separate particles bigger than 20 µm, MP were diafiltrated with 3 L water under continuous stirring, using a Solvent Resistant Stirred

Cell (Milipore, USA) filtration system with a 20-µm stainless steel sieve (Advantech, USA), the retentate collected and particles recovered by centrifugation at 2000 g for 2 min. Particles >200 µm were eliminated by filtration through a 200-µm stainless steel sieve (Advantech, USA). Intactness of MP before and after filtration (see **Supplemental Figure 1**) was verified with an Axioskop microscope, equipped with Axiocam ICc 5 (Carl Zeiss, Munich, Germany) and 20x amplification objective. Images were collected with ProgRes CapturePro v2.8.8 software (Jenoptik AG, Jena, Germany). Both NP and MP suspensions were aliquoted and freeze-dried.

Preparation of IFA emulsion was performed by dissolving OVA and poly(I:C) in PBS and mixing with IFA for 30 min in a 1:1 ratio by using a vortex mixer.

2.4.2. Characterization of NP and MP

Size and polydispersity index (PDI) of NP in 5 mM Hepes pH 7.4 were determined by dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern, UK). Zeta potential (ZP) was determined by laser Doppler velocimetry using the same apparatus. Size distribution of MP was determined by light obscuration (LO) using a PAMAS SVSS system (PAMAS GmbH, Rutesheim, Germany) equipped with HCB-LD-25/25 sensor and 1-ml syringe. Each sample was measured three times, each measurement consisting of three runs of 0.2 ml at a flow rate of 10 ml/min.

Encapsulation efficiency (EE) was calculated by fluorescence of OVA-AF488 (excitation 495 nm, emission 520 nm) or poly(I:C)-rhodamine (excitation 546 nm, emission 576 nm) detected in the supernatant with Infinite® M 1000 Pro (Tecan, Switzerland) microplate reader.

To study release kinetics NP/MP containing fluorescently labeled OVA and poly(I:C) were resuspended in PBS, containing 0.01% Tween 20 and 0.01% sodium azide, at 10 mg PLGA/ml, and maintained at 37ºC under tangential shaking at 100 rpm in a GFL 1086 shaking water bath (Burgwedel, Germany) for 30 days. At regular time intervals, 250 µl aliquots were taken and centrifuged for 20 min at 18000 g. Supernatants were stored at 4ºC until fluorescence intensity was determined (Infinite® M 1000 Pro, Tecan, Switzerland) [37]. OVA concentrations on remaining supernatant samples from the final day were also analyzed by BCA assay (Pierce, Rockford, IL, USA) after dissolving particles in DMSO and 0.5 M NaOH + 0.5% SDS as described [38] to validate fluorescence measurements, with comparable results (**Supplemental Table 1**).

2.5. Analysis of particle uptake by DC

Particle uptake by DC was determined by plating out D1 cells in 96-well plates (105 cells/well) and pre-cooling on ice (10 min). Pre-cooled cells were cultured for 1 h at 4ºC (on ice) or at 37ºC in the presence of PLGA particles containing OVA-AF488. After

incubation, cultured cells were washed and centrifuged twice with cold saline buffer to remove unbound particles and cells fixed with 4% paraformaldehyde (100 µl/well). Fixation was blocked by addition of 100 µl/well fetal calf serum (FCS) and washing with cold PBS. Cells were kept at room temperature and stained with rat anti-mouse CD45.2-APC fluorescent antibodies to allow detection of cells positive for particle association, based on OVA-AF488 fluorescence (excitation 495 nm, emission 520 nm) as analyzed using a BD LSRII flow cytometer. Data were acquired using the BD FACS DIVA software and analyzed with Flow Jo software (treestar).

2.6. MHC class I Ag presentation

DC were incubated for 2 h with PLGA-OVA formulations at the indicated concentrations, washed and followed by an overnight incubation (37ºC) in the presence of B3Z $CD8^+$ T cell hybridomas to measure MHC class I presentation as described previously [39]. To determine the relative maximum B3Z T cell activation, DC were loaded with the minimal epitope SIINFEKL and the extinction value set as 100% (OD590nm = 2.53 = 100%).

2.7. Vaccination studies

Animals were vaccinated with PLGA-OVA/poly(I:C) formulations, soluble OVA/poly(I:C) in PBS, or OVA/poly(I:C) emulsified in IFA by s.c. injection into the right flank on day 0 and day 28. Priming of cytotoxic $CD8^+$ T cells was assessed seven days after the 1st and 14 days after the 2nd vaccination by transferring splenocytes prepared from congenic Ly5.1 C57BL/6 animals which were pulsed with SIINFEKL (OVA8, vaccine specific target cells) or ASNENMETM (FLU9, vaccine non-specific control target cells). Target cells were labeled with either 10 µM OVA8- or 0.5 µM FLU9-CFSE. Cells were mixed 1:1 and 107 total cells were injected intravenously into the vaccinated animals. 18 h later, animals were sacrificed and single cell suspensions were prepared from isolated spleens [9]. Injected target cells were distinguished by APC-conjugated rat anti-mouse CD45.1 mAb (BD Pharmingen, San Diego, USA). *In vivo* cytotoxicity was determined by flow cytometry after 18 h using **equation 1**:

$$\%\,\text{OVA - specific killing} = \left(1 - \left[\left(\frac{OVA_8 - CFSE - peak\ area}{FLU_9 - CFSE - peak\ area}\right)^{vaccinated\ animals} \times \left(\frac{OVA_8 - CFSE - peak\ area}{FLU_9 - CFSE - peak\ area}\right)^{non-vaccinated\ animals}\right]\right) \times 100 \quad (1)$$

OVA-specific $CD8^+$ T cells present in the spleens were analyzed by co-staining with APC-conjugated SIINFEKL/H2-Kb tetramers, AF488-conjugated anti-mouse CD8α

mAb and V500-conjugated rat anti-mouse CD3 mAb. Flow cytometry was performed as described above.

2.8. Detection of antibody responses

Antibody responses were determined by collecting serum samples on day 21 (1 week before 2nd vaccination) and on day 35 (1 week after 2nd vaccination). IgG1, IgG2a and IgG2b titers against OVA were determined by ELISA. In brief, high absorbent 96-wells Nunc immunoplates were coated with 5 µg/ml OVA in PBS and incubated with titrated serum samples in 10% FCS in PBS. Antibodies were detected using streptavidine conjugated rabbit anti-murine IgG1, IgG2a and IgG2b mAb, followed by addition of horse-radish-peroxidase conjugated biotin. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as a substrate and the color conversion was stopped after 10 min with 0.16 M H2SO4, which was then measured on a spectrophotometer by absorbance at 450 nm (OD450nm). To determine immune polarization the IgG2a/IgG1 ratios were determined using OD450nm values determined at 1:100 dilution if values applied were ≥ 2-fold OD450nm of the negative control (sera from non-immunized mice).

3. Results and Discussion

Particulate vaccines are promising modalities as the immune system reacts more vigorously to vaccines presented in a particulate form compared to soluble ones [9, 10]. However, the exact parameters needed to achieve robust immune responses against particulate vaccines are still a matter of debate [29-31]. This may be related to different parameters that can direct the type and potency of the immune response [40, 41], such as the route of administration upon vaccination and Ag release kinetics [27, 42, 43]. The latter is critical as sustained Ag release is essential to properly stimulate DC [44]. We have recently shown that low-burst release of encapsulated Ag is crucial for efficient MHC class I Ag presentation and $CD8^+$ T cell activation [39]. Of importance, there exists a strong correlation between (particle) size and the efficiency of Ag uptake, processing and presentation by APC [29, 32, 42].

APC may take up and process Ag with similar dimensions to pathogens, such as viruses and bacteria, with the size influencing the mechanisms of uptake and processing by APC [45]. It has been reported that particles in the range of 20-200 nm are efficiently taken up by DC and facilitate the induction of cellular immune responses, whereas particles of 0.5-5 µm mainly generate humoral responses; limited uptake of 10 µm or larger particles was observed, leading to defective immune activation [29, 31, 46]. Also, nano-sized particles supported DC maturation by TLR9 triggering in contrast to micro-sized particles [31]. These studies suggest that the efficient internalization of particles significantly dictates the ensuing vaccine induced immune response. In contrast, others have reported that vaccinations with MP also induce CTL responses, comparable to IFA- or Montanide-based delivery systems [17, 47].

Using PLGA particles co-encapsulating protein Ag and a TLR3L, we compared NP (which can be engulfed by DC) with > 20-µm MP (which are too large to be taken up) for their capacity to induce MHC class I cross-presentation *in vitro* and improve immune responses *in vivo*. To study the effect of particle uptake on the subsequent immune response, we designed NP and MP of similar compositions and with similar release properties. So, we engineered MP (PLGA 50:50) to match the release properties of NP by adding salt to the inner water phase to increase porosity and accelerate drug diffusion and release (see Supplemental **Figure 2** for inner phase compositions) [48-51]. MP were also diafiltrated to eliminate particles smaller than 20 µm that could be internalized by DC, as well as particles larger than 200 µm, and size distribution before and after filtration was determined (**Supplemental Table 2** & **Supplemental Figure 3**). Number-based mean-diameter of MP increased from 5±1 µm before filtration (BF) to 17±5 µm after filtration (AF), showing that the filtration step effectively decreased the number of particles smaller than 20 µm. Importantly, volume-based mean-diameters indicate that less than 1% of the total volume, and consequently less than 1% of the OVA content, corresponded to particles smaller than 20 µm, making it unlikely that residual particles <20 µm would have a significant effect on Ag uptake, MHC class I Ag presentation and $CD8^+$ T cell activation. Physicochemical characterization of all formulations was done in terms of encapsulation efficiency (EE), drug loading (DL), size, PDI and ZP as summarized in **Table 1** (for NP size distributions see **Supplemental Figure 4**).

Table 1: Physical characteristics of OVA/poly(I:C)-loaded PLGA NP and MP in terms of size (Z-average obtained by DLS for NP; and number (n) and volume (v) average obtained by LO for MP), PDI, ZP, EE and DL.*

Formulation	Size	PDI	ZP (mV)	OVA		Poly I:C	
				EE (%)	DL (%)	EE (%)	DL (%)
NP 50: 50	357 ± 25 nm	0.16 ± 0.03	-41 ± 7	52 ± 7	2.01 ± 0.27	66 ± 8	0.63 ± 0.07
NP 75: 25	400 ± 16 nm	0.25 ± 0.02	-25 ± 3	75 ± 1	2.87 ± 0.03	59 ± 7	0.56 ± 0.07
MP 50: 50	17 ± 5 µm (n) 112 ± 26 µm (v)	n/a	-14 ± 3	86 ± 2	0.68 ± 0.01	81 ± 2	0.16 ± 0.00

* Values represent mean +/- standard deviation of 3 independently prepared batches

PDI: polydispersity index; ZP: Zeta-potential; EE: encapsulation efficiency; DL: drug loading.

As release studies showed faster Ag and TLR3L release from NP 50:50 compared to MP 50:50, NP were also prepared with PLGA 75:25, which is more hydrophobic and therefore degrades slower, to achieve particles comparable in size to NP 50:50 and closer in release kinetics to MP 50:50. Release studies showed sustained release over 30 days (**Figure 1**) with NP 75:25 exhibiting similar long-term release characteristics to MP, but remaining closer to NP 50:50 in the early stages (<10 days), due to higher burst release. These profiles contrast with a previous study by Joshi et al. (2013) where drastic differences in release kinetics were observed: whereas the initial (burst) release

accounted for nearly total Ag and TLRL release from 300-nm NP, the release from MP with a size of about 17 μm was only 10% after 15 days [31]. Besides the difference in size, this could have contributed to the distinct immunological properties of their NP and MP formulations.

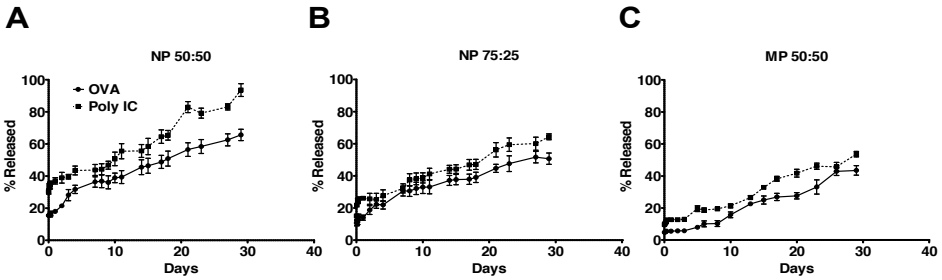


Figure 1: *In vitro* release kinetics of OVA and poly(I:C) from NP and MP. OVA (closed circles) and poly(I:C) (closed squares) release in PBS/0.01% Tween-20/0.01% NaN3 (pH 7.4) of A) NP 50:50, B) NP 75:25, and C) MP 50:50 were monitored for 30 days at 37°C. Data are presented as average ± SD of 3 independent batches.

Efficiency of particle association with DC was studied by culturing DC in the presence of the 3 different particle formulations at 4ºC (binding) and 37ºC (binding & internalization) with various concentrations of encapsulated Ag. DC showed very high capacity to bind and internalize NP compared to MP (**Figure 2A**), and we observed consistently that both NP 50:50 and 75:25 were engulfed with higher efficiency than MP (**Figure 2B & C**). In line with these results, we have recently shown that NP are internalized by $CD11c^+$ DC *in vivo* at the site of injection [52]. Internalized NP function as an intracellular Ag depot gradually releasing the encapsulated Ag into MHC class I processing and cross presentation pathways, leading to sustained intracellular release [12, 53].

In contrast, MP are poorly internalized, likely because of their large size at the early phases of PLGA degradation, releasing the encapsulated Ag and TLRL locally via an initial burst followed by a more gradual release due to polymer hydrolysis (**Figure 1**). Consequently, MP essentially deliver soluble Ag and TLRL to DC and function as a local Ag depot under the skin at the site of injection. We and others have previously shown that DC poorly cross-present protein Ag when it is delivered in soluble form [9, 10]. Therefore, if the aim is to induce a robust $CD8^+$ T cell response through vaccination, micron-sized particulate vaccines might not be the right candidates.

MHC class I Ag cross-presentation by DC of OVA encapsulated in NP or in MP was studied *in vitro*. Soluble OVA is poorly cross-presented in MHC class I processing routes, unless very high amounts are added to DC cultures [9]. We observed that NP performed 8 - 10 fold better compared to MP in routing Ag into MHC class I processing pathways, whereas NP 50:50 and NP 75:25 showed similar efficiency, possibly due to the similar release kinetics at early time-points (**Figure 3A**). $CD8^+$ T cell activation was calculated relative to that induced by 5 nM SIINFEKL as 100%, and no Ag as baseline

(see **Figure 3B** for raw data). Our observations are supported by the report of Joshi et al., who observed that the efficiency of particle uptake and upregulation of MHC class I and CD86 expression on BMDC was correlated with small particle size [31].

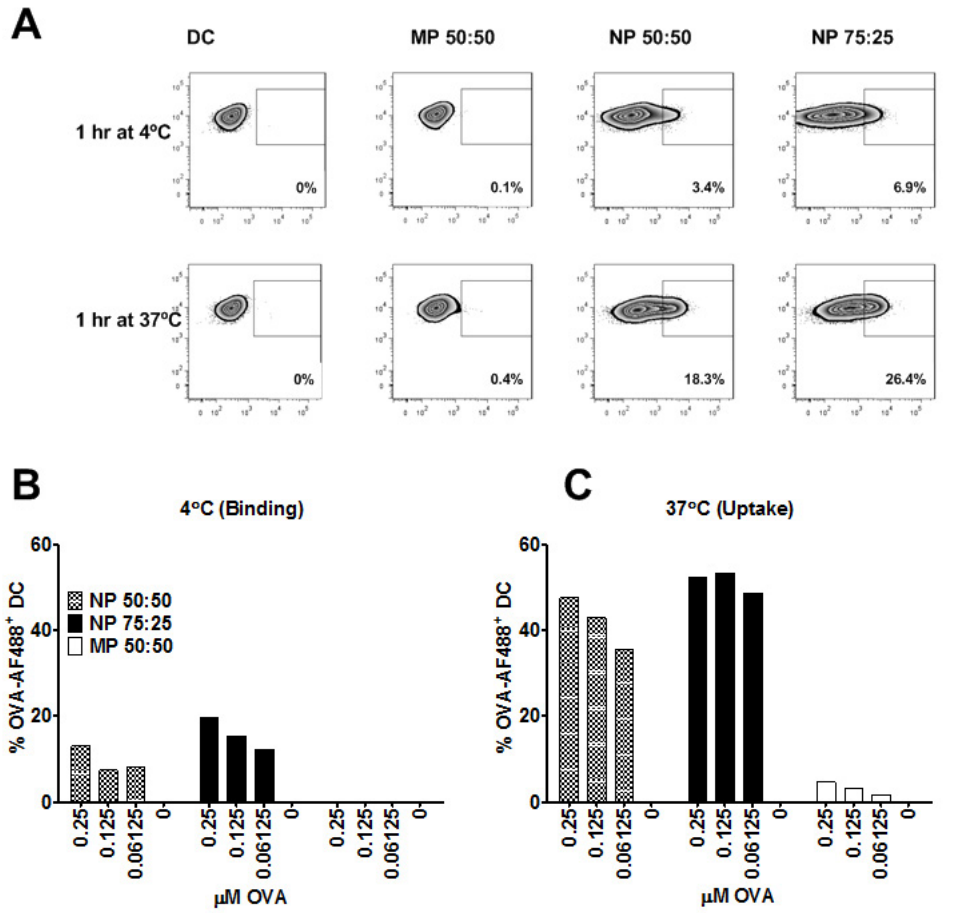


Figure2: Binding and uptake of NP-encapsulated protein Ag by dendritic cells compared to MP-encapsulated Ag. A) D1 dendritic cells cells were incubated for 1 h with titrated amounts (µM) of OVA (and poly(I:C)) encapsulated in NP 50:50, NP 75:25 and MP 50:50 also containing OVA-AF488 dye. Ag incubation with DC was performed in parallel at **B)** 4°C (binding) and **C)** 37°C (binding & internalization), followed by extensive washing to remove unbound Ag and cell fixation with 4% paraformaldehyde. Cells were analyzed by flow cytometry to determine green fluorescence. Percentages of DC positive for OVA-AF488 were quantified at different Ag concentrations. Data shown are measurements from one experiment.

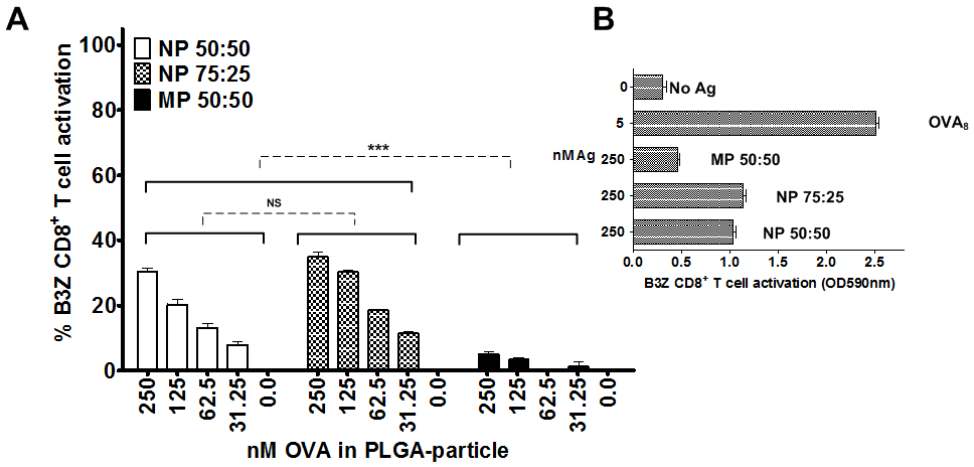


Figure3: Efficient MHC class I cross-presentation of protein Ag incorporated in NP but not MP. A) D1 cells were pulsed for 2 h with titrated amounts (nM) of OVA (and poly(I:C)) encapsulated in NP 50:50, NP 75:25 or MP 50:50. MHC class I presentation of processed OVA protein antigen was detected by co-culture with H-2Kb/SIINFEKL-specific B3Z CD8+ T cells. Normalized values in panel A were calculated based on the OD590nm values obtained by using DC loaded with SIINFEKL cultured together with B3Z CD8+ T cells (OD590nm = 2.53 = 100% CD8+ T cell activation). **B)** OD590nm values obtained with the highest concentration of OVA (250 nM) (and poly(I:C)) encapsulated NP 50:50, NP 75:25, MP 50:50 or SIINFEKL (5 nM, known to result in maximal CD8+ T cell activation in this assay). Data shown are means of triplicate measurements ± SD as % from one representative example out of at least three independent experiments.

We subsequently analyzed whether the enhanced MHC class I presentation observed *in vitro* would translate into better CD8+ T cell priming *in vivo*. For this purpose, we vaccinated animals with NP and MP formulations encapsulating OVA and Poly(I:C) or Ag and adjuvant formulated in IFA. Mice were sacrificed 7 days later to determine the number of Ag-specific CD8+ T cells in the spleen. Since NP 50:50 showed similar CD8+ T cell priming *in vivo* as NP 75:25 (data not shown), the ensuing *in vivo* studies comparing NP and MP were performed based on particle formulations using the same polymer composition (PLGA 50:50).

The *in vivo* vaccine potency of NP and MP formulations was analyzed in comparison to IFA. It is well known that plain PLGA particles have poor immune-activating properties: the absence of TLRL in PLGA-based vaccine protocols results in defective immune activation [8, 24, 54]. For that reason poly(I:C) was co-encapsulated with the Ag in our particles to achieve a strong activation of DC and promote the production of IL-12 [9]. Vaccinations with NP led to considerably higher numbers of Ag-specific CD8+ T cells compared to MP ($p = 0.01$) and IFA ($p = 0.04$), with almost no difference being observed between vaccination with MP and non-vaccinated mice (Figure 4). NP also performed better than IFA, suggesting that internalization of the particles may be of importance in inducing a stronger cellular immune response in comparison to sustained release from a local Ag depot, though the mechanisms by which IFA delivers Ag are still not fully understood.

Analysis of cytokine production showed that vaccination with NP resulted in the highest production of IFN-γ (**Supplemental Figure 5**) but did not induce much IL-2 (data not shown). Under these conditions we also analyzed the $CD8^+$ T cell *in vivo* cytotoxicity. In line with the relatively higher number of specific $CD8^+$ T cells induced with NP, animals vaccinated with NP showed effective OVA-specific cytotoxicity *in vivo* (**Supplemental Figure 6**).

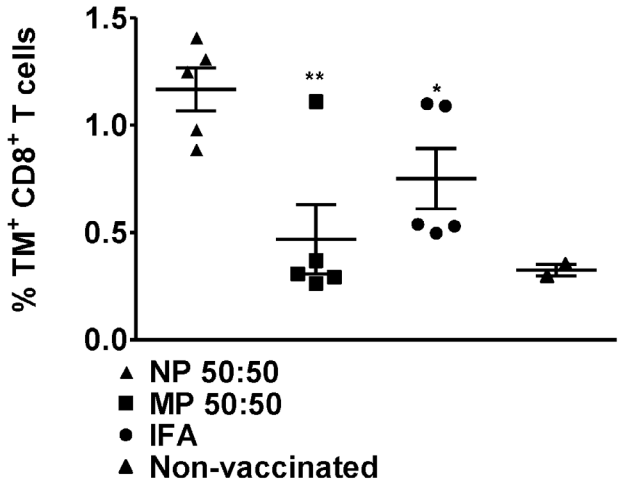


Figure 4: NP vaccine shows effective $CD8^+$ T cell priming potency. Naïve animals received a single s.c. vaccination with the 50 µg OVA and 20 µg poly(I:C) formulated in NP 50:50 (closed triangles), MP 50:50 (open squares) or in IFA (closed circles). Non-vaccinated animals were used as control (open triangles). Mice were sacrificed on day 7 after vaccination and the % of SIINFEKL-TM^+ $CD8^+$ T cells were measured by flow cytometry. Results shown are representative of one experiment out of two and present averages ± SEM from n = 3 - 5 mice per group, * = $p < 0.05$ and ** = $p < 0.01$ using an unpaired student t test in relation to the group immunized with NP 50:50. Each symbol represents the specific T cell response in an individual mouse.

Co-encapsulation of OVA and TLRL has previously shown to induce anti-OVA (IgG) humoral responses, as well as polarization of the immune response [24, 37, 55]. Blood samples were collected on day 21 or 35 after vaccination and titers of IgG1, IgG2a and IgG2b antibodies were determined. NP and IFA, but not MP, induced IgG1 production and low titers of IgG2a and IgG2b after single vaccination (prime) (**Figure 5A** & **B** & **C)**. A second vaccination (boost) considerably enhanced the IgG1 titers for IFA and NP, but again not MP (**Figure 5D**). Significant IgG2a titers were induced after boost with NP but poorly by the other vaccines (**Figure 5E**). IgG2b titers were induced to a similar level by vaccinations with IFA and NP (**Figure 5F**).

IgG2a is the IgG-subtype associated with TH1 responses in mice and analysis of the IgG1/IgG2a ratio allows one to determine the immune-polarization [56]. Vaccinations with NP resulted in a more balanced TH1/TH2 antibody response characterized by similar titers of IgG1 and IgG2a (IgG1/IgG2a ≈ 1).

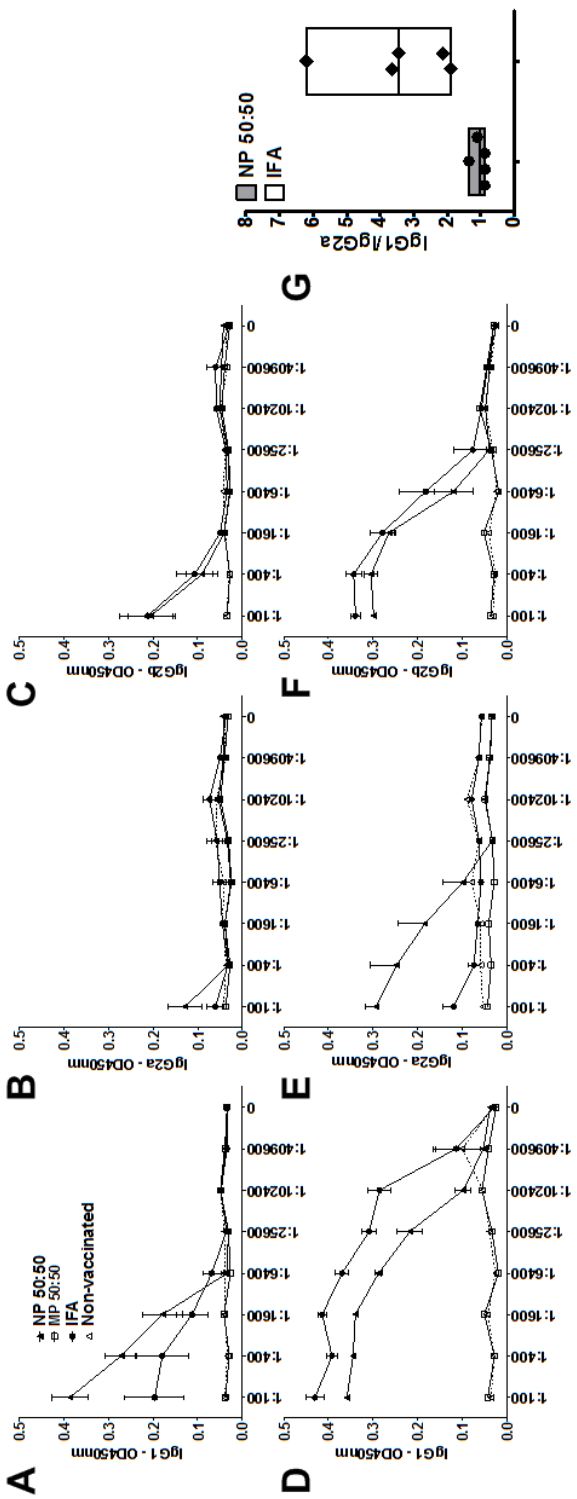


Figure 5: Induction of balanced TH1/TH2-associated humoral responses by vaccinations with NP. A) Animals were vaccinated on day 0 and day 28 with 50 µg OVA and 20 µg poly(I:C) formulated in NP 50:50 (closed triangles), MP 50:50 (open squares) or in IFA (closed circles). Non-vaccinated animals were used as control (open triangles). Serum samples were collected on day 21 (3 weeks after prime, panel **A, B** and **C)** and 35 (1 week after boost, panel **D, E** and **F)** and the titers for IgG1, IgG2a and IgG2b determined via ELISA. G) Immune polarization was calculated for NP 50:50 (closed circles) and IFA (closed diamonds) based on the IgG1/IgG2a ratio determining ([IgG1 OD450nm vaccinated mice/OD450nm non-immunize mice]/[IgG2a OD450nm vaccinated mice/OD450nm non-immunized mice]) at the lowest serum dilution (1:100). Results shown are averages ± SEM from 5 mice per group and time-point, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.01$ two-way ANOVA with Bonferroni post tests.

In contrast, vaccinations with IFA led to a predominant TH2 response (IgG1/IgG2a > 2) (**Figure 5G**), which might contribute to the differences in $CD8^+$ T cell responses detected after vaccinations. A predominant humoral TH2 response will likely be accompanied by a weak $CD8^+$ T cell response [57]. The higher production of IgG2a after vaccination is linked to the increased uptake by DC of particles encapsulating the protein and adjuvant [31], compared to soluble protein and poly(I:C). In addition, direct stimulation of B cells by the NP might also contribute better IgG2a responses compared to IFA-based vaccine formulations [21, 58].

PLGA MP are reported to have a negative effect on protein structural integrity, leading to denaturation and aggregation [59]. Differences in Ag integrity of OVA in NP compared to OVA in MP might also play a role in the immune responses we report here, since a larger and deeper inner core of MP likely results in more extensive local acidification as compared to the much smaller NP [60]. However, release of denatured OVA from MP should induce at least equal but probably stronger *in vivo* T cell [61] and B cell responses [62, 63] compared to native OVA. In support of this we have shown that proteolytic cleavage of whole proteins into smaller peptide strands improves the activation of T cells by human DC [64]. Still, we cannot exclude that the inability of MPs to induce antibodies cross-reacting with native OVA may be due in part to a more dramatic loss of protein structure compared to the NP.

4. Conclusions

Our results show that the ability of DC to internalize Ag- and TLRL-containing PLGA particles is a crucial factor to achieve effective MHC class I cross-presentation, prime $CD8^+$ T cells and to elicit both a T and B cell response after direct vaccinations. We report here that NP are more efficiently internalized by DC *in vitro*, resulting in superior vaccine potency compared to MP when aiming to stimulate cellular immune responses. Furthermore, NP co-encapsulating Ag and TLRL outperformed IFA as an adjuvant, by more efficiently boosting $CD8^+$ T cell activation and (IgG2a) antibody production. In conclusion, because of the superior responses induced in comparison to IFA, our data supports the application of biodegradable PLGA NP delivery systems as a substitute for mineral oil emulsions for protein vaccine-based immunotherapy.

Acknowledgements

The authors thank Ahmed Allam and Amir Ghassemi for the contribution to particle formulation studies, and Reza Nejadnik for support in particle characterization. This study was supported by grants from Immune System Activation (ISA) Pharmaceuticals and the Leiden University Medical Center.

Conflict of Interest

The authors declare that they have no conflict of interest.

5. References

1. Ada, G., Overview of vaccines and vaccination. Mol Biotechnol, 2005. 29(3): p. 255-72.
2. Arens, R., T. van Hall, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Prospects of combinatorial synthetic peptide vaccine-based immunotherapy against cancer. Semin Immunol, 2013. 25(2): p. 182-90.
3. Melief, C.J., Cancer immunotherapy by dendritic cells. Immunity, 2008. 29(3): p. 372-83.
4. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, H.P. Merkle, and M. Groettrup, Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine, 2006. 24(11): p. 1847-57.
5. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, F. Essahsah, L.M. Fathers, R. Offringa, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, S.H. van der Burg, and C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med, 2009. 361(19): p. 1838-47.
6. Atanackovic, D., N.K. Altorki, E. Stockert, B. Williamson, A.A. Jungbluth, E. Ritter, D. Santiago, C.A. Ferrara, M. Matsuo, A. Selvakumar, B. Dupont, Y.T. Chen, E.W. Hoffman, G. Ritter, L.J. Old, and S. Gnjatic, Vaccine-induced $CD4^+$ T cell responses to MAGE-3 protein in lung cancer patients. J Immunol, 2004. 172(5): p. 3289-96.
7. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner, Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. Eur J Immunol, 2007. 37(8): p. 2063-74.
8. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup, TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, 2008. 26(13): p. 1626-37.
9. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, F. Ossendorp, and J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer Immunol Immunother, 2013. 62(7): p. 1161-73.
10. Braun, M., C. Jandus, P. Maurer, A. Hammann-Haenni, K. Schwarz, M.F. Bachmann, D.E. Speiser, and P. Romero, Virus-like particles induce robust human T-helper cell responses. Eur J Immunol, 2012. 42(2): p. 330-40.
11. Rosalia, R.A., L.J. Cruz, S. van Duikeren, A.T. Tromp, A.L. Silva, W. Jiskoot, T. de Gruijl, C. Lowik, J. Oostendorp, S.H. van der Burg, and F. Ossendorp, CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses. Biomaterials, 2014.
12. Shen, H., A.L. Ackerman, V. Cody, A. Giodini, E.R. Hinson, P. Cresswell, R.L. Edelson, W.M. Saltzman, and D.J. Hanlon, Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology, 2006. 117(1): p. 78-88.

13. Tran, K.K., X. Zhan, and H. Shen, Polymer blend particles with defined compositions for targeting antigen to both class I and II antigen presentation pathways. Adv Healthc Mater, 2014. 3(5): p. 690-702.
14. Tacken, P.J., I.J. de Vries, R. Torensma, and C.G. Figdor, Dendritic-cell immunotherapy: from ex vivo loading to *in vivo* targeting. Nat Rev Immunol, 2007. 7(10): p. 790-802.
15. Hailemichael, Y., Z. Dai, N. Jaffarzad, Y. Ye, M.A. Medina, X.F. Huang, S.M. Dorta-Estremera, N.R. Greeley, G. Nitti, W. Peng, C. Liu, Y. Lou, Z. Wang, W. Ma, B. Rabinovich, R.T. Sowell, K.S. Schluns, R.E. Davis, P. Hwu, and W.W. Overwijk, Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat Med., 2013. 19(4): p. 465-72. doi: 10.1038/nm.3105. Epub 2013 Mar 3.
16. Parker, R., S. Deville, L. Dupuis, F. Bertrand, and J. Aucouturier, Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile. Procedia in Vaccinology, 2009. 1(1): p. 140-147.
17. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. Int J Cancer, 2011. 129(2): p. 407-16.
18. Mundargi, R.C., V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J Control Release, 2008. 125(3): p. 193-209.
19. Newman, K.D., P. Elamanchili, G.S. Kwon, and J. Samuel, Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells *in vivo*. J Biomed Mater Res, 2002. 60(3): p. 480-6.
20. Putney, S.D. and P.A. Burke, Improving protein therapeutics with sustained-release formulations. Nat Biotechnol, 1998. 16(2): p. 153-7.
21. Kasturi, S.P., I. Skountzou, R.A. Albrecht, D. Koutsonanos, T. Hua, H.I. Nakaya, R. Ravindran, S. Stewart, M. Alam, M. Kwissa, F. Villinger, N. Murthy, J. Steel, J. Jacob, R.J. Hogan, A. Garcia-Sastre, R. Compans, and B. Pulendran, Programming the magnitude and persistence of antibody responses with innate immunity. Nature, 2011. 470(7335): p. 543-7.
22. Badiee, A., N. Davies, K. McDonald, K. Radford, H. Michiue, D. Hart, and M. Kato, Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205. Vaccine, 2007. 25(25): p. 4757-66.
23. Zhang, X.Q., C.E. Dahle, N.K. Baman, N. Rich, G.J. Weiner, and A.K. Salem, Potent antigen-specific immune responses stimulated by codelivery of CpG ODN and antigens in degradable microparticles. J Immunother, 2007. 30(5): p. 469-78.
24. Pulendran, B. and R. Ahmed, Immunological mechanisms of vaccination. Nat Immunol, 2011. 12(6): p. 509-17.
25. Demento, S.L., N. Bonafe, W. Cui, S.M. Kaech, M.J. Caplan, E. Fikrig, M. Ledizet, and T.M. Fahmy, TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. J Immunol, 2010. 185(5): p. 2989-97.
26. Link, A., F. Zabel, Y. Schnetzler, A. Titz, F. Brombacher, and M.F. Bachmann, Innate immunity mediates follicular transport of particulate but not soluble

protein antigen. J Immunol, 2012. 188(8): p. 3724-33.
27. Johansen, P., T. Storni, L. Rettig, Z. Qiu, A. Der-Sarkissian, K.A. Smith, V. Manolova, K.S. Lang, G. Senti, B. Mullhaupt, T. Gerlach, R.F. Speck, A. Bot, and T.M. Kundig, Antigen kinetics determines immune reactivity. Proc Natl Acad Sci U S A, 2008. 105(13): p. 5189-94.
28. Simon, L.C. and C.M. Sabliov, The effect of nanoparticle properties, detection method, delivery route and animal model on poly(lactic-co-glycolic) acid nanoparticles biodistribution in mice and rats. Drug Metab Rev, 2013.
29. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
30. Jain, S., P. Malyala, M. Pallaoro, M. Giuliani, H. Petersen, D.T. O'Hagan, and M. Singh, A two-stage strategy for sterilization of poly(lactide-co-glycolide) particles by gamma-irradiation does not impair their potency for vaccine delivery. J Pharm Sci, 2011. 100(2): p. 646-54.
31. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. AAPS J, 2013. 15(1): p. 85-94.
32. Tran, K.K. and H. Shen, The role of phagosomal pH on the size-dependent efficiency of cross-presentation by dendritic cells. Biomaterials, 2009. 30(7): p. 1356-62.
33. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V.S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med, 1997. 185(2): p. 317-28.
34. Schuurhuis, D.H., A. Ioan-Facsinay, B. Nagelkerken, J.J. van Schip, C. Sedlik, C.J. Melief, J.S. Verbeek, and F. Ossendorp, Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J Immunol, 2002. 168(5): p. 2240-6.
35. Sanderson, S. and N. Shastri, LacZ inducible, antigen/MHC-specific T cell hybrids. Int Immunol, 1994. 6(3): p. 369-76.
36. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, F. Ossendorp, and J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer immunology, immunotherapy : CII, 2013.
37. Slutter, B., S. Bal, C. Keijzer, R. Mallants, N. Hagenaars, I. Que, E. Kaijzel, W. van Eden, P. Augustijns, C. Lowik, J. Bouwstra, F. Broere, and W. Jiskoot, Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine, 2010. 28(38): p. 6282-91.
38. Sah, H., A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres. J Pharm Sci, 1997. 86(11): p. 1315-8.
39. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient $CD8^{(+)}$ T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45.
40. Kreutz, M., P.J. Tacken, and C.G. Figdor, Targeting dendritic cells--why bother?

Blood, 2013. 121(15): p. 2836-44.
41. Schliehe, C., C. Redaelli, S. Engelhardt, M. Fehlings, M. Mueller, N. van Rooijen, M. Thiry, K. Hildner, H. Weller, and M. Groettrup, CD8- dendritic cells and macrophages cross-present poly(D,L-lactate-co-glycolate) acid microsphere-encapsulated antigen *in vivo*. J Immunol, 2011. 187(5): p. 2112-21.
42. Fifis, T., A. Gamvrellis, B. Crimeen-Irwin, G.A. Pietersz, J. Li, P.L. Mottram, I.F. McKenzie, and M. Plebanski, Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol, 2004. 173(5): p. 3148-54.
43. Gutierro, I., R.M. Hernandez, M. Igartua, A.R. Gascon, and J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine, 2002. 21(1-2): p. 67-77.
44. Demento, S.L., W. Cui, J.M. Criscione, E. Stern, J. Tulipan, S.M. Kaech, and T.M. Fahmy, Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials, 2012. 33(19): p. 4957-64.
45. Xiang, S.D., A. Scholzen, G. Minigo, C. David, V. Apostolopoulos, P.L. Mottram, and M. Plebanski, Pathogen recognition and development of particulate vaccines: does size matter? Methods, 2006. 40(1): p. 1-9.
46. Sharp, F.A., D. Ruane, B. Claass, E. Creagh, J. Harris, P. Malyala, M. Singh, D.T. O'Hagan, V. Petrilli, J. Tschopp, L.A. O'Neill, and E.C. Lavelle, Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci U S A, 2009. 106(3): p. 870-5.
47. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug Deliv, 2010. 17(7): p. 490-9.
48. Zeestraten, E.C., F.M. Speetjens, M.J. Welters, S. Saadatmand, L.F. Stynenbosch, R. Jongen, E. Kapiteijn, H. Gelderblom, H.W. Nijman, A.R. Valentijn, J. Oostendorp, L.M. Fathers, J.W. Drijfhout, C.J. van de Velde, P.J. Kuppen, S.H. van der Burg, and C.J. Melief, Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. Int J Cancer, 2013. 132(7): p. 1581-91.
49. Yeo, Y. and K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res, 2004. 27(1): p. 1-12.
50. Leelarasamee, N., S.A. Howard, C.J. Malanga, L.A. Luzzi, T.F. Hogan, S.J. Kandzari, and J.K. Ma, Kinetics of drug release from polylactic acid-hydrocortisone microcapsules. J Microencapsul, 1986. 3(3): p. 171-9.
51. Mansour, H.M., M. Sohn, A. Al-Ghananeem, and P.P. Deluca, Materials for pharmaceutical dosage forms: molecular pharmaceutics and controlled release drug delivery aspects. Int J Mol Sci, 2010. 11(9): p. 3298-322.
52. Cruz, L.J., R.A. Rosalia, J.W. Kleinovink, F. Rueda, C.W. Lowik, and F. Ossendorp, Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8 T cell response: A comparative study. J Control Release, 2014. 192C: p. 209-218.
53. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, R. Torensma, and C.G. Figdor, Targeted PLGA nano- but not microparticles

specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. J Control Release, 2010. 144(2): p. 118-26.
54. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander, Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. J Drug Target, 2009. 17(8): p. 652-61.
55. Krishnamachari, Y. and A.K. Salem, Innovative strategies for co-delivering antigens and CpG oligonucleotides. Adv Drug Deliv Rev, 2009. 61(3): p. 205-17.
56. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, Jr., M.P. Beckmann, L.S. Park, K.A. Schooley, R.L. Coffman, T.R. Mosmann, and W.E. Paul, Lymphokine control of *in vivo* immunoglobulin isotype selection. Annu Rev Immunol, 1990. 8: p. 303-33.
57. Romagnani, S., Th1/Th2 cells. Inflamm Bowel Dis, 1999. 5(4): p. 285-94.
58. Eckl-Dorna, J. and F.D. Batista, BCR-mediated uptake of antigen linked to TLR9 ligand stimulates B-cell proliferation and antigen-specific plasma cell formation. Blood, 2009. 113(17): p. 3969-77.
59. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
60. Samadi, N., A. Abbadessa, A. Di Stefano, C.F. van Nostrum, T. Vermonden, S. Rahimian, E.A. Teunissen, M.J. van Steenbergen, M. Amidi, and W.E. Hennink, The effect of lauryl capping group on protein release and degradation of poly(D,L-lactic-co-glycolic acid) particles. J Control Release, 2013. 172(2): p. 436-43.
61. Schirmbeck, R., W. Bohm, and J. Reimann, Injection of detergent-denaturd ovalbumin primes murine class I-restricted cytotoxic T cells in vivo. Eur J Immunol, 1994. 24(9): p. 2068-72.
62. Huang, C.F., T.C. Wu, Y.H. Chu, K.S. Hwang, C.C. Wang, and H.J. Peng, Effect of neonatal sublingual vaccination with native or denatured ovalbumin and adjuvant CpG or cholera toxin on systemic and mucosal immunity in mice. Scand J Immunol, 2008. 68(5): p. 502-10.
63. Peng, H.J., Z.N. Chang, L.C. Tsai, S.N. Su, H.D. Shen, and C.H. Chang, Heat denaturation of egg-white proteins abrogates the induction of oral tolerance of specific Th2 immune responses in mice. Scand J Immunol, 1998. 48(5): p. 491-6.
64. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, A.L. Silva, W. Jiskoot, T. van Hall, P.A. van Veelen, G. Janssen, K. Franken, L.J. Cruz, A. Tromp, J. Oostendorp, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65.

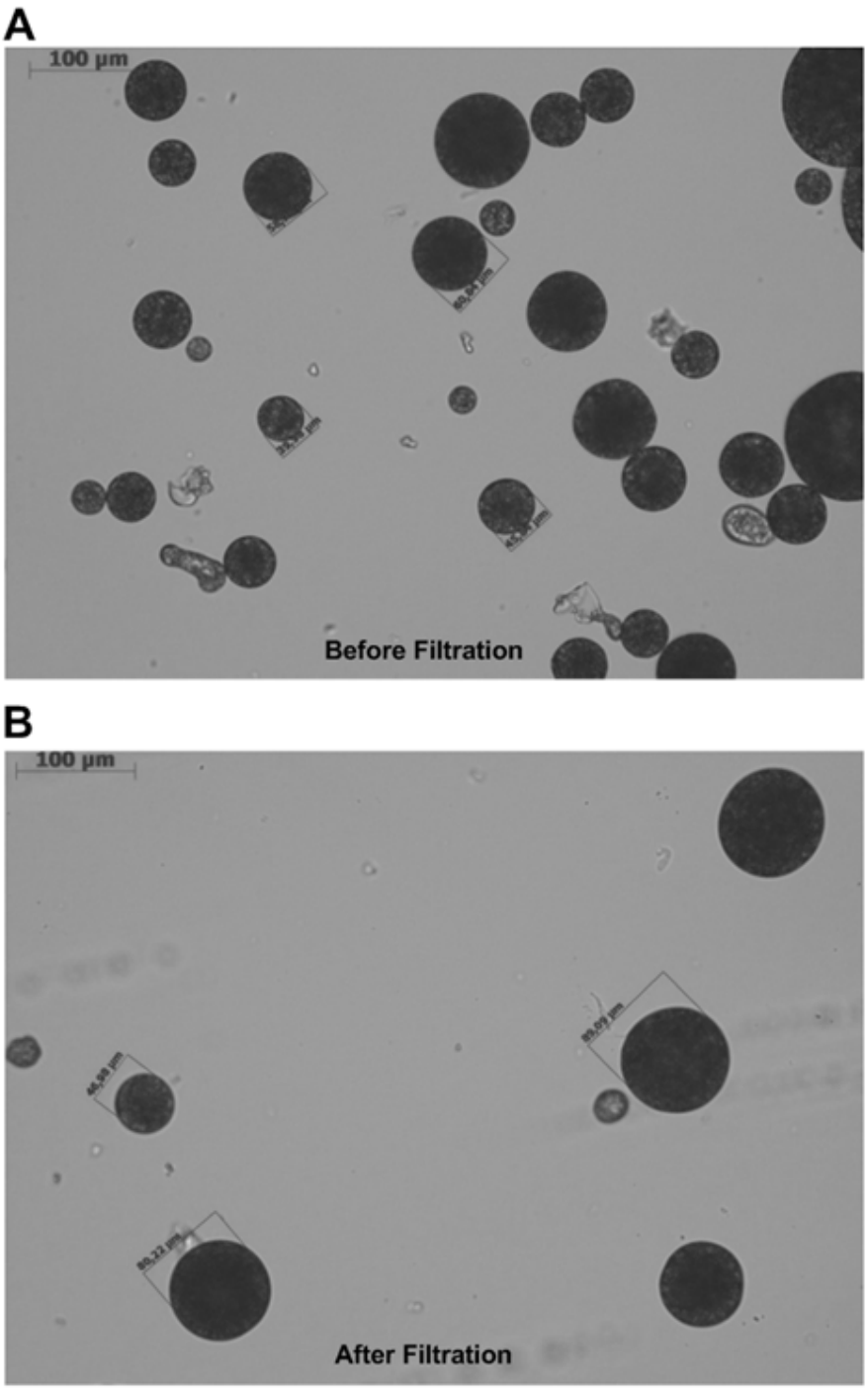
Supplemental Material

Supplemental Table 1: Protein concentration of sample from the last day of release study (day 40). Protein concentration was measured by BCA assay and by fluorescence in supernatants (SN) and expressed as percentage of total encapsulated OVA.

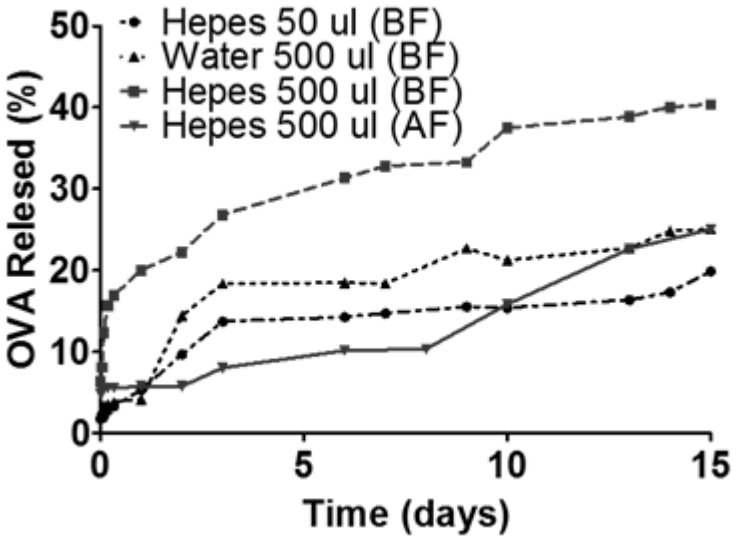
Particles	BCA Assay (% in SN)	Fluorescence (% in SN)
NP 50:50	82 ± 14	85 ±14
NP 75:25	53 ± 10	52 ± 9
MP 50:50	48 ± 10	40 ± 3

Supplemental Table 2: Distribution of MP diameters (µm) determined by LO before (BF) and after filtration (AF) in terms of percentage of total number and volume. Mean values are presented in the last row.

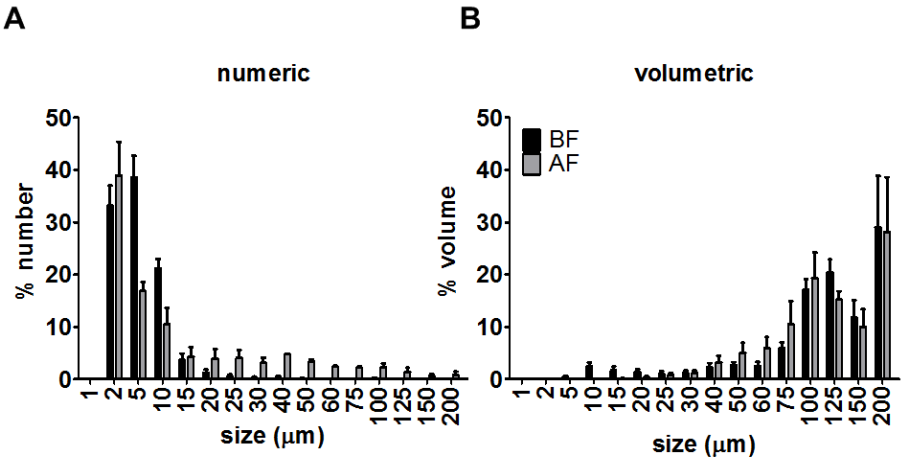
	% Number		% Volume	
Diameter (µm)	BF	AF	BF	AF
1-2	33.2 ± 6.7	39.0 ± 11.0	0.0 ± 0.0	0.0 ± 0.0
2-5	38.6 ± 7.1	16.9 ± 2.9	0.5 ± 0.3	0.0 ± 0.0
5-10	21.2 ± 3.0	10.5 ± 5.4	2.4 ± 1.4	0.1 ± 0.1
10-15	3.7 ± 2.1	4.3 ± 3.2	1.7 ± 1.2	0.1 ± 0.1
15-20	1.3 ± 1.0	4.0 ± 3.1	1.4 ± 1.1	0.4 ± 0.3
20-25	0.6 ± 0.6	4.1 ± 2.7	1.1 ± 0.8	0.8 ± 0.7
25-30	0.3 ± 0.4	3.2 ± 1.7	1.2 ± 0.9	1.2 ± 0.9
30-40	0.4 ± 0.5	4.8 ± 0.1	2.3 ± 1.4	3.2 ± 2.1
40-50	0.2 ± 0.2	3.4 ± 0.6	2.7 ± 0.9	5.0 ± 3.5
50-60	0.1 ± 0.1	2.4 ± 0.3	2.5 ± 1.4	5.9 ± 3.8
60-75	0.1 ± 0.1	2.2 ± 0.3	6.0 ± 1.9	10.6 ± 7.6
75-100	0.2 ± 0.2	2.4 ± 1.2	17.1 ± 3.7	19.3 ± 8.5
100-125	0.1 ± 0.1	1.4 ± 1.5	20.4 ± 4.3	15.3 ± 2.7
125-150	0.0 ± 0.0	0.6 ± 0.8	11.8 ± 5.7	10.0 ± 5.9
150-200	0.0 ± 0.0	0.8 ± 1.1	29.0 ± 17.1	28.2 ± 18.1
Mean diameter	5 ± 1.0	17 ± 5.0	114 ± 16.0	112 ± 26.0



Supplemental Figure 1: Representative images of MP preparations analyzed by optical microscopy (20x magnification) before and after stirred-cell filtration.

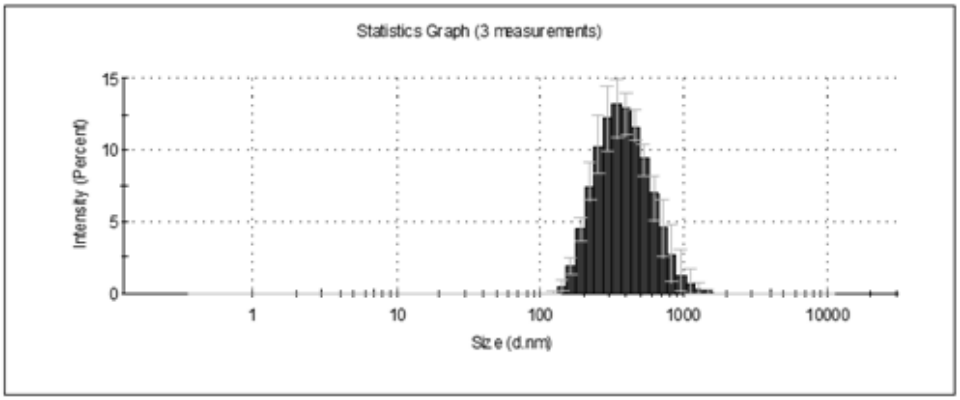


Supplemental Figure 2: OVA release after filtration (AF) and before filtration (BF) of MP with different inner phase composition using PLGA 50:50 observed for 15 days. Hepes 50 µl (BF) (open diamonds) corresponds to an inner phase of 50 µl of 20 mg/ml OVA solution in 25 mM Hepes pH 7.4; and Water 500 µl (BF) (open triangles) to an inner phase of 500 µl of 2 mg/ml OVA in water. Hepes 500 µl (BF) (closed squares) and Hepes 500 µl (AF) (closed circles) correspond to an inner phase of 500 µl of 2 mg/ml OVA in 25 mM Hepes pH 7.4, before and after stirred-cell filtration, respectively. Data presented correspond to 1 batch.

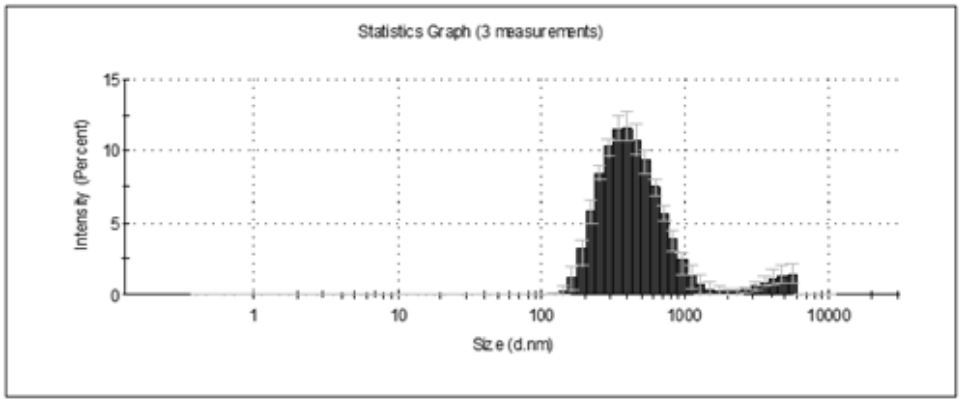


Supplemental Figure 3: Size distribution of MP before filtration (BF) and after filtration (AF) determined by light obscuration. **A)** Number distribution. **B)** Volume distribution. Data are presented as average ± standard deviation of n=3 independent batches.

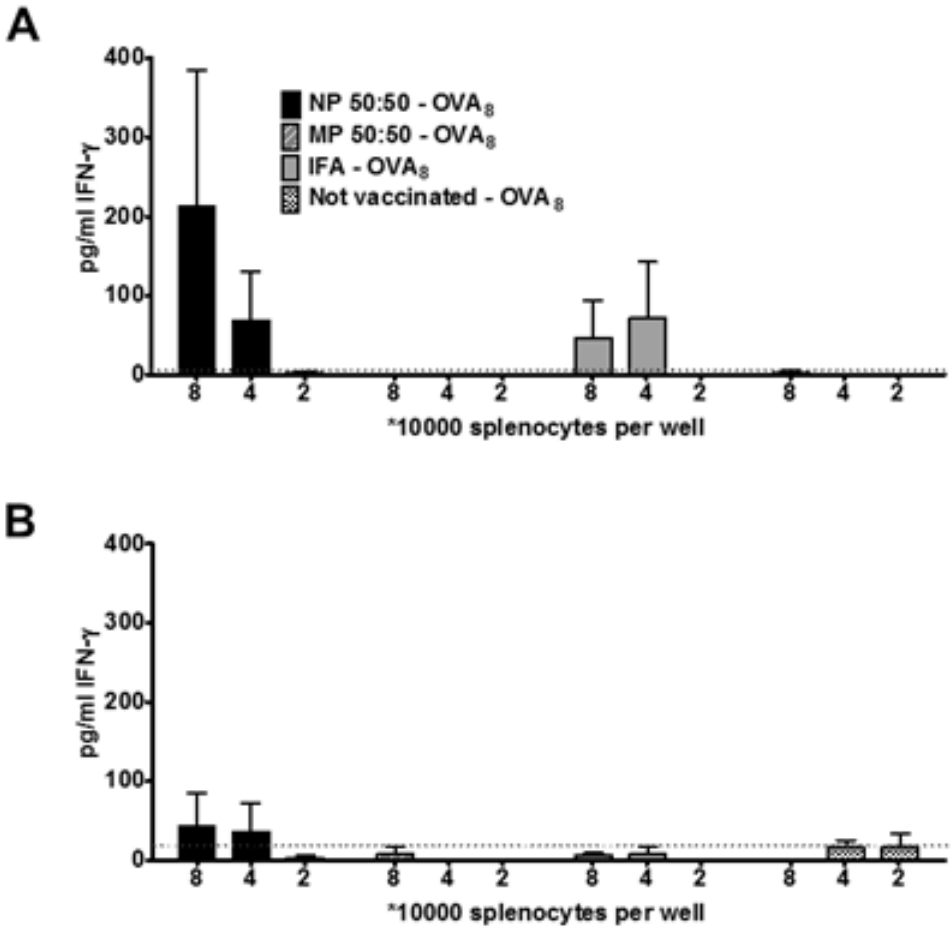
A) NP 50:50



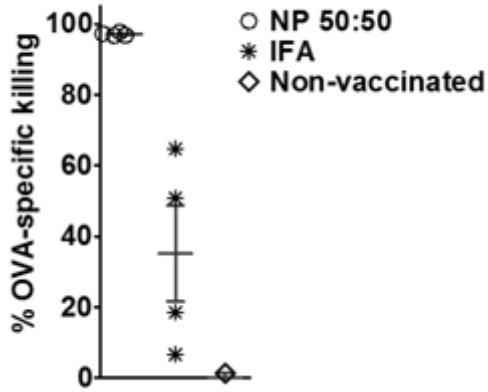
B) NP 75:25



Supplemental Figure 4: Intensity-weighted size distribution of NP determined by DLS. **A)** NP 50:50. **B)** NP 75:25. Data are presented as average ± standard deviation of n=3 independent batches.



Supplemental Figure 5: IFN-γ production after vaccination with NP. Animals were vaccinated with 50 µg OVA and 20 µg poly(I:C) formulated in NP 50:50, MP 50:50 or in IFA. A) Mice were sacrificed on day 7 post-vaccination, spleens harvested and single cell suspensions re-stimulated with 1 µM SIINFEKL (OVA8). 72 h later the amount of Ag-specific IFN-γ produced was determined by ELISA. B) Animals vaccinated twice (day 0 and 28) were sacrificed on day 42 and the ex vivo IFN-γ production analyzed 72 h later. Red dotted lines indicate average background production of cytokines in the absence of specific stimulation.



Supplemental Figure 6: PLGA-OVA/poly(I:C) NP induce cytotoxic $CD8^+$ T cells *in vivo*. *In vivo* cytotoxic capacity of primed OVA-specific T cells were determined in animals which received a single s.c. vaccinations with the 50 µg OVA and 20 µg poly(I:C) formulated in NP 50:50 (open circles) or IFA (asterisks), and in non-vaccinated mice (open diamonds). Vaccinated mice received CFSE-labeled OVA8 (specific) or FLU9 (control) short peptide loaded target cells (Ly5.1. splenocytes) 7 days after vaccination. Animals were sacrificed 18 hr later and the % target cells determined flow cytometry and calculated as described in M&M. Results shown are from one experiment and present averages ± SEM from n = 4 mice per group.

Chapter 4

Efficient *ex vivo* induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles

Rodney A. Rosalia, Ana Luisa Silva, Marcel Camps, Ahmed Allam, Wim Jiskoot, Sjoerd H. van der Burg, Ferry Ossendorp, Jaap Oostendorp

Cancer Immunology, Immunotherapy 2013 62(7): 1161-73

Abstract

Protein antigen (Ag)-based immunotherapies have the advantage to induce T cells with a potentially broad repertoire of specificities. However, soluble protein Ag is generally poorly cross-presented in MHC class I molecules and not efficient in inducing robust cytotoxic $CD8^+$ T cell responses. In the present study, we have applied poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NP) which strongly improve protein Ag presentation by dendritic cells (DC) in the absence of additional TLR ligands or targeting devices. Protein Ag loaded DC were used as antigen presenting cells (APC) to stimulate T cells *in vitro* and subsequently analyzed *in vivo* for their anti-tumor effect via adoptive transfer, a treatment strategy widely studied in clinical trials as a therapy against various malignancies. In a direct comparison with soluble protein Ag, we show that DC presentation of protein encapsulated in plain PLGA-NP results in efficient activation of $CD4^+$ and $CD8^+$ T cells as reflected by high numbers of activated $CD69^+$ and $CD25^+$, interferon (IFN)-γ and interleukin (IL)-2-producing T cells. Adoptive transfer of PLGA-NP-activated $CD8^+$ T cells in tumor-bearing mice displayed good *in vivo* expansion capacity, potent Ag-specific cytotoxicity and IFN-γ cytokine production, resulting in curing mice with established tumors. We conclude that delivery of protein Ag through encapsulation in plain PLGA-NP is a very efficient and simple procedure to stimulate potent anti-tumor T cells.

Keywords: PLGA, OVA, Ag cross-presentation, Adoptive immunotherapy

Précis
This paper shows that DC loaded with protein encapsulated in biodegradable and clinically applied polymer particles efficiently activate $CD8^+$ T cells *in vitro* which upon adoptive transfer *in vivo* show potent anti-tumor immune responses.

1. Introduction

The adaptive immune system plays a major role in anti-tumor control. Induction of a specific immune response against tumor-associated antigen (Ag) is a potential approach for targeted immunotherapy of cancer. The first step in the initiation of an effective anti-tumor response is the uptake of tumor-associated Ag by dendritic cells (DC) and their subsequent presentation to naïve T cells [1,2,3]. DC are highly efficient antigen presenting cells (APC) and play a central role in initiating and regulating adaptive immunity. DC internalize and process exogenous protein Ag and present processed peptide epitopes in the grooves of MHC class I and II molecules to prime $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ helper T (Th) cells, respectively [4]. CTL are capable of direct clearance of malignant cells [5]. Th cells have shown to be vital in CTL priming through CD40-CD40L interactions with DC [6,7]. In addition, activated Th cells secrete cytokines like IL-2 important for CTL proliferation [8,9].

Full-length protein Ag comprise all potential naturally occurring Th and CTL epitopes

and can be clinically applied irrespective of the patient's HLA haplotype. For that reason, protein-based tumor-associated Ag is currently being applied in a variety of immunotherapeutic approaches against cancer [10,11]. However, recent studies have indicated that cross-presentation of protein Ag is an inefficient process leading to poor CTL responses [12,13]. Therefore, improving cross-presentation of protein Ag by DC is essential to further exploit cancer immunotherapy.

Nanoparticles (NP) prepared from the polymer poly(lactic-co-glycolic acid) (PLGA) are promising clinical grade carriers for improving Ag delivery to DC [14,15,16]. PLGA polymers were originally reported for their use as sutures and implants for surgery [17] and since then they have been applied for the preparation of particles for drug delivery purposes, including the delivery of anti-cancer agents [18,19,20]. Internalization of protein Ag-loaded PLGA particles by DC is very efficient, resulting in adequate MHC class I cross-presentation and CTL proliferation *in vitro* [21]. Despite highly efficient DC uptake and cross-presentation *in vitro*, experimental tumor models have shown that the therapeutic effect of PLGA particle-based protein vaccination *in vivo* is strictly dependent on co-encapsulation of Toll like receptor ligands (TLRL) [22]. The necessity for the addition of TLRL for *in vivo* responses is most likely related to observations showing that PLGA-polymers on their own exhibit poor DC or macrophage stimulatory capacity in comparison to TLR4L [23,24]. However TLRL are dispensable for T cell activation *in vitro*, as reported by two previous studies using biodegradable polymer based artificial APC as a method to stimulate T cells *in vitro*. Applying an elegant method to formulate artificial APC using PLGA, T cells were stimulated *in vitro* with efficient proliferative and cytokine producing capacity [25,26]. However, the *in vivo* effector functions of the *in vitro* stimulated T cells were not studied in those reports.

In the present study, the intrinsic capacity of plain protein Ag-loaded PLGA-NP to induce anti-tumor effector T cells with potent functionality *in vivo* is reported. DC, the immune system's natural and most potent APC, express known and yet unknown co-stimulatory molecules and produce various cytokines vital for optimal T cell priming [27,28]. Using murine DC, we performed a detailed analysis of the ability of protein Ag-loaded PLGA-NP, lacking any additional TLRL or targeting moiety, to induce potent tumor-specific effector T cells. For this analysis, we have used a murine model for adoptive T cell transfer therapy, a treatment modality that has been successfully tested in various (pre-)clinical studies against various types of cancer [29,30]. In this murine adoptive T cell transfer therapy model, we show that protein Ag encapsulated in PLGA efficiently and rapidly induces highly activated specific effector $CD8^+$ T cells with a type I cytokine profile that vigorously expand *in vivo* in tumor-bearing mice and have the potency to eradicate established aggressive tumors.

2. Materials and Methods

2.1. Cells

D1 cells, a GM-CSF dependent immature dendritic cell line derived from spleen of WT C57BL/6 (H-2b) mice, were cultured as described previously [35]. Freshly isolated DC (BMDC) were cultured from mouse bone marrow (BM) cells by collecting femurs from WT C57BL/6 strain and cultured as published previously by our group [36]. After 10 days of culture, large numbers of typical DC were obtained which were at least 90% positive for murine DC marker CD11c (data not shown). B3Z $CD8^+$ T-cell hybridoma cell line, specific for the H-2Kb-restricted OVA257–264 CTL epitope SIINFEKL, expressing a β-galactosidase construct under the regulation of the NF-AT element from the IL-2 promoter, was cultured as described before [37] OT-IIZ, a $CD4^+$ T-cell hybridoma cell line, specific for the I-Ab-restricted OVA OVA323–339 Th epitope, expressing the same β-galactosidase construct, was produced in house. The weakly immunogenic and highly aggressive OVA-transfected B16 tumor cell line (B16-OVA), syngeneic to the C57BL/6 strain, was cultured as described [38].

2.2. Preparation and characterization of protein Ag-loaded PLGA-NP

PLGA-NP were prepared using 7-17 kDa PLGA 50:50 (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany) by applying a modified "water-in-oil-in-water" solvent evaporation method as described [39]. In brief, 50 µl of 20 mg/ml pure, endotoxin-free ovalbumin (OVA, Worthington LS003048) dissolved in 25 mM Hepes buffer (pH 7.4) was emulsified with 1 ml of dichloromethane (DCM) containing 25 mg of PLGA with an ultrasonic processor for 15 s at 70 W (Branson Instruments, CT, USA). The secondary emulsion was prepared with 2 ml of 1% (w/v) polyvinyl alcohol (PVA) in water. The double emulsion was then transferred into 25 ml of a 0.3% (w/v) PVA solution, and stirred at 37 °C for 1 h, and the resulting NP were harvested and washed twice with Milli-Q water by centrifugation at 8000 g for 10 min. The NP suspension was aliquoted in cryovials and lyophilized for 24 h. Prior to use, lyophilized NP particle size distribution was determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern UK) after resuspension of the particles in Milli-Q water. The zeta potential of the particles was also measured with the NanoSizer ZS by laser Doppler velocimetry. The OVA content of the particles was determined with a BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, and encapsulation efficiency (%EE) was determined according to **equation 1**. 500 µg lyophilized PLGA-OVA were resuspended in 350 µl sterile MQ water and endotoxin

content were determined with Bacterial endotoxins methode D. Chromogenic kinetic method' an assay according to European Pharmacopeia 2.6.14 seventh edition.

$$\%\,EE = \frac{\text{protein mass in NP}}{\text{total protein mass}} \times 100 \quad (1)$$

2.2.1. In vitro release study of PLGA-encapsulated protein

For release studies, protein-loaded PLGA NP were prepared as described, but with the addition of 1% (w/w total OVA) of Ovalbumin-Alexa Fluor® 488 (PLGA-OVA-Alexa488) (Invitrogen). Encapsulation of OVA-Alexa488 proceeds with similar efficiency as the regular OVA with no dye conjugated (See **Table 1**).

Table 1: PLGA-OVA NP characteristics

Formulation	Size (nm)	PDI	ZP (mV)	Protein loading (µg OVA/mg PLGA)	OVA encapsulation efficiency (%)	Endotoxin level (IU/ml)
PLGA-OVA	274 ± 19	0.18 ± 0.02	-27 ± 1	25 ± 1	62 ± 2	0.03 ± 0.00
PLGA-OVA Alexa	338 ± 12	0.22 ± 0.10	-27 ± 5	20 ± 1	49 ± 4	0.03 ± 0.03
PLGA empty	311 ± 52	0.14 ± 0.06	-30 ± 7	n/a	n/a	n/a

PLGA-OVA-Alexa488 were resuspended in 1x PBS pH 7.4 at a concentration of 10 mg PLGA/ml and maintained at 37oC in a water bath under constant tangential shaking at 100 rpm in a GFL 1086 shaking water bath (Burgwedel, Germany). At regular time intervals, 250 µl samples of the suspension were taken, centrifuged for 20 min at 18,000×g and supernatants were stored at 4ºC until fluorescence intensity, was determined by fluorescence spectrometry (Tecan, Infinite M 1000). Concentration of OVA-Alexa488 in the supernatant was assessed against a calibration curve containing known concentrations of OVA-Alexa488. Protein release profiles were generated for each NP formulation in terms of cumulative antigen release (%) over time. Release was determined according to **equation 2**.

$$\%\,R = \frac{\text{protein mass in supernatant}}{\text{protein mass in supernatant} + \text{protein mass in NP}} \times 100 \quad (2)$$

2.3. Enzyme-linked Immunosorbent Assay (ELISA)

DC (100,000/well) were plated into a 96-well round bottom plate and incubated for 24 hr with titrated amounts of Ag. Supernatants were harvested and tested for IL-12 p40 using an ELISA assay kit (BD OptEIA™ MOUSE IL-12 Cat. Nr 555165) following manufacturer's instructions.

2.4. MHC class I or class II-restricted Ag presentation and T cell proliferation

DC were incubated for 24 h with soluble OVA (sOVA) or OVA encapsulated in PLGA-NP (PLGA-OVA) at the indicated concentrations. Cells were washed followed by overnight incubation at 37 °C in the presence of either B3Z - to measure MHC class I Ag presentation of SIINFEKL (OVA257-264) in H-2Kb - or OT-IIZ cells - to assess MHC class II Ag presentation of ISQAVHAAHAEINEAGR (OVA323-339) in I-Ab. A colorimetric assay using chlorophenol red-β-D-galactopyranoside (CPRG) as substrate was used to detect IL-2 induced lacZ activity. OVA-specific proliferation of naïve $CD8^+$ and $CD4^+$ T cells was performed by culturing OT-I or OT-II splenocytes in the presence of DC loaded with titrated amounts of PLGA-OVA or sOVA. After 24 h incubated cells were pulsed with [3H]-thymidine and cultured further overnight. Samples were then counted on a TopCountTM microplate scintillation counter (Packard Instrument Co., Meridan, CT, USA).

2.5. Analysis of T cell phenotype and T cell cytokine profile

DC were loaded for 24 h with 0.25 μM OVA in PLGA (PLGA-OVA) or soluble OVA (sOVA), washed extensively and used as APC to stimulate spleen suspensions from OT-I and OT-II mice. DC and splenocytes were co-cultured for 24 h in the presence of 7.5 μg/ml Brefeldin A. Total cells were harvested, washed twice with PBA buffer (0.01 M sodium phosphate, 0.15 M NaCl, 1% (w/v) BSA, and 0.01% (w/v) sodium azide) followed by staining with PerCP rat anti-mouse CD8α monoclonal antibodies (mAb) and AF-conjugated rat anti-mouse CD3 mAb. To assess T cell activation, cells were stained with FITC-conjugated rat anti-mouse CD69 mAb or PeCy7-conjugated rat anti-mouse CD25 mAb. To study the T cell cytokine profile, $CD8^+$ T cells were stained as above and subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Intracellular IFN-γ in the T cells was stained with APC-conjugated rat anti-mouse IFN-γ. Similarly, IL-2 and IL-4 were stained using PE-conjugated rat anti-mouse IL-2, IL-4 respectively. TNF-α was stained with FITC-conjugated rat anti-mouse TNF-α mAb. All antibodies were purchased from BD

Pharmingen. Flow cytometry analysis was performed using a LSRII flow cytometer (BD Pharmingen) and analyzed with FlowJo software (Treestar).

2.6. *In vivo* cytotoxicity

To obtain OVA-specific effector $CD8^+$ T cells, single cell suspensions were prepared from spleen and lymph nodes of OT-I mice, washed twice and resuspended in IMDM supplemented with 10% (v/v) FCS. Whole single cell suspensions were cultured in 6-wells plates with Ag (0.25 μM) loaded DC for 24 h at a ratio of 25:1. DC and splenocyte cultures were incubated for 24 hr at 37ºC. Purified $CD8^+$ T cells were obtained by a negative selection protocol using the Mouse CD8 T Cell Lymphocyte Enrichment Set - DM (BD Biosciences) according to the manufacturer's instructions. This protocol yielded $CD8^+$ T cell purities of at least 90% (data not shown). 2.5 x 106 Purified $CD8^+$ T cells were transferred to syngeneic WT C57BL/6 animals that were rested for 24 h after adoptive cell transfer. To obtain OVA-specific target cells, splenocytes from naïve congeneic C57BL/6 Ly5.1 mice were pulsed for 1 h with 1 μM of SIINFEKL-peptide and co-stained with 10 μM CFSE (CFSE-high) (Molecular Probes, Eugene, OR). As a negative control, 1 μM of the immunodominant ASNENMETM-peptide derived from the influenza virus nucleoprotein co-stained with 0.5 μM CFSE (CFSE-low) was used. Specific and non-specific target cells were mixed 1:1 and injected intravenously (i.v.; 10 x 106 cells of each population). Eighteen hours after cells were transferred, mice were sacrificed and spleen cells were harvested to prepare single cell suspensions that were then subjected to flow cytometric analysis. Injected cells were distinguished by APC-conjugated rat anti-mouse CD45.1 mAb. The percentage specific killing was calculated as follow: 100 - ([(% SIINFEKL-peptide pulsed in treated/% ASNENMETM-pulsed in treated)/(% SIINFEKL-peptide pulsed in non-treated/% ASNENMETM-pulsed in non-treated)] x 100).

2.7. Adoptive transfer OVA-specific T cells in B16-OVA tumor bearing and naïve mice

WT C57BL/6 mice were injected subcutaneously (s.c.) in the right flank with 2 x 105 B16-OVA melanoma cells. Seven days after tumor injection, when tumors were palpable, mice were treated by intravenous infusion of 2.5 x 106 purified effector $CD8^+$ T cells derived from OT-I mice, *ex vivo* stimulated for 24 h in the presence of DC loaded with either PLGA-OVA or sOVA. Tumor growth was measured 1 - 3 times a week and survival was monitored daily. Tumor size (mm2) was calculated by (length) × (width). Mice with tumor sizes that equaled or exceeded 140 mm2 were sacrificed. Tail vein blood samples were collected on day 10, 17 and 31 after $CD8^+$ T cell transfer. Blood samples were prepared by erythrocyte lysis, followed by 2 washing steps with PBA buffer. Transferred $CD8^+$ T cells were analyzed by co-staining with APC-conjugated rat anti-mouse Thy1.1 mAb, FITC-conjugated anti-mouse CD8α mAb and AF-conjugated rat anti-mouse CD3 mAb in combination with APC-Cy7-conjugated anti-mouse CD44

antibody and PB-conjugated anti-mouse CD62L antibody. OVA-specific $CD8^+$ T cell mediated cytokine production was detected by overnight stimulation of peripheral blood cells with SIINFEKL-peptide in the presence of 7.5 µg/ml Brefeldin A. Medium was used as a negative control to correct for baseline cytokine production. Cytokine profile was analyzed by intracellular cytokine staining as described above.

2.8. Statistical analysis

Graph Pad Prism software was used for statistical analysis. Values and percentages of specific $CD8^+$ T cells and secreted cytokine production were analyzed by two-tailed unpaired Student t test. Differences in animal survival between the different groups were calculated using Log-rank (Mantel-Cox) test.

3. Results

3.1. Nanoparticle characterization and protein antigen load and release

We prepared several batches of PLGA-OVA NP with similar characteristics. Particles used in our study had an average size of 327 ± 65 nm (mean ± SD; n = 7) and a polydispersity index (PDI) of 0.19 ± 0.07. Encapsulation efficiency of OVA in NP was determined to be 59 ± 5%. Empty particles used as control particles in this study had a comparable size (311 ± 52 nm) and PDI (0.15 ± 0.05). Endotoxin levels were determined for the prepared batches and was shown to be below 0.04 IU/ml in particle suspensions prepared as described in material and methods. (see **Table 1)**. Release kinetics of OVA from the PLGA-OVA particles were analyzed over a period of 35 days. The validity of using OVA-Alexa 488 fluorescence as a measure of (unlabeled) OVA release was confirmed by measuring the OVA content of the nanoparticles and the total amount released at the end of the release study by BCA assay, which gave very similar values as the fluorescence method (results not shown).

The NP had a burst release of the encapsulated OVA of 28.1 ± 0.2%. At the end of the analysis, we could detect 80.4 ± 2.2% of released OVA in suspension indicating that after 35 days about 20% of the originally encapsulated OVA was still associated with NP showing the slow release character of these NP (see **Supplemental Figure 1**).

3.2. Efficient protein MHC class I and class II Ag presentation by DC loaded with protein encapsulated in PLGA-NP

The efficiency of Ag (cross)-presentation of encapsulated protein Ag in comparison to soluble protein Ag was studied *in vitro*. DC were incubated for 24 h with titrated amounts of Ag, as indicated in μM, either encapsulated in PLGA-NP (PLGA-OVA) or in soluble form (sOVA). Ag presentation by MHC class I or II was assessed using the $CD8^+$ (B3Z) and $CD4^+$ (OT-IIZ) T cell hybridomas. DC loaded with PLGA-OVA very efficiently triggered B3Z T cells (**Figure 1A**). In contrast, DC pulsed with sOVA poorly stimulated B3Z $CD8^+$ T cells unless very high concentrations (≥ 64 μM) of sOVA were used (data not shown). MHC class I cross-presentation of protein Ag was strictly dependent on encapsulation in PLGA-NP, as a mixture of the sOVA with empty PLGA-NP did not induce $CD8^+$ T cell activation (**Figure 1B**). In addition, DC loaded with PLGA-OVA resulted in at least 100-fold enhanced activation of OT-IIZ $CD4^+$ T cells in comparison to DC loaded with sOVA, indicating that also MHC class II presentation was dramatically improved by encapsulation (**Figure 1C**). Next to Ag presentation, we analyzed proliferation of naïve $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells induced by DC loaded with PLGA-OVA or sOVA. Co-culture of Ag pulsed DC with either OT-I or OT-II T cells for 72 h, including overnight incubation in the presence of [3H]-thymidine for the last 18 h showed that PLGA-OVA was at least 1000-fold more efficient than sOVA in inducing OT-I T cell proliferation (**Figure 1D**) and 100-fold better than sOVA in inducing OT-II T cell proliferation (**Figure 1E**). Similar to the used D1 dendritic cells, freshly isolated BMDC loaded with PLGA-OVA were superior in comparison to sOVA-loaded BMDC in the stimulation of OT-I and OT-II T cells resulting in improved T cell proliferation (**Supplemental Figure 2**). In addition, we analyzed DC maturation by surface expression of CD86 and determining the amount of IL-12 in culture supernatants after incubation with the NP after 24 hr incubation. Our data show that the empty or OVA-loaded PLGA NP do not detectably activate and mature DC (**Supplemental Figure 3**) in contrast to LPS (TLR4L) or PolyI:C (TLR3L). This indicates that encapsulation of soluble protein antigen in plain PLGA NP strongly enhances antigen presentation by DC irrespective of DC maturation.

3.3. Activation of T cells by DC loaded with PLGA-NP-encapsulated protein Ag

We analyzed whether PLGA-NP based delivery of protein Ag could induce T cell activation and production of pro-inflammatory cytokines. Naïve OVA-specific $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells were stimulated for 24 h in the presence of PLGA-OVA- or sOVA-loaded DC and analyzed for cells expressing the early activation marker CD69. Both $CD8^+$ (**Figure 2A**) and $CD4^+$ (**Figure 2B**) T cells showed strongly enhanced

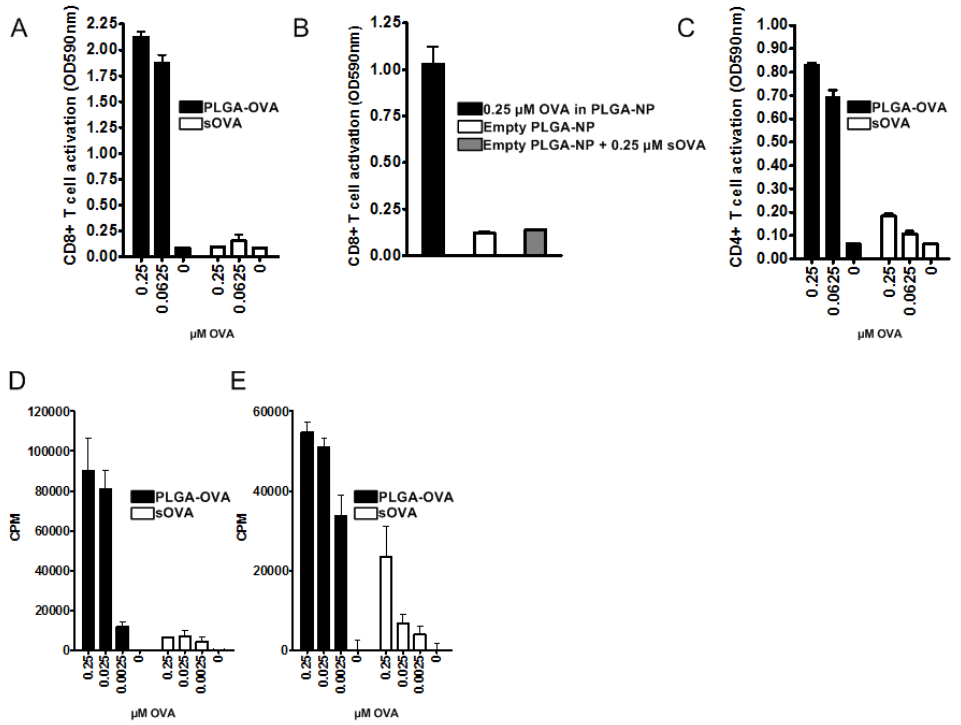


Figure 1: Efficient MHC class I and- class II presentation of OVA Ag incorporated in PLGA-NP. A) D1 cells were pulsed for 24 h with titrated amounts (µM) of OVA, either in soluble form sOVA or encapsulated in PLGA-NP (PLGA-OVA). MHC class I presentation was detected by co-culture with H-2Kb/SIINFEKL-specific B3Z CD8⁺ T cells; **B)** D1 cells were pulsed for 24 h with 0.25 µM OVA in PLGA-OVA, empty PLGA-NP, or a mixture of empty PLGA-NP with 0.25 µM sOVA, washed and co-cultured with B3Z CD8⁺ T cells to assess MHC class I Ag presentation; **C)** D1 cells were pulsed for 24 h with titrated amounts of PLGA-OVA or sOVA, washed, and co-cultured with I-Ab/ISQAVHAAHAEINEAGR-specific OT-IIZ CD4⁺ T cells to assess MHC class II Ag presentation. BMDC were loaded with titrated amounts of PLGA-OVA or sOVA. Ag loaded DC were subsequently used to activate naïve OT-I; **D)** or OT-II T **(E)** cells for 72 h. T cell proliferation was measured in triplicate by 3[H]-thymidine uptake. Data shown are means of triplicate measurements ± SD from one representative example out of at least three independent experiments.

single IFN-γ producers and a relatively smaller population of IL-2 single producers. The cytokines IL-4 and TNF-α could not be detected after *in vitro* stimulation of either CD8⁺ or CD4⁺ T cells with PLGA-OVA pulsed DC (data not shown).

3.4. DC loaded PLGA-NP-encapsulated protein Ag induces CD8⁺ T cells with *in vivo* cytotoxic capacity

To assess their cytotoxic capacity, CD8⁺ T cells stimulated *in vitro* by PLGA-OVA-loaded DC were studied for their ability to lyse Ag-pulsed target cells *in vivo*. Following stimulation, the purified CD8⁺ T cells were transferred into recipient mice. After 24 h SIINFEKL-loaded target and control-target cells were injected and 18 h later mice were sacrificed and spleen single cell suspensions were analyzed by flow cytometry. In line with the observed activation status and cytokine profile, CD8⁺ T cells stimulated with PLGA-OVA-loaded DC demonstrated cytotoxicity against SIINFEKL-loaded target cells. In contrast, CD8⁺ T cells co-cultured in the presence of sOVA-loaded DC were not capable of killing target cells (**Figure 3A** and **B**).

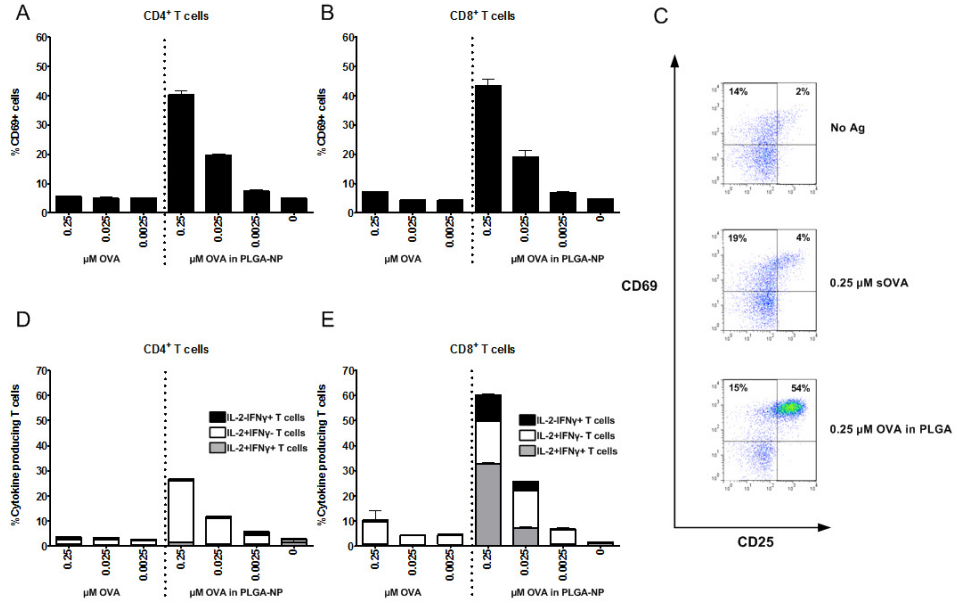


Figure 2: DC pulsed with PLGA-OVA, but not sOVA, induce strong activation of T cells. D1 cells were pulsed for 24 h with titrated amounts of sOVA or PLGA-OVA. Ag loaded DC were washed to remove excess Ag, and co-cultured for an additional 24 h with OT-I or OT-II splenocytes. Cells were harvested and analyzed by flow cytometry for the cell surface expression of A) CD69 on CD8⁺ T cells and B)CD4⁺ T cells ; C) Expression of CD25 and CD69 was analyzed on CD8⁺ T cells which were stimulated for 24 h with DC which were loaded with either PLGA-OVA or sOVA. Immature DC without Ag served as negative control. Intracellular production of IL-2 and IFN-γ by D) CD8⁺ T cells and E) CD4⁺ T cells was analyzed by flow cytometry after 24 hr stimulation with DC pulsed with titrated amounts of PLGA-OVA or sOVA. One representative experiment out of three independent experiments is shown. Data shown are means of triplicate measurements ± SD.

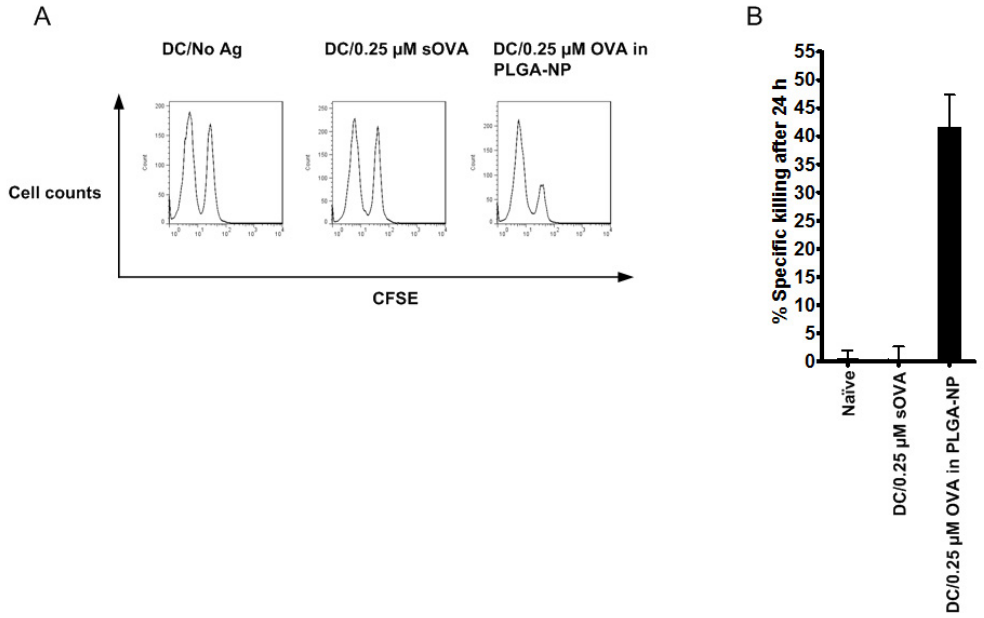


Figure 3: Enhanced *in vivo* **cytotoxicity of** *ex vivo* **PLGA-OVA-stimulated CD8⁺ T cells. A)** Mice were transferred with purified CD8⁺ OT-I T cells which were *in vitro* stimulated with D1 cells loaded with PLGA-OVA and sOVA. Differentially CFSE-labeled SIINFEKL-peptide loaded and control target cells were i.v. administered. After 18 h the spleens from recipient animals were harvested and analyzed by flow cytometry for percentage of specific killing of target cells; **B)** Experiment was performed twice and averages ± SEM of n = 7 mice for each condition are shown in bar graphs.

inoculated s.c. with OVA-expressing B16 melanoma tumor cells. After 7 days, animals were treated by adoptive T cell transfer therapy by a single i.v. injection of 2.5 x 106 purified OVA-specific CD8⁺ T cells stimulated for 24 h *in vitro* in the presence of DC loaded with either PLGA-OVA or sOVA. Tumor growth and animal survival in CD8⁺ T cell transferred mice were compared to those in non-treated animals. Animals were developing palpable tumors within 10 days after s.c. tumor inoculation. In tumor-bearing mice that were treated by adoptive transfer with OVA-specific CD8⁺ T cells stimulated in the presence of PLGA-OVA-loaded DC, we observed a clear therapeutic effect which resulted in delayed tumor growth in comparison to non-treated animals and animals treated with CD8⁺ T cells stimulated with sOVA loaded DC. We observed regression of tumors in the range of 2 - 4 mm2 in some animals by day 14, which were undetectable on day 18 after tumor challenge (insert in **Figure 4A**). By day 22, four animals within this group had tumor recurrences which eventually grew out. Nevertheless, 8 out of 12 tumor-challenged mice treated with PLGA-OVA induced CD8⁺ T cells remained tumor free for the duration of the experiment (**Figure 4A**). By contrast, in tumor challenged animals that received sOVA induced CD8⁺ T cells, tumors reappeared in 11 out of 12 mice and grew out, albeit at a decreased rate when compared to non-treated animals (**Figure 4B**). In all non-treated animals, tumors grew out fast and all mice were sacrificed

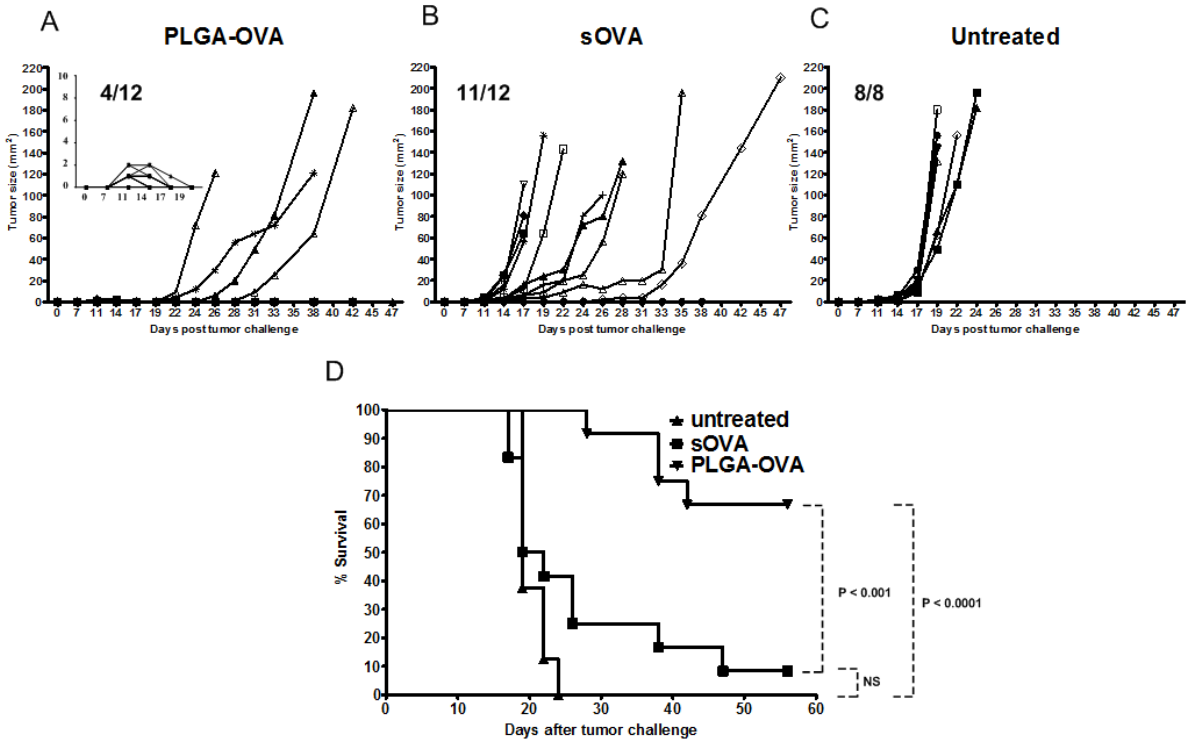


Figure 4: CD8⁺ T cells primed by PLGA-OVA loaded DC eradicate established tumors. Animals were inoculated s.c. on the right flank with 2 x 105 B16-OVA tumor cells and rested for 1 week followed by a single i.v. injection of 2.5 x 106 purified CD8⁺ T cells which were *ex vivo* stimulated with Ag loaded DC as described above. Tumor growth was monitored in individual animals treated with DC/PLGA-OVA induced CD8⁺ T cells (**A**; n = 12 animals), DC/sOVA induced CD8⁺ T cells (**B**; n = 12 animals) or in untreated animals (**C**; n = 8 animals). Insert in A) represents tumor growth curves in the initial 19 days after tumor challenge; **D**) Animal survival per group was assessed and differences between the different groups were calculated using Log-rank (Mantel-Cox) test. $p < 0.001$ for animals treated with DC/PLGA-OVA compared to DC/sOVA induced CD8⁺ T cells. $p < 0.0001$ for animals treated with DC/PLGA-OVA compared to untreated animals. NS = $p > 0.05$ for animals treated with DC/sOVA compared to untreated animals.

by day 24, because of maximum allowed tumor burden (**Figure 4C**). Tumors that did grow in animals that received PLGA-OVA induced $CD8^+$ T cells had a significantly slower average growth rate when compared to tumors from mice that received sOVA induced $CD8^+$ T cells. Consequently, the survival of mice treated with PLGA-OVA induced $CD8^+$ T cells was significantly higher when compared to mice treated with $CD8^+$ T cells stimulated in the presence of sOVA-loaded DC (**Figure 4D**).

3.6. $CD8^+$ T cells stimulated with PLGA-NP-encapsulated protein Ag efficiently expand and produce type I cytokines *in vivo*

We measured by flow cytometry the actual numbers of OVA-specific $CD8^+$ T cells ($CD8^+Thy1.1^+$ OT-I cells) in peripheral blood of tumor challenged mice up to a month after adoptive transfer. Ten days after adoptive transfer of equal amounts of purified OVA-specific $CD8^+$ T cells, mice that had received PLGA-OVA induced cells showed 5-fold higher levels of $CD8^+$ T cells than animals that had received sOVA stimulated $CD8^+$ T -cells (**Figure 5A**). The percentage of PLGA-OVA induced $CD8^+$ T cells remained significantly higher at day 17 and 31 after transfer. (**Figure 5B**). A similar *in vivo* expansion capacity of PLGA-OVA induced $CD8^+$ T cells was observed upon transfer in naïve mice, i.e. not challenged with tumors (data not shown). Furthermore, we analyzed the production of cytokines by $CD8^+$ T cells in peripheral blood by intracellular staining. To this end, peripheral blood mononuclear cells were harvested from mice at day 10, 17 and 31 after adoptive transfer and stimulated overnight in the presence of SIINFEKL-peptide. IFN-γ-, IL-2- and TNF-α-producing $CD8^+$ T cells were detectable by flow cytometry. On day 10 after adoptive transfer, we observed significantly higher percentages of IFN-γ-producing $CD8^+$ T cells in mice that had received PLGA-OVA induced $CD8^+$ T cells when compared to mice that had received sOVA induced $CD8^+$ T cells. We were unable to detect IFN-γ-producing $CD8^+$ T cells in tumor-bearing animals which were not treated using adoptive T cell transfer therapy (data not shown). Although, the percentage of IFN-γ producing $CD8^+$ T cells declined at day 17 and 31 after adoptive transfer, the IFN-γ-producing $CD8^+$ T cells remained significantly higher throughout the analysis period (**Figure 5C**). A trend in increased levels of IL-2- and TNF-α-producing $CD8^+$ T cells could be observed in mice transferred with PLGA-OVA but not with sOVA induced $CD8^+$ T cells (data not shown). In addition, the phenotype of peripheral blood OVA-specific T cells at day 17 and 31 after adoptive transfer of PLGA-OVA induced $CD8^+$ T cells was analyzed. The majority of the cells possessed a central memory phenotype based on high expression of CD62L (L-selectin) and CD44 (**Figure 5D**) showing the superior functionality of the T cells expanded with this simple expansion protocol with NP encapsulated protein antigen.

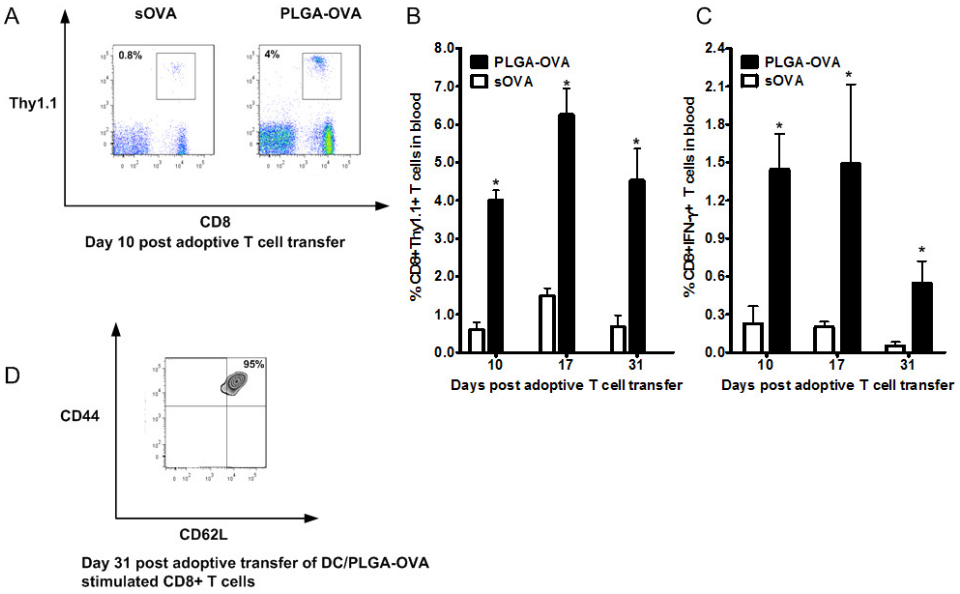


Figure 5: PLGA-OVA stimulated CD8⁺ T cells expand and persist in the peripheral blood and have higher capacity to produce IFN-γ. A) Tumor bearing animals received a single i.v. injection of CD8⁺ T cells stimulated with PLGA-OVA-loaded DC. Tail vein blood samples were taken on day 10 after adoptive transfer of the CD8⁺ T cells and numbers of CD8⁺Thy1.1⁺ T cells were measured by flow cytometry; **B)** *In vivo* persistence of i.v. transferred CD8⁺Thy1.1⁺ T cells in tumor bearing animals was monitored for 4 weeks in blood on day 17 and 31; **C)** Intracellular IFN-γ production by CD8⁺ T cells was analyzed on day 10, 17 and 31 after adoptive transfer; **D)** Memory phenotype of transferred DC/PLGA-OVA *in vitro* stimulated CD8⁺Thy1.1⁺ T cells was determined by analysis of CD44 and CD62L surface expression. Results shown are averages ± SEM from n = 3-12 mice per group, dependent on the number of animals alive at each time-point post tumor challenge. * = $p < 0.05$ for animals treated with DC/PLGA-OVA compared to DC/sOVA induced CD8⁺ T cells using a un-paired student t test.

4. Discussion

In this study we analyzed the phenotype and *in vivo* functionality of T cells stimulated *in vitro* by DC loaded with plain PLGA-NP encapsulated protein Ag with no additional immune stimulatory agent or targeting moiety. We showed that encapsulation of protein Ag in plain PLGA-NP not only enhanced Ag (cross-)presentation by DC but also improved the functionality of the induced T cells to cure animals from tumors upon adoptive T cell transfer. OVA antigen in PLGA-NP was more efficiently processed and presented in MHC class I and II by DC and resulted in potent activation and proliferation of OVA-specific CD8⁺ and CD4⁺ T cells, high production of type I cytokines and tumor

control resulting in an overall survival of 75% of tumor-bearing animals.

PLGA-based particles as vaccine delivery systems were pioneered already more than 30 years ago [14]l lactic-co-glycolic acid. Several studies have shown that efficient anti-tumor immune responses *in vivo* by PLGA-particles require not only encapsulated Ag, but also a co-encapsulated adjuvant such as a TLRL, surface coating of particles with mannan or protamine to stimulate immunity or DC targeting moieties, for example anti-DEC205 antibodies [22,36,37,38].

$CD8^+$ T cells induced in our study were applied for adoptive T cell transfer purposes. Adoptive T cell transfer therapy potency in (pre)clinical setting is enhanced upon efficient *ex vivo/in vitro* stimulation/manipulation of donor T cells [30,39,40]. Using a similar murine model, efficient stimulation of T cells with potent effector functions was reported using artificial APC systems [26]. They constructed PLGA-based artificial APC expressing MHC class I molecules containing a specific CTL short-peptide epitope, which also provides T cell co-stimulation in the form of CD28 and CD3 triggering and releases IL-2 [41,42]. We propose that our simple approach with natural APC is equally efficient and has the advantage that our method is not restricted to the known MHC class I and II-presented T cells epitopes. In addition, the use of natural DC as APC might facilitate priming of T cells via more co-stimulatory pathways [43,44,45] and additional DC-mediated cytokines required for optimal type I pro-inflammatory T cell activation, for example IL-12 [46], and avoids sub-optimal formation of the immunological synapse as has been described for other bead-based artificial APC systems [47].

Adoptive T cell therapy has yielded promising results as a cancer immunotherapy in the last decade [30,48]. Standard adoptive transfer protocols mandate that T cells are cultured for 2 – 14 days in the presence of specific Ag and exogenous cytokines [39,49,50,51] for optimal stimulation and expansion. In contrast, we opted for a short 24 h stimulation of T cells without addition of any exogenous cytokines. We favor short incubation with DC loaded with PLGA-OVA, which potently activates T cells, because longer incubation periods might tip the balance to activation induced cell death (AICD) [52,53]. In addition, our protocol allowed us to transfer T cells that were not skewed based on the cytokines added to the cultures [54,55] nor negatively affected by the added cytokines [42,56,57].

In our culture systems we used two types of DC: D1 cells, a well characterized murine splenic DC cell line, originally isolated from WT C57BL/6 animals [58] and bone marrow derived DC. Both $CD11c^+$ myeloid types of DC were cultured as immature cells in GM-CSF containing media. D1 DC do not exhibit substantial functional differences with BMDC, they possess equal capacity to prime T cells and upon transfer to recipient animals show similar efficiency to induce protective anti-tumor immunity [32]. We compared $CD4^+$ and $CD8^+$ T cell proliferation by PLGA-NP encapsulated protein Ag and we observed similar observations using either D1 cells or WT BMDC as APC. Therefore, easily cultured myeloid types of DC are well suited for the T cell activation protocol with NP encapsulated Ag we describe here.

In this study splenocytes from OT-I mice, which contain high numbers of OVA-specific

T cells, were used to activate and adoptively transfer into recipient animals. We are aware that in clinical settings, majority of patients which were treated with adoptive T cell transfer therapy exhibit lower precursor frequencies of TAA-specific T cells that require stimulation and expansion to yield sufficient numbers for adoptive transfer. Our system still works by DC/PLGA-OVA stimulation of cell cultures containing lower amounts (between 1 - 10%) of OVA-specific T cells regarding T cell activation and cytokine production (data not shown). On the other hand, higher precursor frequencies of both $CD4^+$ and $CD8^+$ Ag-specific T cells have been observed in draining lymph nodes of cervical cancer patients [59,60]. These cells were able to produce type I pro-inflammatory cytokines and proliferate upon specific stimulation suggesting that these cells might be suitable for future adoptive T cell transfer protocols. Indeed, in a melanoma patient case report, it was shown that $CD4^+$ T cells isolated in relatively higher precursor frequencies could be successfully stimulated and transferred to the recipient back to the patient resulting in a clinical response [61].

The PLGA-OVA particles used in our study are devoid of any additional TLRL or immunostimulatory agents. We observed no differences in T-cell proliferation by MyD88 KO BMDC loaded with PLGA-OVA (data not shown), it is therefore unlikely that any unanticipated TLR-stimulation plays a role in our system. The enhanced T cell proliferation and activation is most likely the result of enhanced uptake of Ag available for efficient processing and MHC presentation. Uptake of particulate matter proceeds via phagocytosis [62,63] of protein Ag-loaded PLGA particles by DC resulting in adequate MHC class I cross-presentation to CTL [64]. DC internalize particles and maintain these intracellularly for up to 72 hr [65]. Prolonged presence of Ag inside cells has been shown to result in sustained MHC class I Ag presentation and efficient priming of $CD8^+$ T cells [66].

Transferred DC/PLGA-OVA stimulated $CD8^+$ T cells were still detectable 31 days post adoptive transfer and the majority of these cells possessed a central memory phenotype correlating with tumor control. Numbers of specific T cells *in vivo* have been shown to directly correlate with tumor-regression [67]. Efficient tumor killing is achieved upon efficient $CD8^+$ T cell activation accompanied with high production of type I pro-inflammatory associated cytokines which is dependent on the method of *in vitro* activation [68,69]. Our data are in line with these reports as peripheral $CD8^+$ T cells from mice which received DC/PLGA-OVA stimulated $CD8^+$ T cells were capable of producing type I pro-inflammatory cytokines upon specific peptide stimulation. Indeed, expansion of IFN-γ producing T cells correlates with clinical effect in patients with human papillomavirus type 16 induced vulvar intraepithelial neoplasia [70].

Numbers of cytokine-producing cells decreased with time in all treated animal groups The decrease in numbers of cytokine-producing OVA-specific $CD8^+$ T cells in time might be related to lack of sufficient OVA-specific $CD4^+$ T cells. Co-transfer of DC/PLGA-OVA *in vitro* stimulated $CD4^+$ T cells may prolong and sustain higher numbers of cytokine producing effector $CD8^+$ T cells [71].

We conclude that protein Ag delivery by PLGA-NP might be an attractive and simple strategy to improve *ex vivo* tumor-specific T cell stimulation for clinical adoptive

T transfer therapy. Apparently, the intrinsic characteristics of PLGA-Ag NP to be efficiently internalized and processed by DC is sufficient to induce effector T cells *in vitro* with expansion capacity *in vivo*, and with strong therapeutic effectiveness. So, encapsulation of tumor associated protein Ag in PLGA-NP may serve as a clinically feasible strategy to generate T cells with optimal effector quality for adoptive transfer-based immunotherapy of cancer.

Acknowledgements

This study was supported by grants from Immune System Activation (ISA) Pharmaceuticals, University of Leiden and the Leiden University Medical Center. We thank Ing. S. Marlina Sibuea for the studies performed to determine the release kinetics of the protein from the PLGA-NP and Ing. W.M.Ramp-Koopmanschap for the analysis of endotoxin levels of the formulated PLGA-OVA batches.

Conflict of Interest

The authors declare that they have no conflict of interest.

5. References

1. Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. Nature (392) 6673:245-252
2. Melief, C. J. (2008) Cancer immunotherapy by dendritic cells. Immunity. (29) 3:372-383
3. Steinman, R. M. and Hemmi, H. (2006) Dendritic cells: translating innate to adaptive immunity. Curr.Top.Microbiol.Immunol. (311) :17-58
4. Petersen, T. R., Dickgreber, N., and Hermans, I. F. (2010) Tumor antigen presentation by dendritic cells. Crit Rev.Immunol. (30) 4:345-386
5. Kawakami, Y., Eliyahu, S., Delgado, C. H., Robbins, P. F., Sakaguchi, K., Appella, E., Yannelli, J. R., Adema, G. J., Miki, T., and Rosenberg, S. A. (1994) Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with *in vivo* tumor rejection. Proc.Natl.Acad.Sci.U.S.A (91) 14:6458-6462
6. Ossendorp, F., Mengede, E., Camps, M., Filius, R., and Melief, C. J. (1998) Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J.Exp.Med. (187) 5:693-702
7. Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature (393) 6684:480-483
8. Blachere, N. E., Morris, H. K., Braun, D., Saklani, H., Di Santo, J. P., Darnell, R. B., and Albert, M. L. (2006) IL-2 is required for the activation of memory $CD8^+$ T cells via antigen cross-presentation. J.Immunol. (176) 12:7288-7300
9. Keene, J. A. and Forman, J. (1982) Helper activity is required for the *in vivo* generation of cytotoxic T lymphocytes. J.Exp.Med. (155) 3:768-782
10. Mischo, A., Bubel, N., Cebon, J. S., Samaras, P., Petrausch, U., Stenner-Liewen, F., Schaefer, N. G., Kubuschok, B., Renner, C., and Wadle, A. (2011) Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad antibody responses in humans, a RAYS-based analysis. Int.J.Oncol. (39) 1:287-294
11. Sang, M., Lian, Y., Zhou, X., and Shan, B. (2011) MAGE-A family: attractive targets for cancer immunotherapy. Vaccine (29) 47:8496-8500
12. Zandvliet, M. L., van, Liempt E., Jedema, I., Veltrop-Duits, L. A., Willemze, R., Guchelaar, H. J., Falkenburg, J. H., and Meij, P. (2010) Co-ordinated isolation of $CD8^{(+)}$ and $CD4^{(+)}$ T cells recognizing a broad repertoire of cytomegalovirus pp65 and IE1 epitopes for highly specific adoptive immunotherapy. Cytotherapy. (12) 7:933-944
13. Zhang, H., Hong, H., Li, D., Ma, S., Di, Y., Stoten, A., Haig, N., Di, Gleria K., Yu, Z., Xu, X. N., McMichael, A., and Jiang, S. (2009) Comparing pooled peptides with intact protein for accessing cross-presentation pathways for protective $CD8^+$ and $CD4^+$ T cells. J.Biol.Chem. (284) 14:9184-9191
14. Newman, K. D., Samuel, J., and Kwon, G. (1998) Ovalbumin peptide encapsulated in poly(d,l lactic-co-glycolic acid) microspheres is capable of inducing a T helper type 1 immune response. J.Control Release (54) 1:49-59

15. Waeckerle-Men, Y., Gander, B., and Groettrup, M. (2005) Delivery of tumor antigens to dendritic cells using biodegradable microspheres. Methods Mol. Med. (109) :35-46
16. Zhang, Z., Tongchusak, S., Mizukami, Y., Kang, Y. J., Ioji, T., Touma, M., Reinhold, B., Keskin, D. B., Reinherz, E. L., and Sasada, T. (2011) Induction of anti-tumor cytotoxic T cell responses through PLGA-nanoparticle mediated antigen delivery. Biomaterials (32) 14:3666-3678
17. Gilding, D. K. and Reed, A. M. (1979) Biodegradable polymers for use in surgeryGÇöpolyglycolic/poly(actic acid) homo- and copolymers: 1. Polymer (20) 12:1459-1464
18. Mahapatro, A. and Singh, D. K. (2011) Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines. J.Nanobiotechnology. (9) :55
19. J ain, A. K., Das, M., Swarnakar, N. K., and Jain, S. (2011) Engineered PLGA nanoparticles: an emerging delivery tool in cancer therapeutics. Crit Rev.Ther. Drug Carrier Syst. (28) 1:1-45
20. Hamdy, S., Haddadi, A., Hung, R. W., and Lavasanifar, A. (2011) Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. Adv. Drug Deliv.Rev. (63) 10-11:943-955
21. Shen, H., Ackerman, A. L., Cody, V., Giodini, A., Hinson, E. R., Cresswell, P., Edelson, R. L., Saltzman, W. M., and Hanlon, D. J. (2006) Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology (117) 1:78-88
22. Schlosser, E., Mueller, M., Fischer, S., Basta, S., Busch, D. H., Gander, B., and Groettrup, M. (2008) TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine (26) 13:1626-1637
23. Demento, S. L., Eisenbarth, S. C., Foellmer, H. G., Platt, C., Caplan, M. J., Mark, Saltzman W., Mellman, I., Ledizet, M., Fikrig, E., Flavell, R. A., and Fahmy, T. M. (2009) Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy. Vaccine (27) 23:3013-3021
24. Yoshida, M., Mata, J., and Babensee, J. E. (2007) Effect of poly(lactic-co-glycolic acid) contact on maturation of murine bone marrow-derived dendritic cells. J.Biomed.Mater.Res.A (80) 1:7-12
25. Han, H., Peng, J. R., Chen, P. C., Gong, L., Qiao, S. S., Wang, W. Z., Cui, Z. Q., Yu, X., Wei, Y. H., and Leng, X. S. (2011) A novel system of artificial antigen-presenting cells efficiently stimulates Flu peptide-specific cytotoxic T cells *in vitro*. Biochem.Biophys.Res.Commun. (411) 3:530-535
26. Steenblock, E. R. and Fahmy, T. M. (2008) A comprehensive platform for *ex vivo* T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells. Mol.Ther. (16) 4:765-772
27. Palucka, K. and Banchereau, J. (2012) Cancer immunotherapy via dendritic cells. Nat.Rev.Cancer (12) 4:265-277
28. Steinman, R. M. (2012) Decisions about dendritic cells: past, present, and future. Annu.Rev.Immunol. (30) :1-22
29. Rosenberg, S. A. and Dudley, M. E. (2009) Adoptive cell therapy for the treatment

of patients with metastatic melanoma. Curr.Opin.Immunol. (21) 2:233-240
30. Restifo, N. P., Dudley, M. E., and Rosenberg, S. A. (2012) Adoptive immunotherapy for cancer: harnessing the T cell response. Nat.Rev.Immunol. (12) 4:269-281
31. Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V. S., Davoust, J., and Ricciardi-Castagnoli, P. (1997) Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J.Exp.Med. (185) 2:317-328
32. Schuurhuis, D. H., Ioan-Facsinay, A., Nagelkerken, B., van Schip, J. J., Sedlik, C., Melief, C. J., Verbeek, J. S., and Ossendorp, F. (2002) Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J.Immunol. (168) 5:2240-2246
33. Sanderson, S. and Shastri, N. (1994) LacZ inducible, antigen/MHC-specific T cell hybrids. Int.Immunol. (6) 3:369-376
34. Moore, M. W., Carbone, F. R., and Bevan, M. J. (1988) Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell (54) 6:777-785
35. Slutter, B., Bal, S., Keijzer, C., Mallants, R., Hagenaars, N., Que, I., Kaijzel, E., van, Eden W., Augustijns, P., Lowik, C., Bouwstra, J., Broere, F., and Jiskoot, W. (2010) Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine (28) 38:6282-6291
36. Hamdy, S., Haddadi, A., Shayeganpour, A., Samuel, J., and Lavasanifar, A. (2011) Activation of antigen-specific T cell-responses by mannan-decorated PLGA nanoparticles. Pharm.Res. (28) 9:2288-2301
37. Mueller, M., Schlosser, E., Gander, B., and Groettrup, M. (2011) Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. Int.J.Cancer (129) 2:407-416
38. Tacken, P. J., Zeelenberg, I. S., Cruz, L. J., van Hout-Kuijer, M. A., van de Glind, G., Fokkink, R. G., Lambeck, A. J., and Figdor, C. G. (2011) Targeted delivery of TLR ligands to human and mouse dendritic cells strongly enhances adjuvanticity. Blood (118) 26:6836-6844
39. Klebanoff, C. A., Gattinoni, L., Palmer, D. C., Muranski, P., Ji, Y., Hinrichs, C. S., Borman, Z. A., Kerkar, S. P., Scott, C. D., Finkelstein, S. E., Rosenberg, S. A., and Restifo, N. P. (2011) Determinants of successful $CD8^+$ T-cell adoptive immunotherapy for large established tumors in mice. Clin.Cancer Res. (17) 16:5343-5352
40. Montagna, D., Turin, I., Schiavo, R., Montini, E., Zaffaroni, N., Villa, R., Secondino, S., Schiavetto, I., Caliogna, L., Locatelli, F., Libri, V., Pession, A., Tonelli, R., Maccario, R., Siena, S., and Pedrazzoli, P. (2012) Feasibility and safety of adoptive immunotherapy with *ex vivo*-generated autologous, cytotoxic T lymphocytes in patients with solid tumor. Cytotherapy. (14) 1:80-90
41. Boyman, O. and Sprent, J. (2012) The role of interleukin-2 during homeostasis and activation of the immune system. Nat.Rev.Immunol. (12) 3:180-190
42. Boyman, O., Purton, J. F., Surh, C. D., and Sprent, J. (2007) Cytokines and T-cell homeostasis. Curr.Opin.Immunol. (19) 3:320-326
43. Denoeud, J. and Moser, M. (2011) Role of CD27/CD70 pathway of activation in

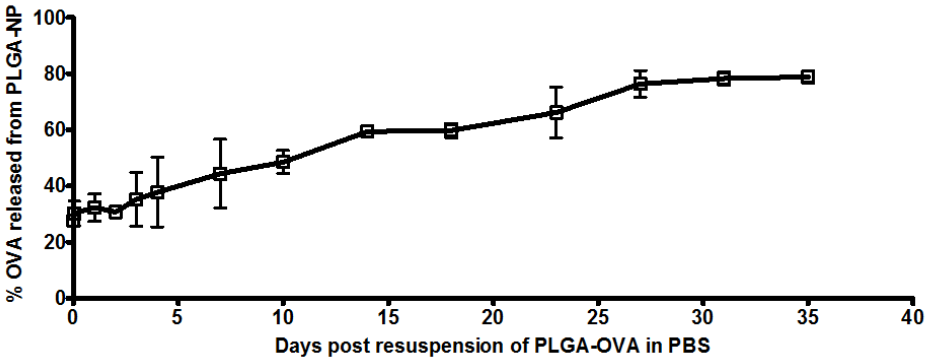
immunity and tolerance. J.Leukoc.Biol. (89) 2:195-203
44. Gerdes, N. and Zirlik, A. (2011) Co-stimulatory molecules in and beyond co-stimulation - tipping the balance in atherosclerosis? Thromb.Haemost. (106) 5:804-813
45. Schreiber, T. H., Wolf, D., Bodero, M., Gonzalez, L., and Podack, E. R. (2012) T Cell Costimulation by TNFR Superfamily (TNFRSF)4 and TNFRSF25 in the Context of Vaccination. J.Immunol. (189) 7:3311-3318
46. Trinchieri, G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat.Rev.Immunol. (3) 2:133-146
47. Turtle, C. J. and Riddell, S. R. (2010) Artificial antigen-presenting cells for use in adoptive immunotherapy. Cancer J. (16) 4:374-381
48. Gattinoni, L., Powell, D. J., Jr., Rosenberg, S. A., and Restifo, N. P. (2006) Adoptive immunotherapy for cancer: building on success. Nat.Rev.Immunol. (6) 5:383-393
49. Dudley, M. E., Wunderlich, J. R., Shelton, T. E., Even, J., and Rosenberg, S. A. (2003) Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. J.Immunother. (26) 4:332-342
50. Ho, W. Y., Nguyen, H. N., Wolfl, M., Kuball, J., and Greenberg, P. D. (2006) *In vitro* methods for generating $CD8^+$ T-cell clones for immunotherapy from the naive repertoire. J.Immunol.Methods (310) 1-2:40-52
51. Le, H. K., Graham, L., Miller, C. H., Kmieciak, M., Manjili, M. H., and Bear, H. D. (2009) Incubation of antigen-sensitized T lymphocytes activated with bryostatin 1 $^+$ ionomycin in IL-7 $^+$ IL-15 increases yield of cells capable of inducing regression of melanoma metastases compared to culture in IL-2. Cancer Immunol. Immunother. (58) 10:1565-1576
52. Budd, R. C. (2001) Activation-induced cell death. Curr.Opin.Immunol. (13) 3:356-362
53. Green, D. R., Droin, N., and Pinkoski, M. (2003) Activation-induced cell death in T cells. Immunol.Rev. (193) :70-81
54. Dobrzanski, M. J., Reome, J. B., Hollenbaugh, J. A., and Dutton, R. W. (2004) Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J.Immunol. (172) 3:1380-1390
55. Garcia-Hernandez, Mde L., Hamada, H., Reome, J. B., Misra, S. K., Tighe, M. P., and Dutton, R. W. (2010) Adoptive transfer of tumor-specific Tc17 effector T cells controls the growth of B16 melanoma in mice. J.Immunol. (184) 8:4215-4227
56. Hinrichs, C. S., Spolski, R., Paulos, C. M., Gattinoni, L., Kerstann, K. W., Palmer, D. C., Klebanoff, C. A., Rosenberg, S. A., Leonard, W. J., and Restifo, N. P. (2008) IL-2 and IL-21 confer opposing differentiation programs to $CD8^+$ T cells for adoptive immunotherapy. Blood (111) 11:5326-5333
57. Waldmann, T. A. (2006) The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat.Rev.Immunol. (6) 8:595-601
58. Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V. S., Davoust, J., and Ricciardi-Castagnoli, P. (1997) Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J.Exp.Med. (185) 2:317-328

59. de Vos van Steenwijk PJ, Heusinkveld, M., Ramwadhdoebe, T. H., Lowik, M. J., van der Hulst, J. M., Goedemans, R., Piersma, S. J., Kenter, G. G., and van der Burg, S. H. (2010) An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. Cancer Res. (70) 7:2707-2717
60. Piersma, S. J., Welters, M. J., van der Hulst, J. M., Kloth, J. N., Kwappenberg, K. M., Trimbos, B. J., Melief, C. J., Hellebrekers, B. W., Fleuren, G. J., Kenter, G. G., Offringa, R., and van der Burg, S. H. (2008) Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a restriction element. Int.J.Cancer (122) 3:486-494
61. Hunder, N. N., Wallen, H., Cao, J., Hendricks, D. W., Reilly, J. Z., Rodmyre, R., Jungbluth, A., Gnjatic, S., Thompson, J. A., and Yee, C. (2008) Treatment of metastatic melanoma with autologous $CD4^+$ T cells against NY-ESO-1. N.Engl.J.Med. (358) 25:2698-2703
62. Prior, S., Gander, B., Blarer, N., Merkle, H. P., Subira, M. L., Irache, J. M., and Gamazo, C. (2002) *In vitro* phagocytosis and monocyte-macrophage activation with poly(lactide) and poly(lactide-co-glycolide) microspheres. Eur.J.Pharm.Sci. (15) 2:197-207
63. Yoshida, M. and Babensee, J. E. (2006) Differential effects of agarose and poly(lactic-co-glycolic acid) on dendritic cell maturation. J.Biomed.Mater.Res.A (79) 2:393-408
64. Shen, H., Ackerman, A. L., Cody, V., Giodini, A., Hinson, E. R., Cresswell, P., Edelson, R. L., Saltzman, W. M., and Hanlon, D. J. (2006) Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology (117) 1:78-88
65. Schliehe, C., Schliehe, C., Thiry, M., Tromsdorf, U. I., Hentschel, J., Weller, H., and Groettrup, M. (2011) Microencapsulation of inorganic nanocrystals into PLGA microsphere vaccines enables their intracellular localization in dendritic cells by electron and fluorescence microscopy. J.Control Release (151) 3:278-285
66. van, Montfoort N., Camps, M. G., Khan, S., Filippov, D. V., Weterings, J. J., Griffith, J. M., Geuze, H. J., van, Hall T., Verbeek, J. S., Melief, C. J., and Ossendorp, F. (2009) Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc.Natl. Acad.Sci.U.S.A (106) 16:6730-6735
67. Coulie, P. G. and Connerotte, T. (2005) Human tumor-specific T lymphocytes: does function matter more than number? Curr.Opin.Immunol. (17) 3:320-325
68. Dobrzanski, M. J., Reome, J. B., Hollenbaugh, J. A., and Dutton, R. W. (2004) Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J.Immunol. (172) 3:1380-1390
69. Garcia-Hernandez, Mde L., Hamada, H., Reome, J. B., Misra, S. K., Tighe, M. P., and Dutton, R. W. (2010) Adoptive transfer of tumor-specific Tc17 effector T cells controls the growth of B16 melanoma in mice. J.Immunol. (184) 8:4215-4227
70. Welters, M. J., Kenter, G. G., de Vos van Steenwijk PJ, Lowik, M. J., Berends-van

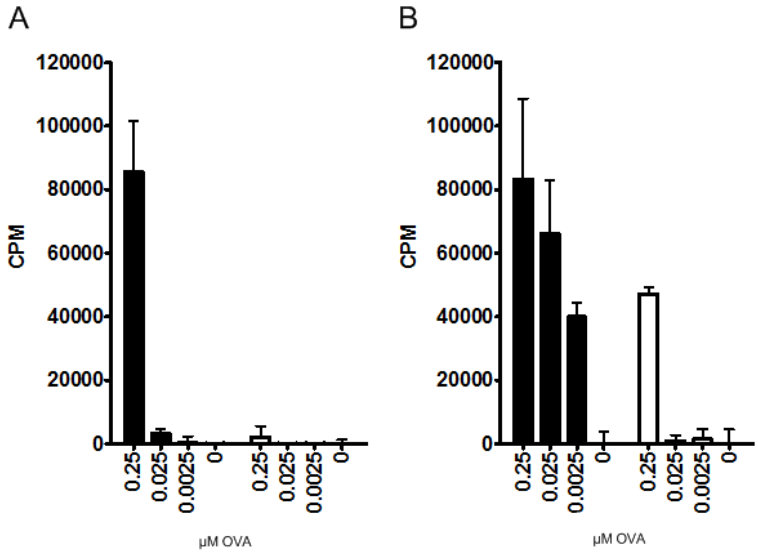
der Meer DM, Essahsah, F., Stynenbosch, L. F., Vloon, A. P., Ramwadhdoebe, T. H., Piersma, S. J., van der Hulst, J. M., Valentijn, A. R., Fathers, L. M., Drijfhout, J. W., Franken, K. L., Oostendorp, J., Fleuren, G. J., Melief, C. J., and van der Burg, S. H. (2010) Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. Proc. Natl.Acad.Sci.U.S.A (107) 26:11895-11899

71. Bos, R. and Sherman, L. A. (2010) $CD4^+$ T-cell help in the tumor milieu is required for recruitment and cytolytic function of $CD8^+$ T lymphocytes. Cancer Res. (70) 21:8368-8377

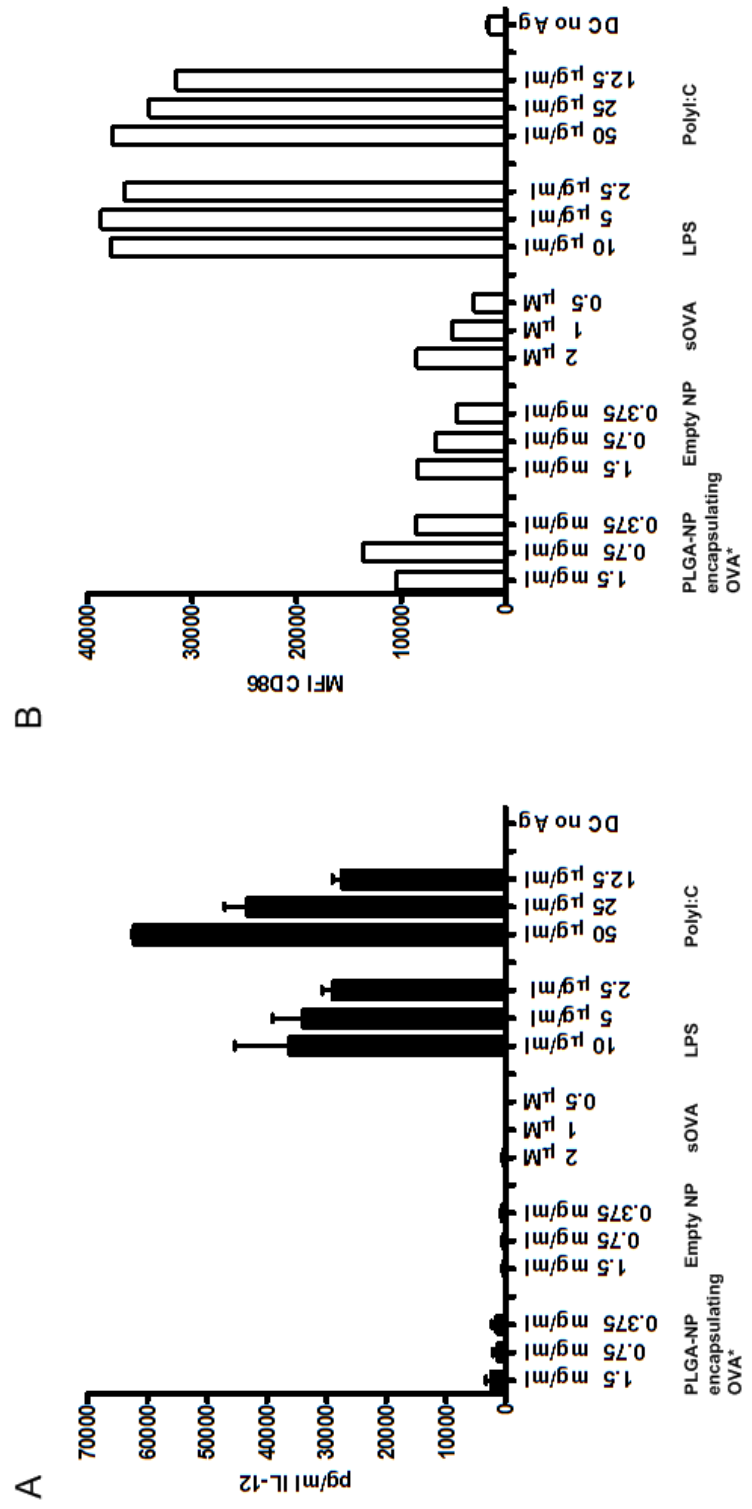
6. Supplemental Material



Supplemental Figure 1. Encapsulated OVA is released gradually from PLGA-NP. PLGA-OVA-Alexa488 with an average OVA content of 14.11 ± 2.89 µg OVA/mg PLGA were re-suspended at a concentration of 10 mg/mL of PBS and incubated at 37ºC under constant shaking. At indicated time points 250 µl samples were collected, centrifuged for 20 min at 18,000xg and the amount of OVA-Alexa488 in the supernatant determined by fluorescence, as described in the material and methods. Average results of two independent release studies with four different batches of PLGA-OVA are shown, mean ± SD.



Supplemental figure 2. Improved CD4⁺ and CD8⁺ T cell proliferation by BMDC loaded with PLGA-OVA in comparison to sOVA-loaded BMDC. WT C57BL/6 BMDC were incubated with titrated amounts of PLGA-OVA or sOVA. Ag loaded BMDC were subsequently used as APC in a co-culture with naïve OT-I **(A)** or OT-II T **(B)** cells for 72 h. T cell proliferation was measured in triplicate by 3[H]-thymidine uptake. Data shown are representative of two independent experiments.



Supplemental Figure 3. PLGA-OVA do not mature DC in comparison to TLRL. DC were incubated in the presence of titrated amounts of PLGA-OVA, empty PLGA-NP, sOVA, a mixture of sOVA and empty PLGA-NP, LPS (Sigma L4130, Escherichia coli 0111:B4) and Poly(I:C (Invivogen, tlrl-picw). After 24 hr supernatants were collected and analyzed via ELISA for IL-12 levels **(A)**. DC were harvested and analyzed by flow cytometry for the expression of CD86 **(B)**. Data shown are representative of three independent experiments. *1.5 mg/ml of PLGA-NP encapsulating OVA (PLGA-OVA) is required to obtain of 0.25 µM OVA

Chapter 5

Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low burst release is crucial for efficient $CD8^+$ T cell activation

Ana Luísa Silva, Rodney A. Rosalia, Arzu Sazak, Myrra G. Carstens, Ferry Ossendorp, Jaap Oostendorp, Wim Jiskoot

European Journal of Pharmaceutics and Biopharmaceutics 2013 83(3): 338-345

Abstract

Overlapping synthetic long peptides (SLP) hold great promise for immunotherapy of cancer. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NP) are being developed as delivery systems to improve the potency of peptide-based therapeutic cancer vaccines. Our aim was to optimize PLGA NP for SLP delivery with respect to encapsulation and release, using OVA24, a 24-residue long synthetic antigenic peptide covering a CTL epitope of ovalbumin (SIINFEKL), as a model antigen. Peptide-loaded PLGA NP were prepared by a double emulsion/solvent evaporation technique. Using standard conditions (acidic inner aqueous phase), we observed that either encapsulation was very low (1 – 30%), or burst release extremely high (> 70%) upon resuspension of NP in physiological buffers. By adjusting formulation and process parameters, we uncovered that the pH of the first emulsion was critical to efficient encapsulation and controlled release. In particular, an alkaline inner aqueous phase resulted in circa 330 nm sized NP with approximately 40% encapsulation efficiency and low (< 10%) burst release. These NP showed enhanced MHC class I restricted T cell activation *in vitro* when compared to high-burst releasing NP and soluble OVA24, proving that efficient entrapment of the antigen is crucial to induce a potent cellular immune response.

Keywords: Peptide antigen, CTL epitope, PLGA nanoparticles, cancer immunotherapy, cellular immune response

1. Introduction

In recent years there is an increased interest in the application of therapeutic vaccination for treatment of cancer [65]. Therapeutic cancer vaccines aim to induce a strong cellular response against tumor associated antigens [66]. Dendritic cells (DC) are professional antigen presenting cells (APCs) that play a major role in the initiation of such an immune response, by continuously sampling the environment for foreign antigens and establishing the communication between the innate and adaptive immune system [67, 68]. Only appropriately activated DC are capable of inducing a robust cytotoxic T cell (CTL) response, which is required for effective immunotherapy of established tumors [3, 69-71]. For this purpose, DC are the major target cells for cancer immunotherapy vaccines [3, 66].

Therapeutic vaccination with overlapping synthetic long peptides (SLP), covering the entire amino acid sequence of tumor associated protein antigens and thus containing all possible MHC class I and II epitopes, has been successfully applied in murine models and clinical therapeutic vaccination trials [66, 72-75]. Moreover, vaccination of patients suffering from human papillomavirus 16 (HPV16) induced premalignant vulvar intraepithelial neoplasia with an HPV16-based SLP vaccine resulted in complete clinical regression of the lesions in some cases [66, 74, 75].

So far, Montanide-based water-in-oil (w/o) emulsions have been applied to formulate

SLP in the majority of clinical therapeutic cancer vaccination trials [74-80]. The use of Montanide-based formulations has some important limitations, including non-biodegradability, causing significant local side effects, poorly controlled antigen release rates and limited scalability because of lack of long-term stability [81-83]. Biodegradable delivery systems based on poly(lactic-co-glycolic acid) (PLGA) offer a promising alternative strategy for peptide-based cancer vaccines. PLGA is well suited for the preparation of micro- and nanoparticles (NP) [54, 84-86], which can protect antigen from proteolytic enzymatic degradation and rapid clearance [87-90], allow co-encapsulation and simultaneous delivery of both antigen and adjuvants, and facilitate antigen uptake by DC [8, 91-93]. PLGA has a long safety record and is Food and Drug Administration (FDA) approved as an excipient, owing to its biodegradability and biocompatibility, with several slow-release formulations currently on the market [92, 94]. PLGA undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid, which are natural by-products of metabolic pathways. Antigen release can be regulated e.g. by varying the lactic acid/glycolic acid ratio [95-97]. PLGA-based particulate systems can be manufactured reproducibly according to Good Manufacturing Practice conditions and have been studied extensively for the delivery of a wide variety of antigens [54, 94, 98-100]. Antigens encapsulated in PLGA microparticles have shown to induce immune responses comparable to those of antigens adjuvanted with Montanide 51 [17, 97]. PLGA is also known to have several disadvantages regarding instability of protein antigens, e.g., due to the hydrophobicity of the polymer and the local acidification of the microenvironment that occurs during degradation of the polymer at physiological pH [101-103]. However, for synthetic peptides, which do not possess a tertiary structure, this is less problematic and several peptides in PLGA microspheres have been successfully launched on the market [94, 100, 103].

The aim of this study was the pharmaceutical characterization of PLGA NP as a suitable delivery system for encapsulation of SLP for cancer immunotherapy. OVA24, a 24-residue long synthetic antigenic peptide covering a CTL epitope of ovalbumin (OVA; SIINFEKL), was studied as a model SLP, because of its proven capability to induce CTL responses *in vitro* and *in vivo* [104]. Efficient entrapment of OVA24 SLP in the polymeric matrix was obtained by exploring and fine tuning of formulation and process parameters. OVA24-loaded PLGA NP were characterized for antigen encapsulation efficiency, antigen burst release, particle size and zeta-potential, and the obtained formulations were immunologically evaluated *in vitro* for their potency to induce $CD8^+$ T cell activation.

2. Materials and methods

2.1. Materials

Synthetic long peptide 24-mer OVA24 (DEVSGLEQLESIINFEKLAAAAAK) [105],

covering the cytotoxic T lymphocyte (CTL) epitope SIINFEKL of ovalbumin (OVA) was synthesized at the interdepartmental GMP facility of the Department of Clinical Pharmacy and Toxicology of Leiden University Medical Center as described previously [73]. Poly(D,L-lactic-co-glycolic acid) [PLGA], Resomer® RG 502H was purchased from Boehringer Ingelheim (Ingelheim, Germany). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), sodium cholate, dichloromethane (DCM), dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) were obtained from Biosolve BV (Valkenswaard, the Netherlands), Polyvinil alcohol (PVA) 4-88 (31 kDa) was purchased from Fluka (Steinheim, Germany). Tween 20 was purchased from Merck Schuchardt (Hohenbrunn, Germany). Sodium hydroxide was purchased from Boom (Meppel, Netherlands). Reversed phase HPLC column ReproSil-Pur C18-AQ 3 µm (150x4 mm) was purchased from Dr. Maisch HPLC GmbH (Ammerbuch-Entringen, Germany). Phosphate-buffered saline (NaCl 8.2 g/L; Na2HPO4.12 H2O 3.1 g/L; NaH2PO4.2H2O 0.3 g/L) (PBS) was purchased from B. Braun (Melsungen, Germany). Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Lonza (Walkersville, USA). All other chemicals were of analytical grade and all aqueous solutions were prepared with milli Q water.

2.2. Nanoparticle preparation

2.2.1. General particle preparation process

Nanoparticles loaded with OVA24 were prepared using a double emulsion with solvent evaporation method [37]. In brief, 50 mg of PLGA dissolved in 1 ml of dichloromethane was emulsified under sonication (30 s, 20 W) with a solution containing 1.4 mg OVA24 (for solution compositions, see results). To this first emulsion (w1/o), 2 ml of an aqueous surfactant solution (for surfactant types, see results) 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 25 ml of extraction medium (0.3% w/v surfactant) 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, and resuspended in deionized water, aliquoted and freeze-dried at -55oC in a Christ Alpha 1-2 freeze-drier (Osterode am Harz, Germany) for 12 hours.

As starting conditions, the method described by Slütter et al. [37] was used, with 1% v/v Tween 20 in 25 mM Hepes pH 7.4 as the aqueous phase for second emulsion, with the following modifications: DMSO used as inner phase instead of PBS pH 7.4 and 5% (w/v) PLGA was used instead of 2.5% .

2.2.2. Optimization of formulation parameters

In order to achieve an optimum formulation, the following parameters involved in the particle preparation were varied, and their effect on peptide encapsulation efficiency was investigated.

a) Surfactant type: investigated by dissolving different surfactant types (PVA; Tween 20; sodium cholate) in the second aqueous phase (w2) during the second emulsion step.
b) Inner solvent: investigated by dissolving the peptide in different solutions (w1), i.e. DMSO; 50% ACN + 0.1% TFA; 50% ACN in 25 mM NaOH; and 50% ACN in 0.25 mM NaOH + 400 µL Hepes pH 8.0 at different concentrations).
c) Volume ratio (w1/o): investigated by varying the volume of the inner aqueous phase w1 (50 µL, 100 µL; 500 µL).

2.3. Nanoparticle characterization

2.3.1. Dynamic light scattering and zeta-potential

The Z-average size and polydispersity index (PDI) of NP were measured by dynamic light scattering, using a Zetasizer (Nano ZS, Malvern Ltd., United Kingdom). The zeta-potential was measured by laser Doppler electrophoresis, using the same device. For that purpose, NP were diluted to 2.5 mg/ml in 1 mM Hepes pH 7.4.

2.3.2. Antigen content and encapsulation efficiency

Peptide encapsulation efficiency was determined by measuring the peptide content of digested particles by reversed phase HPLC. For that purpose, 200 µL NP suspension (containing 10 mg NP) was freeze-dried overnight. The lyophilizate was then dissolved in 250 µL DMSO and the solution was agitated at 50°C for 30 min. Next, 750 µL 50% ACN with 0.1% TFA were added and the mixture was agitated at 50°C for an extra 60 min, to allow dissolution of the peptide and degradation/precipitation of PLGA, which was then eliminated by centrifugation for 10 min at 18000 g. The supernatant containing the peptide was collected and 50 µL were injected into a HPLC system equipped with a C18 column (Dr. Maisch Reprosil-Pur C18-AQ, 3 mg, 150 x 4.6 mm) and an ultraviolet detector (Waters 486). Mobile phases applied were 5% ACN in water with 0.1% TFA (solvent A), and 95% ACN in water with 0.085% TFA (solvent B). Separation was performed by applying a linear gradient from 0% to 78% solvent B over 20 min, at a flow rate of 1 ml/min, and peptide detection was performed by absorbance

at 220 nm. Peptide concentration in each sample was calculated using a calibration curve created with known concentrations of OVA24.

2.4. *In vitro* release studies

To determine burst release (BR) at time zero (t_0), freeze-dried NP were resuspended in 1x PBS, 1x IMDM cell culture medium, or 5% w/v glucose solution at a concentration of 10 mg PLGA NP/mL and the mixture was vortexed for 30 seconds at room temperature to allow complete resuspension of the particles. Then particles were centrifuged for 10 min at 18000 g, the supernatant recovered and the pellet resuspended. Both fractions were freeze-dried overnight, and extraction was performed as described previously, before being analyzed by HPLC for peptide quantification.

For longer release studies, peptide-loaded PLGA NP were prepared as described, resuspended in 1x PBS pH 7.4 at a concentration of 10 mg and maintained at 37oC in a water bath under constant tangential shaking at 100/min in a GFL 1086 shaking water bath (Burgwedel, Germany). At regular time intervals, 250 µl samples of the suspension were taken, centrifuged for 10 min at 18000 g, and the supernatant recovered. To eliminate undesirable PLGA degradation products affecting detection by HPLC, the supernatant was freeze-dried over night, and extraction was performed as described, with the final supernatant being analyzed by HPLC for peptide quantification. Peptide release profiles were generated for each NP formulation in terms of cumulative antigen release (%) over time.

2.5. *In vitro* antigen presentation

D1 cells, a long term growth factor-dependent immature splenic DC line derived from C57BL/6 (H-2Kb) mice, were cultured as described previously [106]. B3Z, cultured as described before, is a $CD8^+$ T-cell hybridoma cell line, specific for the H-2Kb–restricted ovalbumin derived CTL epitope SIINFEKL, that expresses a β-galactosidase construct under the regulation of the NF-AT element from the IL-2 promoter [107]. DC were incubated for 2.5 h with soluble OVA24 (sOVA24) or OVA24 encapsulated in PLGA NP at the indicated concentrations. Cells were washed three times with medium before the T-cell hybridoma B3Z cells were added followed by overnight incubation at 37 °C. MHC class I Ag presentation of SIINFEKL (OVA257–264) in H-2Kb was detected by activation of SIINFEKL-specific $CD8^+$ B3Z T cells. Upon T-cell receptor (TCR) ligation, lacZ protein is produced under the control of the IL-2 promoter thus allowing measurement of the IL-2 production indirectly by a colorimetric assay using chlorophenol red-β-d-galactopyranoside (CPRG) as substrate to detect lacZ activity in cell lysates. Color conversion is determined by measuring absorbance (optical density, OD) at 590 nm.

2.6. Statistical analysis

Graph Pad Prism software was used for statistical analysis. Burst release in different physiological media and the effect of inner solvent and emulsion volume on burst release between different formulations in PBS were analyzed by two-tailed unpaired Student's t-test. $CD8^+$ T cell activation of SIINFEKL-specific $CD8^+$ T cells by a two-tailed paired Student's t-test. Effect of the inner phase composition on apparent pH and the effect of Hepes concentration in the inner phase on release were analyzed using two-way ANOVA.

3. Results

3.1. Antigen encapsulation and burst release in PLGA NP

In this study, a 24-mer SLP covering a well-known CTL epitope (SIINFEKL) of ovalbumin, here designated as OVA24, was used as a model antigen to study the encapsulation of SLP in PLGA NP by a double emulsion with solvent evaporation method, as function of formulation and process parameters. As starting point, a slightly modified version of the standard double emulsion method described by Slütter et al. [37] was applied (see section 2.2.1), in which OVA24 was dissolved in DMSO and Tween 20 used as surfactant in the outer phase (**Table 1**, formulation 1). Since this method led to very low encapsulation efficiencies (ca. 1%), DMSO as inner phase was replaced by 50% ACN/0.1% TFA, which resulted in a marginal improvement (see **Table 1**, formulation 2). Attempts to dissolve OVA24 in the PLGA-containing dichloromethane phase failed (results not shown). Therefore, several process parameters were investigated in order to increase the encapsulation efficiency of OVA24 in PLGA NP. First, commonly used surfactants for making NP, sodium cholate and PVA [37, 85], were tested in replacement of Tween 20 (**Table 1**, formulations 3-7). The type of surfactant used to stabilize the second emulsion had a dramatic positive effect on the encapsulation efficiency. Using 50% ACN/0.1% TFA (with an apparent pH of 2.0) as first emulsion medium and PVA as surfactant in the second emulsion step resulted in the highest encapsulation efficiency (EE) (up to about 30%, see **Table 1**, formulations 4-7). PVA concentrations of 1% and 2% (w/v) PVA yielded comparable EE and particle size (**Table 1**, formulations 6 and 7). PVA concentrations below 1% led to a lower encapsulation efficiency and a larger particle size (**Table 1**, formulations 4 and 5). For our further studies, OVA24-loaded PLGA NP prepared with 50% ACN/0.1% TFA as inner solvent and 1% PVA as surfactant in the second emulsion (**Table 1**, formulation 6) were selected.

Table 1: Effect of first and second emulsion compositions on encapsulation efficiency of OVA24 SLP in PLGA NP.

Formulation	1st emulsion medium	2nd emulsion medium	DL (%) *	EE (%)	Z-average size (nm)	PDI	ZP (mV)
1	DMSO	1% Tween 20	0.01 ± 0.004	1 ± 0.3	319 ± 90	0.23 ± 0.07	-36 ± 7.0
2	50 µL 50% ACN 0.1% TFA	1% Tween 20	0.06 ± 0.02	4 ± 1	277 ± 5	0.20 ± 0.01	-27 ± 2.0
3	50 µL 50% ACN 0.1% TFA	1% NaCholate	0.17 ± 0.04	9 ± 2	203 ± 3	0.10 ± 0.03	-32 ± 1.1
4	50 µL 50% ACN 0.1% TFA	0.3 % PVA	0.37 ± 0.08	16 ± 1	606 ± 4	0.17 ± 0.02	-6 ± 1.5
5	50 µL 50% ACN 0.1% TFA	0.5 % PVA	0.62 ± 0.20	22 ± 7	470 ± 36	0.22 ± 0.05	-12 ± 2.1
6	50 µL 50% ACN 0.1% TFA	1 % PVA	0.86 ± 0.31	26 ± 11	372 ± 44	0.17 ± 0.01	-10 ± 1.9
7	50 µL 50% ACN 0.1% TFA	2 % PVA	0.61 ± 0.27	22 ± 3	345 ± 28	0.15 ± 0.03	-10 ± 2.4
8	100 µL 50% ACN 25 mM NaOH	1 % PVA	0.26 ± 0.22	9 ± 8	360 ± 45	0.22 ± 0.01	-12 ± 0.8
9	100 µL 50% ACN 0.25 mM NaOH	1 % PVA	0.71 ± 25	26 ± 9	379 ± 25	0.19 ± 0.02	-12 ± 0.7
10	500 µL 50% ACN 0.1% TFA	1 % PVA	0.82 ± 0.34	29 ± 12	234 ± 3	0.11 ± 0.03	-15 ± 1.3
11	500 µL 50% ACN 25 mM NaOH	1 % PVA	1.03 ± 0.15	37 ± 5	303 ± 68	0.11 ± 0.05	-11 ± 0.1
12	100 µL 50% ACN 0.25 mM NaOH + 400 µL 1 mM Hepes pH 8.0	1 % PVA	0.55 ± 0.21	20 ± 8	384 ± 7	0.18 ± 0.03	-11 ± 0.2
13	100 µL 50% ACN 0.25 mM NaOH + 400 µL 5 mM Hepes pH 8.0	1 % PVA	0.50 ± 0.20	18 ± 6	427 ± 46	0.20 ± 0.05	-10 ± 1.7
14	100 µL 50% ACN 0.25 mM NaOH + 400 µL 10 mM Hepes pH 8.0	1 % PVA	0.44 ± 0.21	16 ± 7	382 ± 69	0.18 ± 0.05	-10 ± 0.6
15	100 µL 50% ACN 0.25 mM NaOH + 400 µL 25 mM Hepes pH 8.0	1 % PVA	0.70 ± 0.09	25 ± 3	298 ± 27	0.12 ± 0.03	-10 ± 0.9
16	100 µL 50% ACN 0.25 mM NaOH + 400 µL 50 mM Hepes pH 8.0	1 % PVA	1.07 ± 0.07	38 ± 3	328 ± 50	0.22 ± 0.07	-14 ± 0.3

* DL = drug loading, EE = encapsulation efficiency, calculated as described in section 2.3.2. PDI = polydispersity index, ZP = zeta-potential. Data are presented as average ± standard deviation of n=3 independent batches.

Formulation 6 was tested for burst release of the peptide in PBS and IMDM (cell culture medium), which are commonly used for *in vitro* and *in vivo* applications. Directly upon resuspension (t=0) in either PBS or IMDM, OVA24-loaded PLGA NP showed a very high release, ranging from 80 to 90% (**Figure 1**), indicating that initial encapsulation results were misleading. In contrast, a release study in isotonic 5% glucose solution (**Figure 1**), did not induce substantial peptide release.

With the purpose to decrease the very high burst release, new solutions were tested as inner phase (w1) in the NP preparation process, where the effect of pH was studied. Instead of using pHs below the isoelectric point (pI) of the peptide (pI = 4.3), we decided to use pHs above it, which would change the charge distribution in the peptide and thereby could affect encapsulation, as well as inner phase volume, which might affect the peptide's distribution between the inner and outer aqueous phases present during formation of the double emulsion. Therefore, instead of 50% ACN/0.1% TFA (apparent pH 2.0), OVA24 was dissolved in 50% ACN/25 mM NaOH (apparent pH 12.5).

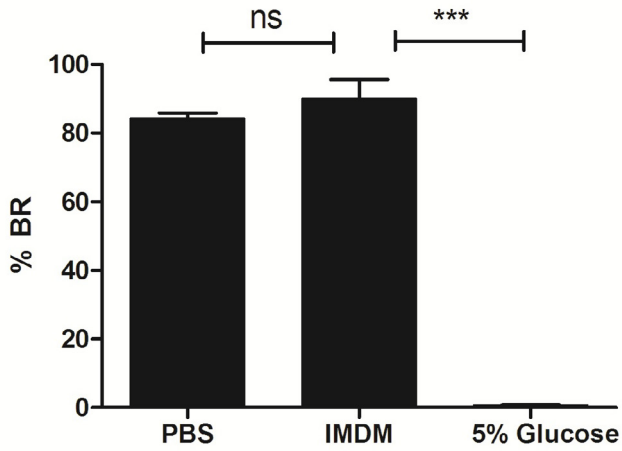


Figure 1: Burst release of OVA24 in different physiological media (PBS, IMDM cell culture medium, 5% glucose) from PLGA NP (**Table 1,** formulation 6). Data are presented as average ± standard deviation of n=3 independent batches. Data was analyzed by two-tailed unpaired Student's t-test. P values are presented as non-significant (ns) = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.0001$.

Improvement was observed in neither encapsulation efficiency nor burst release when using an inner alkaline phase volume of 100 µL (**Table 1**, formulation 9; **Figure 2a**). However, a concomitant increase of inner emulsion volume, by using an inner phase volume of 500 µL (**Table 1**, formulation 11), drastically reduced burst release from circa 90% to less than 10%, while improving encapsulation efficiency. Still, when at acidic pH, a higher inner emulsion volume (**Table 1**, formulation 10) did not affect encapsulation efficiency or burst release compared to previous values, confirming the central effect of pH in the matter. Indeed we observed that the peptide was very acidic, most likely due to remaining TFA from synthesis, drastically decreasing pH of solutions upon dissolution, with only the higher volumes containing enough base/buffer molecules to

neutralize the acid contained in the peptide (**Figure 2b**), and keep the pH above 7.

The size and zeta-potential of the prepared OVA24-loaded NP was measured in 1 mM Hepes pH 7.4. Irrespective of the preparation method, the particles were negatively charged, with zeta-potentials ranging from -10 to -15 mV (see **Table 1**), when prepared using 1% PVA as surfactant. Though the inner emulsion ratio is generally thought to influence final particle size, with a larger ratio typically yielding bigger particles [84], we did not observe significant differences in average size (**Table 1**), with final particle sizes ranging from 300 to 400 nm for higher inner phase volumes and polydispersity indices below 0.3.

Since it is well known that extreme pHs may harm peptide structure and stability, we decided to lower the working pH. Therefore, NaOH concentrations were decreased from 25 mM (apparent pH 12.5) to 0.25 mM NaOH (yielding an apparent pH of 10.5), and after dissolving the peptide in 50% ACN 0.25 mM NaOH, pH was adjusted by diluting 5 fold in Hepes buffer pH 8.0 at different Hepes concentrations (**Table 1**, formulations 12-16). We observed that a buffer concentration of at least 50 mM was necessary to maintain a basic pH in presence of peptide (**Figure 2b**), with the final pH having a direct effect on burst release (**Figure 2c**). Since the formulation with a relatively mild inner phase with an apparent pH of 8.0 (see **Table 1**, formulation 16) showed a comparable EE and burst release as the formulation with an apparent inner phase pH of 10.5 (**Table 1**, formulation 11), formulation 16 was adopted for further functional studies. With this formulation we obtained particles with an encapsulation efficiency of 38%, with an average size of 328 nm, and zeta potencial of -13.6 mV.

3.2. Release kinetics

Since the particles are meant to be delivered to DC and taken up by DC rapidly after administration, short term release properties of the peptide-loaded particles (T**able 1**, formulation 16) were assessed for 24 h. It can be observed that after 1 h OVA24 shows approximately 30% release in PBS at 37ºC under shaking 100/min (**Figure 3**). Over the next 24 h, no further release was observed. The slight decline in initial concentration of OVA24 was due to aggregation and, hence, partial precipitation during the centrifugation step. Indeed, free OVA24 dissolved in PBS at 37°C under agitation aggregated over time, as detected by DLS (results not shown). Adding Tween 80 (0.1 % w/v) to the release medium did not help. We did not further study this phenomenon, but it obviously limited the duration of the release study.

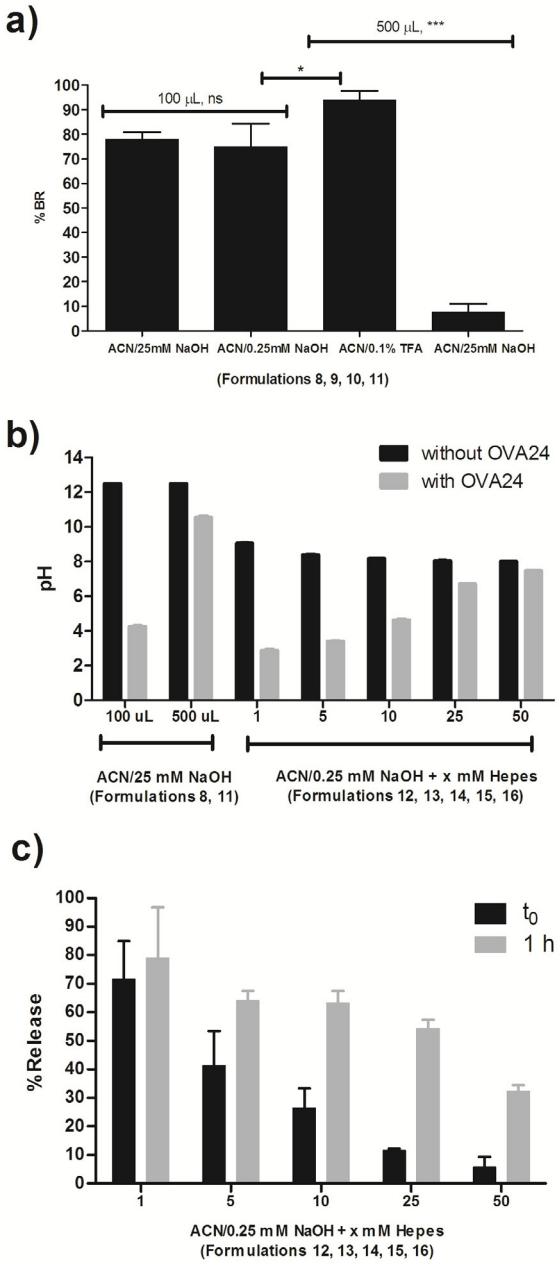


Figure 2: A) Effect of inner solvent and emulsion volume on burst release of OVA24 in PLGA NP, assessed at to upon resuspension in PBS (Formulations 8-11). **B)** Effect of the inner phase composition on apparent pH before and after the addition of OVA24 (P<0.0001) (formulations 8, 11- 16). **C)** Effect of Hepes concentration in the inner phase on release, assessed at t0 (burst release) and 1 h after resuspension (P<0.0001) (formulations 12-16). Data are presented as average ± standard deviation of n=3 independent batches. Formulation numbers are according to Table 1. P values are presented as non-significant (ns) = P > 0.05; * = P < 0.05; ** = P < 0.01; *** = P < 0.0001.

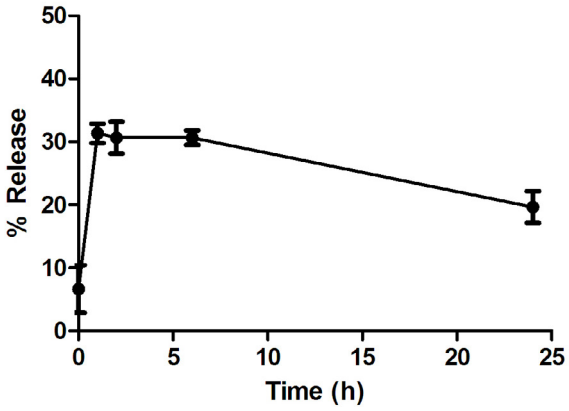


Figure 3: Release kinetics of OVA24 from PLGA NP (Table 1, formulation 16) in PBS at 37oC under shaking 100/min. Data are presented as average ± standard deviation of n=3 independent batches.

3.3. *In vitro* antigen presentation of SLP antigen encapsulated in NP

The effect of encapsulation of OVA24 on the efficiency of DC uptake and processing into MCH class I for antigen cross-presentation resulting into activation of $CD8^+$ T cells was tested *in vitro* (**Figure 4**). For that purpose, the different formulations were incubated with DC for 2.5 h, washed to remove excess unbound antigen, followed by co-culture in the presence of OVA-specific B3Z $CD8^+$ T cells. OVA24-containing PLGA NP with low (<10%) burst release (**Table 1**, formulation 16) were compared with those exhibiting high (>75%) burst release (**Table 1**, formulation 9), against soluble OVA24, empty particles, and a mixture of soluble OVA24 and empty particles. Encapsulation of OVA24 in PLGA-NP resulted in significantly enhanced activation of B3Z $CD8^+$ T cells compared to soluble OVA24. Although both tested NP formulations enhanced MHC class I antigen cross-presentation, we observed that delivery of OVA24 via encapsulation in PLGA NP with low (<10%) burst release resulted in improved T cell responses in comparison to OVA24-containing PLGA NP with high (ca. 75%) burst release. The addition of empty NP to soluble OVA24 did not show an effect on T cell activation in comparison to soluble OVA24 alone, indicating the strict necessity of peptide encapsulation in NP for enhanced processing in MHC class I antigen presentation pathways. Taken together, these data demonstrate that effective entrapment of antigen in PLGA with low burst release is crucial for efficient antigen cross-presentation by DC.

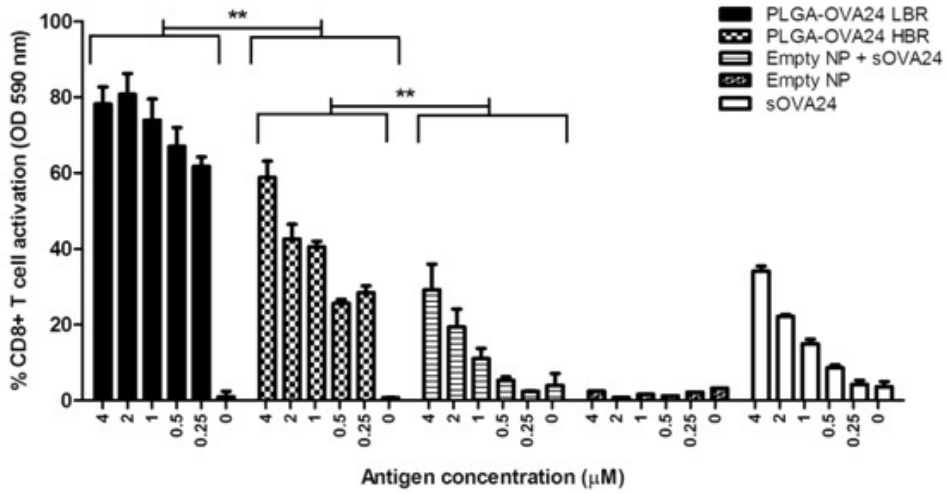


Figure 4: Normalized CD8⁺ T cell activation of SIINFEKL-specific CD8⁺ T cells (B3Z) after co-culturing with DC incubated for 2.5 hours with titrated amounts of different OVA24 SLP formulations: low-burst releasing (LBR) PLGA NP loaded with OVA24 (Table 1, formulation 16), high-burst releasing (HBR) PLGA NP loaded with OVA24 (Table 1, formulation 9), soluble OVA24 mixed with empty NP (sOVA24 + Empty NP), empty NP, and soluble OVA24 (sOVA24). Data are presented as average ± SD of triplicate measurements. Representative results from one out of 3 experiments are shown. Graphs depict normalized T cell activation data based on the maximal OD 590 nm value/T cell activation measured upon incubation of DC with 1 μM of the minimal H2-Kb epitope OVA8/SIINFEKL-peptide (OD 590 nm value of 0.918 = 100%) as positive control. P values are presented as * = $P < 0.05$; ** = $P < 0.01$.

4. Discussion

The aim of this study was to develop a method to efficiently encapsulate OVA24 in PLGA, and perform a full characterization of the obtained formulation. This approach may result in antigen-bearing biodegradable particles that can be actively taken up by DC, generating specific T cell immunity and improving the efficacy of synthetic peptide-based cancer vaccines.

Whereas hydrophilic and hydrophobic peptides have been successfully encapsulated in PLGA NP and/or microparticles in the past [85, 97, 100, 108], efficient entrapment of the moderately hydrophobic OVA24 proved to be challenging. OVA24 does not readily dissolve in aqueous solutions and is insoluble in solvents like DCM or chloroform. However, it can be dissolved in a suitable matrix such as 50% v/v ACN in water, as well as in the commonly used solvent DMSO, prior to dilution in aqueous solutions. Standard encapsulation procedures generally used for encapsulation of hydrophilic or hydrophobic antigens led to low encapsulation efficiencies and high burst release once NP were resuspended in isotonic solutions at physiological pH. Therefore,

several formulation parameters, particularly inner and outer emulsion compositions, were studied in order to increase encapsulation. The positive effect of using PVA in the outer aqueous phase on EE may be due to fact that it is not a classical surfactant with a distinct hydrophobic tail and hydrophilic headgroup, but a polymer that coats the surface, stabilizing the emulsion. In contrast, both Tween 20 and cholate are able to form micelles at concentrations above their critical micelle concentrations (0.07 and 0.6% w/v for Tween 20 and cholate, respectively), that may capture and solubilize the peptide during the emulsification process and thereby favor its extraction in the external water phase. A measurable difference between NP formulated with Tween 20 or sodium cholate (**Table 1**, formulations 1-3) and those formulated with PVA (**Table 1**, formulations 5-16) was that the negative zeta-potential of the former formulations was significantly higher. While the relatively high zeta-potential values obtained with cholate can be explained by the negative charge of the surfactant, we can only speculate that the nonionic surfactant Tween 20, due to its lower molecular size, does not shield the negative charge of the PLGA as much as PVA coating does.

The observed high burst release in the formulations with acidic inner phase led us to the notion that the majority of the OVA24 molecules might not be encapsulated in the NP's polymeric matrix, but instead were adsorbed to their surface, masking real encapsulation efficiencies. This would explain the rapid release of peptide from the NP in presence of salt by disruption of electrostatic interactions between the peptide and the PLGA surface, due to the presence of counter ions that may shield the charges, and/or alter the peptide's solubility. Indeed, the high burst release is highly consistent with observed release profiles for a tumor necrosis factor alpha blocking peptide adsorbed to PLGA NP [99], occurring once exposed to salt-based isotonic solutions at physiological pH. Increasing particle hydrophobicity by using different types of PLGA with higher molecular weights and lactic acid/glycolic acid ratio (PLGA 75:25), as well as using DMSO as solvent, creating an oil-in-water emulsion, instead of water-in-oil-in-water to reduce porosity [109, 110], did not result in a decrease of the burst release (data not shown), concurring with the adsorption hypothesis, in opposition to diffusion due to high porosity. Moreover, resuspension in either water or isotonic 5% (w/v) glucose solution did not lead to substantial burst release (**Figure 1**), which provides further evidence that the instant release was not due to high particle porosity and peptide diffusion due to osmosis.

A clear correlation between burst release and pH of the inner phase was observed (**Figure 2**), confirming that the pH of the inner phase is of primary importance for efficient peptide encapsulation. The effect of the higher inner phase volume on the burst release is mainly pH related, by providing a higher number of base/buffer molecules able to neutralize the acidic peptide and maintain an alkaline pH, whereas a higher acidic inner volume showed no effect (**Table 1**, formulation 10).

In order to better understand the physicochemical characteristics of the peptide and the effect of pH of the inner (w1) phase on the observed encapsulation efficiency and burst release, the sequence of the peptide was analyzed using ProtParam, the online protein identification and analysis software that is available through the ExPASy World Wide Web server [111] Using this tool, the theoretical isoelectric point of the OVA24 peptide

was determined as 4.3, and the grand average of hydropathicity (GRAVY) as 0.087 [112]. According to this index, amino acids are separated into hydrophilic (negative GRVAY value) and hydrophobic (positive GRAVY value). The amino acids with GRAVY values closer to zero correspond to the least hydrophobic and hydrophilic ones, which are respectively alanine (1.8) and glycine (-0.4). The slightly positive value of the OVA24 peptide confirms its slight hydrophobic nature.

Hydrophobic protein domains have shown to adsorb to polymer surfaces by electrostatic and/or hydrophobic interactions, and particle surface properties can influence adsorption [113]. Though for the OVA24/PLGA system the mechanism of encapsulation versus surface localization is still not clear, we hypothesize that efficient encapsulation at a higher pH of the inner phase may result from a more favorable partitioning of the peptide between internal/external aqueous phases and interfaces. A closer look at the peptide sequence and position of its charged residues (D-E-VSGLE-QLE-SIIN-FE-K^+LAAAAAK$^+$) allows us to divide it into two sections, with distinct characteristics. At low pHs, below the peptide's pI, the DEVSGLEQLESIINFE part should be mostly neutral, with the exception of the N-terminal amine group, and therefore rather hydrophobic. On the other hand, the K^+LAAAAAK$^+$ sequence might act as a charged headgroup, thereby rendering the molecule surface active and prone to migrate to the surface. Free peptide in the external phase may also adsorb to the particles through electrostatic interactions between positively charged residues and negatively charged PLGA NP surfaces. Once exposed to saline solutions at higher pHs, the 'hydrophobic tail' will become negatively charged, hence more hydrophilic, by which the molecule may loose its surface activity, as well as repel from negatively charged PLGA, whereas the electrostatic interactions between the positively charged residues and PLGA may also be displaced by the presence of counter ions, which could explain the release in PBS pH 7.4, but not in water (i.e., during washing of the particles during preparation) or isotonic glucose solutions with acidic pHs.

Previous studies with the short synthetic peptide SIINFEKL in PLGA microparticles showed nearly total release of the peptide within 24 hours in PBS [54]. Studies with longer peptides (13 to 43 amino acids) or recombinant human growth hormone in microparticles showed 20-70% release over the first 24 h [114-117], whereas insulin also shows 30-40% release from PLGA NP within 24 h [118]. With our method we were able to encapsulate OVA24 with nearly 40% EE in NP showing minimal burst release and a total peptide release of circa 30% over 24 h. The better retention of SLP will allow delivery of encapsulated peptide to DC, which is a great improvement compared to release over the same period of time of high releasing formulations. This is especially important if we consider subsequent development of this particulate system for delivery of SLP, which may include co-encapsulation of adjuvants, such as Toll-like receptor ligands (TLRL), or even surface modification with targeting moieties that require covalent binding to the particles at controlled pH such as described by Cruz et al. [85] without losing most of the antigen during the manufacturing process or shortly after administration.

The effect of OVA24 encapsulation on antigen cross-presentation to activate $CD8^+$ T cells was tested *in vitro* with conclusive results. OVA24-containing PLGA NP with low

burst release showed significantly higher capacity of $CD8^+$ T cell activation comparing to those with high burst release (**Figure 4**). Likewise, soluble peptide mixed with empty particles did not show any improvement when compared to soluble peptide, further proving the need of effective entrapment of antigen in PLGA to increase antigen cross presentation by DC. Furthermore, we showed that not only encapsulation of OVA24 in PLGA NP is required, but also the release characteristics are of vital importance, with low burst release being fundamental to induce a potent cellular immune response. This is probably due to enhanced uptake and/or processing by DC of particulate antigen. When incubated with low-burst releasing particles, DC may efficiently uptake the antigen cargo still encapsulated in PLGA particles, whereas with high-burst releasing particles, DC will take up less encapsulated peptide, thereby lowering the overall efficiency by which the peptide is internalized and routed into MHC class I antigen presentation pathways. The improved MHC class I antigen presentation observed with low-burst releasing particles is indicative of sustained release of the peptide inside the DC, similar to other antigen delivery systems to DC [119].

5. Conclusion

In this study we described a method for efficient encapsulation of a model SLP in PLGA NP, resulting in a particle delivery system able to enhance $CD8^+$ T cell activation *in vitro*. This encapsulation method, employing an apparent inner phase pH above the pI of the encapsulated SLP, may be a promising approach for encapsulation of peptides with amphiphilic and/or hydrophilic properties, and may be considered as a firm basis for the development of NP formulations for SLP-based immunotherapy of cancer. Preliminary studies showed that the method is applicable to other SLP as well (unpublished results). Additionally, this study shows the importance of thorough characterization of peptide encapsulation process in PLGA NP to achieve a successful formulation. In conclusion, this study has shown that encapsulation and release characteristics are strongly dependent on the pH of the first emulsion, whereas a direct comparison between NP with similar physicochemical characteristics in terms of charge, size and antigen loading, but different release profiles, uncovered the importance of low burst release to induce a potent cellular immune response.

Acknowledgements

This study was supported by grants from Immune System Activation (ISA) Pharmaceuticals and the Leiden University Medical Center. We thank Jeroen van Smeden for his help with the statistical analysis.

Conflict of Interest

The authors declare that they have no conflict of interest.

6. References

1. Ada, G., Overview of vaccines and vaccination. Mol Biotechnol, 2005. 29(3): p. 255-72.
2. Arens, R., T. van Hall, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Prospects of combinatorial synthetic peptide vaccine-based immunotherapy against cancer. Seminars in immunology, 2013. 25(2): p. 182-90.
3. Melief, C.J., Cancer immunotherapy by dendritic cells. Immunity, 2008. 29(3): p. 372-83.
4. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, . . . M. Groettrup, Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine, 2006. 24(11): p. 1847-57.
5. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
6. Atanackovic, D., N.K. Altorki, E. Stockert, B. Williamson, A.A. Jungbluth, E. Ritter, . . . S. Gnjatic, Vaccine-induced CD4^{+} T cell responses to MAGE-3 protein in lung cancer patients. J Immunol, 2004. 172(5): p. 3289-96.
7. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner, Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. European journal of immunology, 2007. 37(8): p. 2063-74.
8. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup, TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, 2008. 26(13): p. 1626-37.
9. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, . . . J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer Immunol Immunother, 2013. 62(7): p. 1161-73.
10. Braun, M., C. Jandus, P. Maurer, A. Hammann-Haenni, K. Schwarz, M.F. Bachmann, . . . P. Romero, Virus-like particles induce robust human T-helper cell responses. European journal of immunology, 2012. 42(2): p. 330-40.
11. Rosalia, R.A., L.J. Cruz, S. van Duikeren, A.T. Tromp, A.L. Silva, W. Jiskoot, . . . F. Ossendorp, CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses. Biomaterials, 2014.
12. Shen, H., A.L. Ackerman, V. Cody, A. Giodini, E.R. Hinson, P. Cresswell, . . . D.J. Hanlon, Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology, 2006. 117(1): p. 78-88.
13. Tran, K.K., X. Zhan, and H. Shen, Polymer blend particles with defined compositions for targeting antigen to both class I and II antigen presentation pathways. Advanced healthcare materials, 2014. 3(5): p. 690-702.
14. Tacken, P.J., I.J. de Vries, R. Torensma, and C.G. Figdor, Dendritic-cell

immunotherapy: from ex vivo loading to *in vivo* targeting. Nature reviews. Immunology, 2007. 7(10): p. 790-802.
15. Hailemichael, Y., Z. Dai, N. Jaffarzad, Y. Ye, M.A. Medina, X.F. Huang, . . . W.W. Overwijk, Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. Nat Med., 2013. 19(4): p. 465-72. doi: 10.1038/nm.3105. Epub 2013 Mar 3.
16. Parker, R., S. Deville, L. Dupuis, F. Bertrand, and J. Aucouturier, Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile. Procedia in Vaccinology, 2009. 1(1): p. 140-147.
17. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. Int J Cancer, 2011. 129(2): p. 407-16.
18. Mundargi, R.C., V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. Journal of controlled release : official journal of the Controlled Release Society, 2008. 125(3): p. 193-209.
19. Newman, K.D., P. Elamanchili, G.S. Kwon, and J. Samuel, Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells *in vivo*. Journal of biomedical materials research, 2002. 60(3): p. 480-6.
20. Putney, S.D. and P.A. Burke, Improving protein therapeutics with sustained-release formulations. Nat Biotechnol, 1998. 16(2): p. 153-7.
21. Kasturi, S.P., I. Skountzou, R.A. Albrecht, D. Koutsonanos, T. Hua, H.I. Nakaya, . . . B. Pulendran, Programming the magnitude and persistence of antibody responses with innate immunity. Nature, 2011. 470(7335): p. 543-7.
22. Badiee, A., N. Davies, K. McDonald, K. Radford, H. Michiue, D. Hart, and M. Kato, Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205. Vaccine, 2007. 25(25): p. 4757-66.
23. Zhang, X.Q., C.E. Dahle, N.K. Baman, N. Rich, G.J. Weiner, and A.K. Salem, Potent antigen-specific immune responses stimulated by codelivery of CpG ODN and antigens in degradable microparticles. Journal of immunotherapy, 2007. 30(5): p. 469-78.
24. Pulendran, B. and R. Ahmed, Immunological mechanisms of vaccination. Nature immunology, 2011. 12(6): p. 509-17.
25. Demento, S.L., N. Bonafe, W. Cui, S.M. Kaech, M.J. Caplan, E. Fikrig, . . . T.M. Fahmy, TLR9-targeted biodegradable nanoparticles as immunization vectors protect against West Nile encephalitis. J Immunol, 2010. 185(5): p. 2989-97.
26. Link, A., F. Zabel, Y. Schnetzler, A. Titz, F. Brombacher, and M.F. Bachmann, Innate immunity mediates follicular transport of particulate but not soluble protein antigen. J Immunol, 2012. 188(8): p. 3724-33.
27. Johansen, P., T. Storni, L. Rettig, Z. Qiu, A. Der-Sarkissian, K.A. Smith, . . . T.M. Kundig, Antigen kinetics determines immune reactivity. Proc Natl Acad Sci U S A, 2008. 105(13): p. 5189-94.
28. Simon, L.C. and C.M. Sabliov, The effect of nanoparticle properties, detection method, delivery route and animal model on poly(lactic-co-glycolic) acid nanoparticles biodistribution in mice and rats. Drug metabolism reviews, 2013.
29. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses.

Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
30. Memish, Z.A., G. Dbaibo, M. Montellano, V.P. Verghese, H. Jain, A.P. Dubey, . . . J.M. Miller, Immunogenicity of a single dose of tetravalent meningococcal serogroups A, C, W-135, and Y conjugate vaccine administered to 2- to 10-year-olds is noninferior to a licensed-ACWY polysaccharide vaccine with an acceptable safety profile. The Pediatric infectious disease journal, 2011. 30(4): p. e56-62.
31. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. AAPS J, 2013. 15(1): p. 85-94.
32. Tran, K.K. and H. Shen, The role of phagosomal pH on the size-dependent efficiency of cross-presentation by dendritic cells. Biomaterials, 2009. 30(7): p. 1356-62.
33. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, . . . P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. The Journal of experimental medicine, 1997. 185(2): p. 317-28.
34. Schuurhuis, D.H., A. Ioan-Facsinay, B. Nagelkerken, J.J. van Schip, C. Sedlik, C.J. Melief, . . . F. Ossendorp, Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J Immunol, 2002. 168(5): p. 2240-6.
35. Sanderson, S. and N. Shastri, LacZ inducible, antigen/MHC-specific T cell hybrids. International immunology, 1994. 6(3): p. 369-76.
36. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, . . . J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer immunology, immunotherapy : CII, 2013.
37. Slutter, B., S. Bal, C. Keijzer, R. Mallants, N. Hagenaars, I. Que, . . . W. Jiskoot, Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine, 2010. 28(38): p. 6282-91.
38. Sah, H., A new strategy to determine the actual protein content of poly(lactide-co-glycolide) microspheres. Journal of pharmaceutical sciences, 1997. 86(11): p. 1315-8.
39. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient $CD8(^+)$ T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45.
40. Kreutz, M., P.J. Tacken, and C.G. Figdor, Targeting dendritic cells--why bother? Blood, 2013. 121(15): p. 2836-44.
41. Schliehe, C., C. Redaelli, S. Engelhardt, M. Fehlings, M. Mueller, N. van Rooijen, . . . M. Groettrup, CD8- dendritic cells and macrophages cross-present poly(D,L-lactate-co-glycolate) acid microsphere-encapsulated antigen *in vivo*. J Immunol, 2011. 187(5): p. 2112-21.
42. Fifis, T., A. Gamvrellis, B. Crimeen-Irwin, G.A. Pietersz, J. Li, P.L. Mottram, . . . M. Plebanski, Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol, 2004. 173(5): p.

3148-54.
43. Gutierro, I., R.M. Hernandez, M. Igartua, A.R. Gascon, and J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine, 2002. 21(1-2): p. 67-77.
44. Demento, S.L., W. Cui, J.M. Criscione, E. Stern, J. Tulipan, S.M. Kaech, and T.M. Fahmy, Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials, 2012. 33(19): p. 4957-64.
45. Xiang, S.D., A. Scholzen, G. Minigo, C. David, V. Apostolopoulos, P.L. Mottram, and M. Plebanski, Pathogen recognition and development of particulate vaccines: does size matter? Methods, 2006. 40(1): p. 1-9.
46. Sharp, F.A., D. Ruane, B. Claass, E. Creagh, J. Harris, P. Malyala, . . . E.C. Lavelle, Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci U S A, 2009. 106(3): p. 870-5.
47. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug delivery, 2010. 17(7): p. 490-9.
48. Zeestraten, E.C., F.M. Speetjens, M.J. Welters, S. Saadatmand, L.F. Stynenbosch, R. Jongen, . . . C.J. Melief, Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. International journal of cancer. Journal international du cancer, 2013. 132(7): p. 1581-91.
49. Yeo, Y. and K. Park, Control of encapsulation efficiency and initial burst in polymeric microparticle systems. Arch Pharm Res, 2004. 27(1): p. 1-12.
50. Leelarasamee, N., S.A. Howard, C.J. Malanga, L.A. Luzzi, T.F. Hogan, S.J. Kandzari, and J.K. Ma, Kinetics of drug release from polylactic acid-hydrocortisone microcapsules. Journal of microencapsulation, 1986. 3(3): p. 171-9.
51. Mansour, H.M., M. Sohn, A. Al-Ghananeem, and P.P. Deluca, Materials for pharmaceutical dosage forms: molecular pharmaceutics and controlled release drug delivery aspects. Int J Mol Sci, 2010. 11(9): p. 3298-322.
52. Cruz, L.J., R.A. Rosalia, J.W. Kleinovink, F. Rueda, C.W. Lowik, and F. Ossendorp, Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8 T cell response: A comparative study. Journal of controlled release : official journal of the Controlled Release Society, 2014. 192C: p. 209-218.
53. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, . . . C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. Journal of controlled release : official journal of the Controlled Release Society, 2010. 144(2): p. 118-26.
54. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander, Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. J Drug Target, 2009. 17(8): p. 652-61.
55. Krishnamachari, Y. and A.K. Salem, Innovative strategies for co-delivering antigens and CpG oligonucleotides. Advanced drug delivery reviews, 2009.

61(3): p. 205-17.
56. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, Jr., M.P. Beckmann, L.S. Park, . . . W.E. Paul, Lymphokine control of *in vivo* immunoglobulin isotype selection. Annual review of immunology, 1990. 8: p. 303-33.
57. Romagnani, S., Th1/Th2 cells. Inflamm Bowel Dis, 1999. 5(4): p. 285-94.
58. Eckl-Dorna, J. and F.D. Batista, BCR-mediated uptake of antigen linked to TLR9 ligand stimulates B-cell proliferation and antigen-specific plasma cell formation. Blood, 2009. 113(17): p. 3969-77.
59. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
60. Samadi, N., A. Abbadessa, A. Di Stefano, C.F. van Nostrum, T. Vermonden, S. Rahimian, . . . W.E. Hennink, The effect of lauryl capping group on protein release and degradation of poly(D,L-lactic-co-glycolic acid) particles. Journal of controlled release : official journal of the Controlled Release Society, 2013. 172(2): p. 436-43.
61. Schirmbeck, R., W. Bohm, and J. Reimann, Injection of detergent-denatured ovalbumin primes murine class I-restricted cytotoxic T cells *in vivo*. European journal of immunology, 1994. 24(9): p. 2068-72.
62. Huang, C.F., T.C. Wu, Y.H. Chu, K.S. Hwang, C.C. Wang, and H.J. Peng, Effect of neonatal sublingual vaccination with native or denatured ovalbumin and adjuvant CpG or cholera toxin on systemic and mucosal immunity in mice. Scand J Immunol, 2008. 68(5): p. 502-10.
63. Peng, H.J., Z.N. Chang, L.C. Tsai, S.N. Su, H.D. Shen, and C.H. Chang, Heat denaturation of egg-white proteins abrogates the induction of oral tolerance of specific Th2 immune responses in mice. Scand J Immunol, 1998. 48(5): p. 491-6.
64. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, . . . C.J. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65.
65. Perrie, Y., A.R. Mohammed, D.J. Kirby, S.E. McNeil, and V.W. Bramwell, Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. International Journal of Pharmaceutics, 2008. 364(2): p. 272-80.
66. Welters, M.J., G.G. Kenter, S.J. Piersma, A.P. Vloon, M.J. Lowik, D.M. Berends-van der Meer, . . . S.H. van der Burg, Induction of tumor-specific $CD4^+$ and $CD8^+$ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 178-87.
67. Steinman, R. and K. Inaba, Immunogenicity: role of dendritic cells. Bioessays, 1989. 10(5): p. 145-52.
68. Steinman, R.M., The dendritic cell system and its role in immunogenicity. Annu Rev Immunol, 1991. 9: p. 271-96.
69. Inaba, K., J.W. Young, and R.M. Steinman, Direct activation of $CD8^+$ cytotoxic T lymphocytes by dendritic cells. The Journal of experimental medicine, 1987. 166(1): p. 182-94.
70. Trumpfheller, C., M.P. Longhi, M. Caskey, J. Idoyaga, L. Bozzacco, T. Keler, . .

. R.M. Steinman, Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. Journal of internal medicine, 2012. 271(2): p. 183-92.
71. Vulink, A., K.J. Radford, C. Melief, and D.N. Hart, Dendritic cells in cancer immunotherapy. Advances in cancer research, 2008. 99: p. 363-407.
72. Zwaveling, S., S.C. Ferreira Mota, J. Nouta, M. Johnson, G.B. Lipford, R. Offringa, . . . C.J. Melief, Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol, 2002. 169(1): p. 350-8.
73. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C.J. Melief, Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med, 1998. 187(5): p. 693-702.
74. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 169-77.
75. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
76. Melief, C.J., Synthetic vaccine for the treatment of lesions caused by high risk human papilloma virus. Cancer J, 2011. 17(5): p. 300-1.
77. Melief, C.J., Treatment of established lesions caused by high-risk human papilloma virus using a synthetic vaccine. Journal of immunotherapy, 2012. 35(3): p. 215-6.
78. Chiang, C.L., L.E. Kandalaft, and G. Coukos, Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. International reviews of immunology, 2011. 30(2-3): p. 150-82.
79. van Driel, W.J., M.E. Ressing, G.G. Kenter, R.M. Brandt, E.J. Krul, A.B. van Rossum, . . . J.B. Trimbos, Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I-II trial. European journal of cancer, 1999. 35(6): p. 946-52.
80. Slingluff, C.L., Jr., G. Yamshchikov, P. Neese, H. Galavotti, S. Eastham, V.H. Engelhard, . . . W.G. Ross, Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. Clinical cancer research : an official journal of the American Association for Cancer Research, 2001. 7(10): p. 3012-24.
81. Aucouturier, J., S. Ascarateil, and L. Dupuis, The use of oil adjuvants in therapeutic vaccines. Vaccine, 2006. 24 Suppl 2: p. S2-44-5.
82. Aucouturier, J., L. Dupuis, S. Deville, S. Ascarateil, and V. Ganne, Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert Rev Vaccines, 2002. 1(1): p. 111-8.
83. Leenaars, P.P., M.A. Koedam, P.W. Wester, V. Baumans, E. Claassen, and C.F. Hendriksen, Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. Laboratory animals, 1998.

32(4): p. 387-406.
84. Astete, C.E. and C.M. Sabliov, Synthesis and characterization of PLGA nanoparticles. J Biomater Sci Polym Ed, 2006. 17(3): p. 247-89.
85. Cruz, L.J., P.J. Tacken, R. Fokkink, B. Joosten, M.C. Stuart, F. Albericio, . . . C.G. Figdor, Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN *in vitro*. J Control Release, 2010. 144(2): p. 118-26.
86. Mundargi, R.C., V.R. Babu, V. Rangaswamy, P. Patel, and T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J Control Release, 2008. 125(3): p. 193-209.
87. Vlieghe, P., V. Lisowski, J. Martinez, and M. Khrestchatisky, Synthetic therapeutic peptides: science and market. Drug discovery today, 2010. 15(1-2): p. 40-56.
88. Woodley, J.F., Enzymatic barriers for GI peptide and protein delivery. Critical reviews in therapeutic drug carrier systems, 1994. 11(2-3): p. 61-95.
89. Schall, N., N. Page, C. Macri, O. Chaloin, J.P. Briand, and S. Muller, Peptide-based approaches to treat lupus and other autoimmune diseases. Journal of autoimmunity, 2012.
90. Witt, K.A., T.J. Gillespie, J.D. Huber, R.D. Egleton, and T.P. Davis, Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability. Peptides, 2001. 22(12): p. 2329-43.
91. De Temmerman, M.L., J. Rejman, J. Demeester, D.J. Irvine, B. Gander, and S.C. De Smedt, Particulate vaccines: on the quest for optimal delivery and immune response. Drug discovery today, 2011. 16(13-14): p. 569-82.
92. Fahmy, T.M., S.L. Demento, M.J. Caplan, I. Mellman, and W.M. Saltzman, Design opportunities for actively targeted nanoparticle vaccines. Nanomedicine, 2008. 3(3): p. 343-55.
93. Elamanchili, P., C.M. Lutsiak, S. Hamdy, M. Diwan, and J. Samuel, "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. J Immunother, 2007. 30(4): p. 378-95.
94. Jain, S., D.T. O'Hagan, and M. Singh, The long-term potential of biodegradable poly(lactide-co-glycolide) microparticles as the next-generation vaccine adjuvant. Expert review of vaccines, 2011. 10(12): p. 1731-42.
95. van der Lubben, I.M., J.C. Verhoef, G. Borchard, and H.E. Junginger, Chitosan and its derivatives in mucosal drug and vaccine delivery. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2001. 14(3): p. 201-7.
96. Jiang, W., R.K. Gupta, M.C. Deshpande, and S.P. Schwendeman, Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Adv Drug Deliv Rev, 2005. 57(3): p. 391-410.
97. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug delivery, 2010. 17(7): p. 490-9.
98. Slutter, B., L. Plapied, V. Fievez, M.A. Sande, A. des Rieux, Y.J. Schneider, . . . V. Preat, Mechanistic study of the adjuvant effect of biodegradable nanoparticles

in mucosal vaccination. J Control Release, 2009. 138(2): p. 113-21.
99. Yang, A., L. Yang, W. Liu, Z. Li, H. Xu, and X. Yang, Tumor necrosis factor alpha blocking peptide loaded PEG-PLGA nanoparticles: preparation and *in vitro* evaluation. Int J Pharm, 2007. 331(1): p. 123-32.
100. Wischke, C. and S.P. Schwendeman, Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. Int J Pharm, 2008. 364(2): p. 298-327.
101. Liu, Y. and S.P. Schwendeman, Mapping Microclimate pH Distribution inside Protein-Encapsulated PLGA Microspheres Using Confocal Laser Scanning Microscopy. Molecular pharmaceutics, 2012. 9(5): p. 1342-50.
102. van de Weert, M., W.E. Hennink, and W. Jiskoot, Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharm Res, 2000. 17(10): p. 1159-67.
103. Jiskoot, W., T.W. Randolph, D.B. Volkin, C.R. Middaugh, C. Schoneich, G. Winter, . . . J.F. Carpenter, Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. Journal of pharmaceutical sciences, 2012. 101(3): p. 946-54.
104. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, R. Offringa, and S.H. van der Burg, $CD8^+$ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. J Immunol, 2007. 179(8): p. 5033-40.
105. Khan, S., M.S. Bijker, J.J. Weterings, H.J. Tanke, G.J. Adema, T. van Hall, . . . F. Ossendorp, Distinct uptake mechanisms but similar intracellular processing of two different toll-like receptor ligand-peptide conjugates in dendritic cells. J Biol Chem, 2007. 282(29): p. 21145-59.
106. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, . . . P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med, 1997. 185(2): p. 317-28.
107. Sanderson, S. and N. Shastri, LacZ inducible, antigen/MHC-specific T cell hybrids. Int Immunol, 1994. 6(3): p. 369-76.
108. Wieber, A., T. Selzer, and J. Kreuter, Characterisation and stability studies of a hydrophilic decapeptide in different adjuvant drug delivery systems: a comparative study of PLGA nanoparticles versus chitosan-dextran sulphate microparticles versus DOTAP-liposomes. International Journal of Pharmaceutics, 2011. 421(1): p. 151-9.
109. Luan, X. and R. Bodmeier, Influence of the poly(lactide-co-glycolide) type on the leuprolide release from in situ forming microparticle systems. Journal of controlled release : official journal of the Controlled Release Society, 2006. 110(2): p. 266-72.
110. Bao, W., J. Zhou, J. Luo, and D. Wu, PLGA microspheres with high drug loading and high encapsulation efficiency prepared by a novel solvent evaporation technique. Journal of microencapsulation, 2006. 23(5): p. 471-9.
111. Gasteiger, E., A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel, and A. Bairoch, ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res, 2003. 31(13): p. 3784-8.
112. Kyte, J. and R.F. Doolittle, A simple method for displaying the hydropathic character of a protein. Journal of molecular biology, 1982. 157(1): p. 105-32.
113. Jung, T., W. Kamm, A. Breitenbach, G. Klebe, and T. Kissel, Loading of tetanus toxoid to biodegradable nanoparticles from branched poly(sulfobutyl-

polyvinyl alcohol)-g-(lactide-co-glycolide) nanoparticles by protein adsorption: a mechanistic study. Pharmaceutical research, 2002. 19(8): p. 1105-13.
114. Sales-Junior, P.A., F. Guzman, M.I. Vargas, S. Sossai, V.A. Patarroyo, C.Z. Gonzalez, and J.H. Patarroyo, Use of biodegradable PLGA microspheres as a slow release delivery system for the Boophilus microplus synthetic vaccine SBm7462. Veterinary immunology and immunopathology, 2005. 107(3-4): p. 281-90.
115. Rafi, M., S.M. Singh, V. Kanchan, C.K. Anish, and A.K. Panda, Controlled release of bioactive recombinant human growth hormone from PLGA microparticles. Journal of microencapsulation, 2010. 27(6): p. 552-60.
116. Burton, K.W., M. Shameem, B.C. Thanoo, and P.P. DeLuca, Extended release peptide delivery systems through the use of PLGA microsphere combinations. Journal of biomaterials science. Polymer edition, 2000. 11(7): p. 715-29.
117. Buyuktimkin, B., Q. Wang, P. Kiptoo, J.M. Stewart, C. Berkland, and T.J. Siahaan, Vaccine-like controlled-release delivery of an immunomodulating peptide to treat experimental autoimmune encephalomyelitis. Molecular pharmaceutics, 2012. 9(4): p. 979-85.
118. Yang, J., H. Sun, and C. Song, Preparation, characterization and *in vivo* evaluation of pH-sensitive oral insulin-loaded poly(lactic-co-glycolicacid) nanoparticles. Diabetes, obesity & metabolism, 2012. 14(4): p. 358-64.
119. van Montfoort, N., M.G. Camps, S. Khan, D.V. Filippov, J.J. Weterings, J.M. Griffith, . . . F. Ossendorp, Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc Natl Acad Sci U S A, 2009. 106(16): p. 6730-5.

Chapter 6

Co-encapsulation of synthetic long peptide antigen and Toll like receptor 2 ligand in poly-(lactic-co-glycolic-acid) particles results in sustained MHC class I cross-presentation by dendritic cells

Rodney A. Rosalia,*, Ana Luisa Silva*, Ivan Stepanek, Annelies van der Laan, Jaap Oostendorp, Wim Jiskoot, Sjoerd Van der Burg, Ferry Ossendorp

*contributed equally to this study

Manuscript in preparation for publication

Abstract

We previously reported the succesfull incorporation of synthetic long peptides (SLPs) in poly(lactic- co-glycolic acid) (PLGA) nanoparticles (NP) as a vaccine delivery vehicle. We showed that a low burst release of the encapsulated SLP was crucial to improve MHC class I presentation and $CD8^+$ T cell activation in comparison to soluble SLP (sSLP) *in vitro*. Using SLP-OVA24aa as vaccine antigen (Ag) and toll-like receptor (TLR) 2 ligand Pam3CSK4 as an adjuvant encapsulated in PLGA-NP (PLGA-SLP and PLGA-SLP/TLR2L), we show in this report that TLR 2 stimulation enhances MHC class I presentation of PLGA-SLP by dendritic cells (DCs), however co-encapsulation of the TLR ligand was not required for this effect. DC loaded with PLGA-SLP(/TLR2L) internalized NP into endo-lysosomal compartments and not the cytosol as occurs with sSLP. Moreover, PLGA-NP encapsulated SLP could be detected for prolonged periods inside endo-lysosomal compartments. Prolonged presence of NP inside DC resulted in MHC class I presentation of encapsulated SLP for up to 96 hr, which led to sustained $CD8^+$ T cell proliferation in *in vivo* adoptive transfer of PLGA-SLP loaded DC. These findings explain the *in vivo* effectiveness of nanoparticle vaccination and shows that PLGA-SLP is a promising delivery vehicle for clinical application as a cancer immunotherapy.

Keywords: Peptide antigen, synthetic long peptides, CTL epitope, PLGA nanoparticles, TLRL, cancer immunotherapy, cellular immune response

1. Introduction

Cancer immunotherapy is a promising treatment modality to enhance the tumor associated antigen (Ag) specific T cell responses in cancer patients. Efficient MHC class I Ag presentation and subsequent $CD8^+$ T cell priming are pre-requisites for optimal clinical efficacy of a cancer immunotherapy vaccine [1, 2]. We recently reported that synthetic long peptides (SLP) considerably facilitate MHC class I presentation in comparison to protein, which was related to faster uptake and processing of SLP by dendritic cells (DC) compared to that of protein Ag [3].

SLP-vaccines emulsified in Montanide(-ISA51) water-in-oil-in-water emulsion have been studied in the clinic against various forms of cancer [4, 5] and other immunological diseases [6, 7]. However, the use of Montanide is associated with considerable adverse effects [8-11]. In addition, montanide has poorly defined adjuvant properties and the release kinetics of the emulsified vaccine-Ag cannot be controlled. Alternative vaccine delivery systems and adjuvants for SLP, being at least as efficient as but having less side effects than Montanide, are therefore highly required like well-defined Toll like receptor ligands (TLRL). In light of this we have previously reported the succesful application of nanoparticles (NP) formulated with the fully biocompatible polymer, poly-(lactic-co-glycolic-acid) (PLGA) as delivery vehicle for SLP vaccines. Encapsulating SLP in PLGA led to a significant enhancement of MHC class I Ag presentation and $CD8^+$

T cell activation compared to soluble SLP [10]P. However, PLGA-NP have low immunostimulatory properties by itself but allows controled co-encapsulation and release of TLRL [12, 13].

The aim of the present paper was to study 1) how PLGA-NP encapsulated SLP is routed and processed into MHC class I molecules and 2) how a defined adjuvant co-encapsulated in NP affects the efficiency and duration of $CD8^+$ T cell activation by DC. For this purpose, NP were formulated together with a TLR2L (Pam3CSK4) as adjuvant, which effectively boosted vaccine-Ag specific immune responseswhen covalently conjugated to SLP [14, 15].

We show here that co-encapsulating TLR2L with SLP in PLGA-NP further enhances the efficiency of MHC class I cross-presentation of SLP by DC compared to plain PLGA-SLP. In addition, loading of DC using PLGA-NP results in sustained MHC class I presentation of SLP compared to soluble SLP. However, the effect of sustained MHC clas I presentation was not related to TLR2L-mediated DC maturation but likely because of the prolonged presence of PLGA-NP encapsulated SLP inside endo-lysosomal compartments upon uptake by DC. These organels are similar to the storage compartments in DC we have described for other antigen targeting system like FcR-mediated uptake [16].

Finally, we show that adoptive transfer of DC loaded with PLGA-SLP/TLR2L stimulated $CD8^+$ T cells over a sustained period of time whereas soluble SLP loaded DC failed to do so. Therefore, this study presents additional evidence for the use of PLGA-NP as a clinically suitable vaccine delivery systems to enhance direct MHC class I Ag presentation and T cell activation but also maintain $CD8^+$ T cell responses over a prolonged time period.

2. Material and methods

2.1. Mice

WT C57BL/6 (CD45.2/Thy1.2; H2-Kb) mice were obtained from Charles River Laboratories (France). TAP1 KO mice (C57BL/6 CD45.2/Thy1.2; H2-Kb) were purchased from the Jackson laboratory (Bar Harbor, ME). All mice were used at 8–12 weeks of age in accordance with national legislation and under supervision of the animal experimental committee of the University of Leiden.

2.2. Materials

The synthetic long peptide DEVSGLEQLESIINFEKLAAAAAK (SLP-OVA24) [17], covering the H2-Kb restricted $CD8^+$ T cell epitope SIINFEKL of ovalbumin (OVA) and SLP-OVA24-Bodipy-FL (Bp) were synthesized at the interdepartmental GMP facility of the Department of Clinical Pharmacy and Toxicology of Leiden University Medical Center as described previously [10]. Poly(D,L-lactic-co-glycolic acid) [PLGA], 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) and methanol (MeOH) were obtained from Biosolve BV (Valkenswaard, the Netherlands), Polyvinyl alcohol (PVA) 4-88 (31 kDa) was purchased from Fluka (Steinheim, Germany). Reversed phase HPLC column ReproSil-Pur C18-AQ 3 µm (150x4 mm) was purchased from Dr. Maisch HPLC GmbH (Ammerbuch-Entringen, Germany). Pam3CSK4 and Pam3CSK4-Rhodamine were purchased from Invivogen (San Diego, USA). Iscove's Modified Dulbecco's Medium (IMDM) was purchased from Lonza (Walkersville, USA). All other chemicals were of analytical grade and all aqueous solutions were prepared with milli Q water.

2.3. Nanoparticle preparation and characterization

Nanoparticles loaded with SLP-OVA24 were prepared using a double emulsion with solvent evaporation method as previously described [10]. In brief, 1.4 mg SLP-OVA24 were dissolved in 100 µL 50% ACN in 0.25 mM NaOH and then added to 400 µL 50 mM Hepes, pH 8.0. This solution was then added to 50 mg of PLGA in 1 ml of dichloromethane and the mixture was emulsified under sonication (30 s, 20 W). To this first emulsion (w1/o), 2 ml of an aqueous surfactant solution (for surfactant types, see results) 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 drop-wise to 25 ml of extraction medium (0.3% w/v surfactant) previously heated to 40°C under agitation, to allow quick solvent evaporation, and left stirring for 1 h. The particles were then collected by centrifugation for 15 min at 15000 g at 10°C, washed, resuspended in Milli Q water, aliquoted and freeze-dried at -55°C in a Christ Alpha 1-2 freeze-drier (Osterode am Harz, Germany) overnight. For particles co-encapsulating SLP-OVA24 and Pam3CSK4, 250 µg of Pam3CSK4 were dissolved in DCM together with PLGA, and for particles containing SLP-OVA24-Bp-FL, circa 10% labeled peptide was added to the peptide solution.

Particle characterization was performed as described [10]. NP were diluted to 2.5 mg/ml in 1 mM Hepes pH 7.4. Size and polydispersity index (PDI) of NP were measured by dynamic light scattering, and zeta-potential was measured by laser Doppler electrophoresis, using a Zetasizer (Nano ZS, Malvern Ltd., United Kingdom). The encapsulation efficiency (EE) was calculated according to **equation 1** and drug loading (DL) by **equation 2**. EE of OVA24 was determined by measuring the peptide content

of digested particles by reversed phase HPLC as described [10]. EE of Pam3CSK4-Rhodamine was determined by measuring fluorescence detected in the supernatant against a calibration curve and expressed as percentage of the total amount added.

$$\%\,EE = \frac{\text{SLP/TLR2L mass in NP}}{\text{initial SLP/TLR2L mass}} \times 100 \quad (1)$$

$$\%\,DL = \frac{\text{encapsulated SLP/TLR2L mass}}{\text{total polymer} + \text{SLP/TLR2L mass}} \times 100 \quad (2)$$

2.4. Cells

Freshly isolated murine DCs were cultured from mouse bone marrow (BM) cells, as described before [18]. The D1 cell line, an immature primary splenic DC line (C57BL/6-derived), was cultured as described elsewhere [19]. B3Z $CD8^+$ T cells (H2-kb/SIINFEKL) are hybridoma cell lines expressing a β-galactosidase construct which upon T-cell activation can be measured by a colorimetric assay [3].

2.5. Murine MHC class I Ag presentation assays

C57BL/6 BMDCs or D1 cells (1*105 cells/well) were plated out in triplicate using 96-well plates (Greiner #655101) and incubated for 2.5 hr with the Ag at the indicated concentrations. Cells were washed 3x with complete medium to remove excess Ag before the B3Z $CD8^+$ T cells were added to assess MHC class I cross-presentation. T cells were cultured in the presence of Ag-loaded DC for 2.5 at 37 °C. In some experiments, BMDC or D1 cells were pre-incubated with epoxomicin (324800, Merck) or bafilomycin A1 (196000, Merck) followed by Ag-incubation as described above in the presence of the compounds. D1 cells were pre-incubated with bafilomycin A1 (196000, Merck) and MHC class I Ag presentation determined as described above. To study sustained MHC class I Ag presentation, 2*106 immature D1 cells were incubated for 2.5 hr with 4 μM SLP in different formulations. After incubation, cells were harvested and transferred to 50 ml Falcon tubes, resuspended in complete medium and centrifuged . This procedure was performed 3x to wash away unbound Ag. Cells were either used directly as Ag presentating cells (APC) (“direct condition”) or plated out again in petri dishes and further cultured for 96 hr (“chase condition”). Ag loaded D1 cells were then harvested and plated out in 96-wells plates (5*104 cells/well) and used as APC in co-culture with B3Z $CD8^+$ T cells to detect capacity to cross-present SLP in MHC class I molecules using a colorometric assay as described before [3].

2.6. Adoptive transfer of Ag loaded DC

C57BL/6 BMDC cells (10*106 cells/petri dish, Corning # 430589) were loaded with 4 μM PLGA-SLP, PLGA-SLP/TLR2L and sSLP on t = - 96 hr and t = -2.5 hr. Cells were washed to remove unbound Ag and either used directly or further cultures in the absence of Ag. On t = - 1 day, splenocytes from Thy1.1$^+$ OT-I mice were harvested and transferred i.v. to recipient animals (10*106 splenocytes/mouse). On t = 0, OVA-specific T cells enriched mice received i.v. 1*106 DC loaded with PLGA-SLP, PLGA-SLP/TLR2L. Tail vein bloodsamples were collected on t = day 3 post-transfer of DC and analyzed for the percentages of Thy1.1$^+$ CD8$^+$ T cells using rat anti-mouse CD90.1-APC, CD3-AF800 and CD8-FITC antibodies (Biolegend). Samples were measured using an LSRII flow cytometer (BD) and analyzed with FlowJo software (Treestar).

2.7. Confocal microscopy

DCs were incubated for 24 hr with 10 μM SLP-OVA24-Bodipy-FL in different formulations at 37°C. Specific murine DC used is described in the figure legends. After incubation cells were washed 3 times to remove excess and unbound Ag, resuspended at a concentration of 2x105 cells in 200 μl complete medium and plated into poly-d-lysine coated glass-bottom dishes (MatTek) followed by mild centrifugation to allow the cells to adhere. Adhered cells were then fixed with 0.2% paraformaldehyde. All imaging experiments were carried out on a Leica TCS SP5 confocal microscope (HCX PL APO 63×/1.4 NA oil-immersion objective, 12 bit resolution, 1024×1024 pixels, pinhole 2.1 Airy discs, zoom factor 1 or 7). Imaging was performed using the 488 nm line from an Argon laser collecting emission between 500 and 600 nm. Dual color images were acquired by sequential scanning, with only one laser per scan to avoid cross talk. The images were analyzed using the Leica software program (LAS AF).

2.8. Analysis of cytokine production by DC using Enzyme-linked Immunosorbent Assay (ELISA)

BMDC were incubated for 24 hr with NP. Supernatants were harvested and tested for IL-12 p70 (BD OptEIA™ MOUSE IL-12 Cat. Nr 555256) following manufacturer's instructions.

2.9. Statistics

Graphpad prism was used as the main statistical software. Statistical analyses applied to determine the significance of differences are described in the figure legends.

3. Results

3.1. Characterization of formulated PLGA-SLP and PLGA-SLP/TLR2L NP

PLGA-NP were formulated using a modified double emulsion method and solvent evaporation technique with SLP-OVA24 as described before, PLGA-SLP [10]. This formulation was adapted by adding the TLR2 ligand (TLR2L) Pam3CSK4 in the organic phase to yield PLGA-NP co-encapsulating SLP and adjuvant, PLGA-SLP/TLR2L. For visualization purposes selected batches were formulated with 10 % SLP-OVA24-Bodipy-FL (SLP-Bp). Fluorescent SLP was added during formulation. Particle characteristics are described in **Table 1**.

Co-encapsulation of TLR2L and/or SLP-Bp did not affect the physical properties of the formulated NP, with several batches showing very similar characteristics (**Table 1**).

Table 1: Characterization of PLGA-SLP/TLR2L NP

Formulation	%DL SLP^{o}	%EE SLP^{o}	%DL Pam3CSK4	%EE Pam3CSK4	Size (nm)	PDI	ZP (mV)
PLGA-SLP*	1.06 ± 0.15	39 ± 6	n/a	n/a	322 ± 44	0.19 ± 0.04	-12 ± 1
PLGA-SLP/ TLR2L*	1.02 ± 0.17	38 ± 6	0.32 ± 0.05	67 ± 10	293 ± 19	0.18 ± 0.04	-13 ±1
PLGA-SLP-Bp**	1.01	37	n/a	n/a	312	0.23	-16
PLGA-SLP/ TLR2L-BP**	0.95	35	0.16	33	304	0.20	-13

DL = drug loading; EE = encapsulation efficiency; PDI = poly dispersity index; variance, an arbitrary measure for the degree of dispersity in particle size within one batch of particles suspension, PDI values below 0.3 was considered monodisperse and accepted for follow up studies [20].ZP = zeta potential; The magnitude of the zeta potential is predictive of the colloidal stability. Nanoparticles with Zeta Potential values greater than $^{+}$25 mV or less than -25 mV typically have high degrees of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van der Waal inter-particle attractions.

^{o}SLP-OVA24 (DEVSGLEQLESIINFEKLAAAAAK).

*Values represent average ±SD of > 4 independently prepared batches.

**Values obtained from 1 batch.

3.2. Enhanced cross-presentation of PLGA-NP encapsulated SLP in the presence of TLR2L

DC loaded with PLGA-SLP cross-presented SLP in the context of MHC class I molecules with higher efficiency compared to sSLP, as published previously [20]. TLR-stimulation is known to improve proteasome activity and thus enhance MHC class I Ag cross-presentation. Indeed, the presence of TLR2L during Ag-loading enhanced MHC class I cross-presentation of all SLP-formulations tested compared to DC loaded in the absence of TLR2L (**Figure 1A**). PLGA-SLP/TLR2L resulted in better $CD8^+$ T cell activation than PLGA-SLP. However, co-encapsulation of TLR2L was dispensable to enhance MHC class I presentation of PLGA-encapsulated SLP *in vitro* as mixtures of PLGA-SLP $^+$ soluble TLR2L (sTLR2L) showed similar potency to PLGA-SLP/TLR2L.

TLR2 stimulation failed to improve the cross-presentation of sSLP to comparable levels as observed with PLGA-encapsulated SLP (**Figure 1A**). This observation stresses the importance of an optimal delivery method to achieve high levels of Ag intracellularly and to improve MHC class I Ag processing.

In summary, combining TLR2L stimulation with PLGA-NP delivery of SLP significantly improves MHC class I presentation compared to Ag loading in the absence of TLR2L. The positive effect of TLR2L was irrespective of co-encapsulation in PLGA-NP.

3.3. Ag-delivery via PLGA-NP results in prolonged MHC class I Ag cross-presentation

The duration of MHC class I presentation and TCR recognition/binding is an important factor determining $CD8^+$ T cell priming. Short TCR-stimulation leads to sub-optimal T cell priming which is associated in impaired effector functions anergy [21, 22]. The long term effects on MHC class I cross-presentation of SLP was studied by stimulating B3Z $CD8^+$ T cells with 96 hr rested Ag-loaded DC. DC were incubated for 2.5 hr in the presence of 4 μM SLP in either soluble or particulate form with or without TLR2L. As a positive control, DC were incubated with SLP-TLR2L conjugates which induce the formation of Ag-storage compartments upon internalization. These compartments facilitate prolonged MHC class I presentation and sustained $CD8^+$ T cell priming as we have previously reported [14, 18]. DC loaded with sSLP failed to activate $CD8^+$ T cells 96 hr post Ag-incubation (**Figure 1B**). Only DC incubated with PLGA-encapsulated Ag and the SLP-TLR2L conjugate were capable of MHC class I SLP cross-presentation after 96 hr (**Figure 1B**).

TLR-stimulation has been shown to slow down the decay of MHC class I molecules thereby prolonging cell-surface expression of MHC class I molecule/peptide complexes

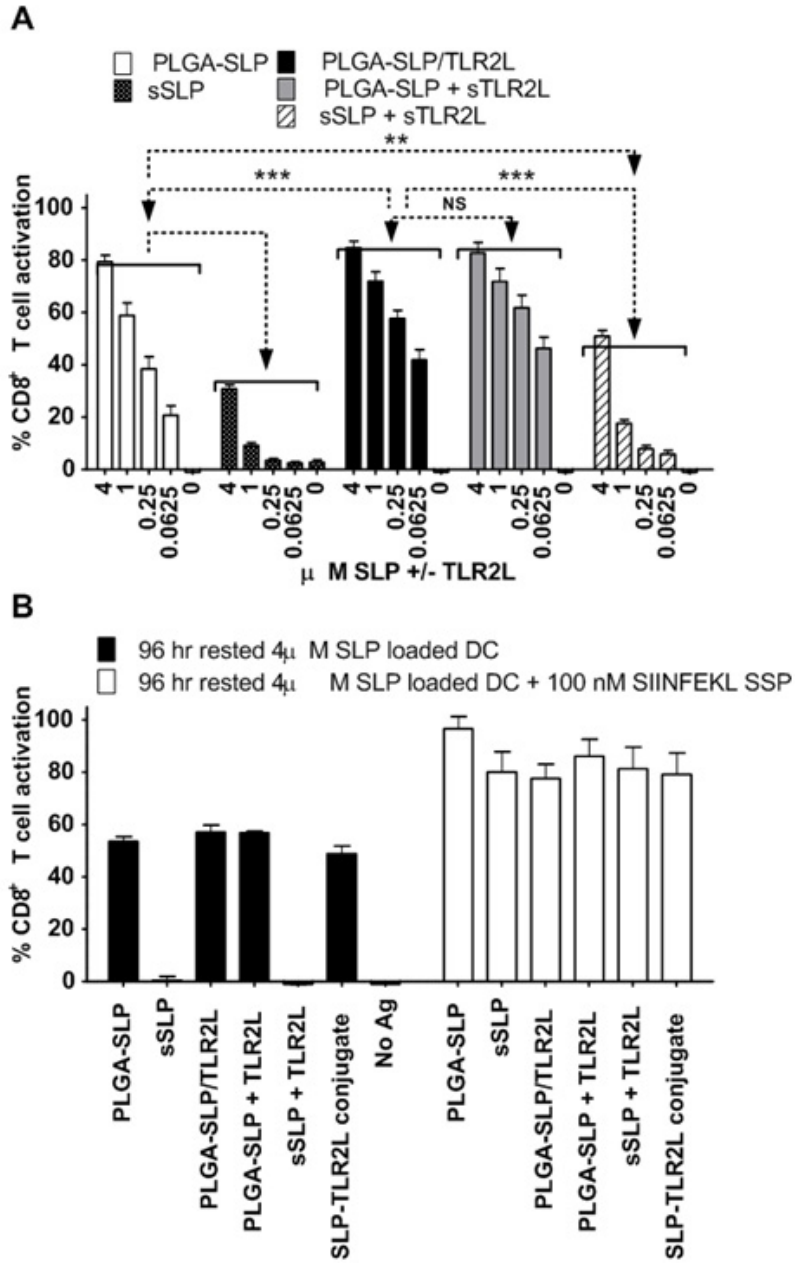


Figure 1. TLR2 stimulation improves MHC class I cross-presentation of PLGA-NP encapsulated SLP. D1 cells were incubated for 2.5 h with titrated amounts of SLP-OVA24 in different formulations with our without Pam3CSK4 (TLR2L) and co-cultured overnight in the presence of **(A)** B3Z CD8+ T cells. T-cell activation was determined as described in Materials and methods. **(B)** D1 cells were incubated with 4 μM Ag for 2.5 hr on t = -96 hr (chase) and on t = -2.5 hr (direct). "Chase" and "direct" Ag loaded DC were harvested at t = 0 hr and their APC capacity to activate B3Z CD8+ T cells compared. Data are shown as mean + SD of three samples from one representative experiment representative of four (A) and three (B) experiments performed. *** $P < 0.001$ using a two-way ANOVA and Bonferroni posttests.

[23]. In our system however, prolonged presence of MHC class I molecules/peptide complexes is unlikely to be the main mechanism facilitating sustained CD8+ T cell activation by PLGA-SLP as plain particles in the absence of additional adjuvants poorly matured DC. PLGA-SLP had no phenotypical (**Figure 2A**) nor functional DC maturating effects (**Figure 2B**) on DC. Addition of Pam3CSK4 to PLGA-SLP formulations, to obtain PLGA-SLP/TLR2L, very efficiciently matured DC (**Figure 2**). However, TLR2L stimulation did not further MHC class I Ag cross-presentation by DC loaded with PLGA-SLP or sSLP loaded DC and then rested for 96 hr in culture in the absence of Ag. Therefore, TLR2-stimulation by itself cannot explain our observations of prolonged Ag presentation by DC pulsed by PLGA-SLP.

In conclusion, Ag delivery via PLGA-NP results in prolonged MHC class I presentation *in vitro* and sustained CD8+ T cell activation. Prolonged MHC class I presentation of SLP was not dependent on DC maturation.

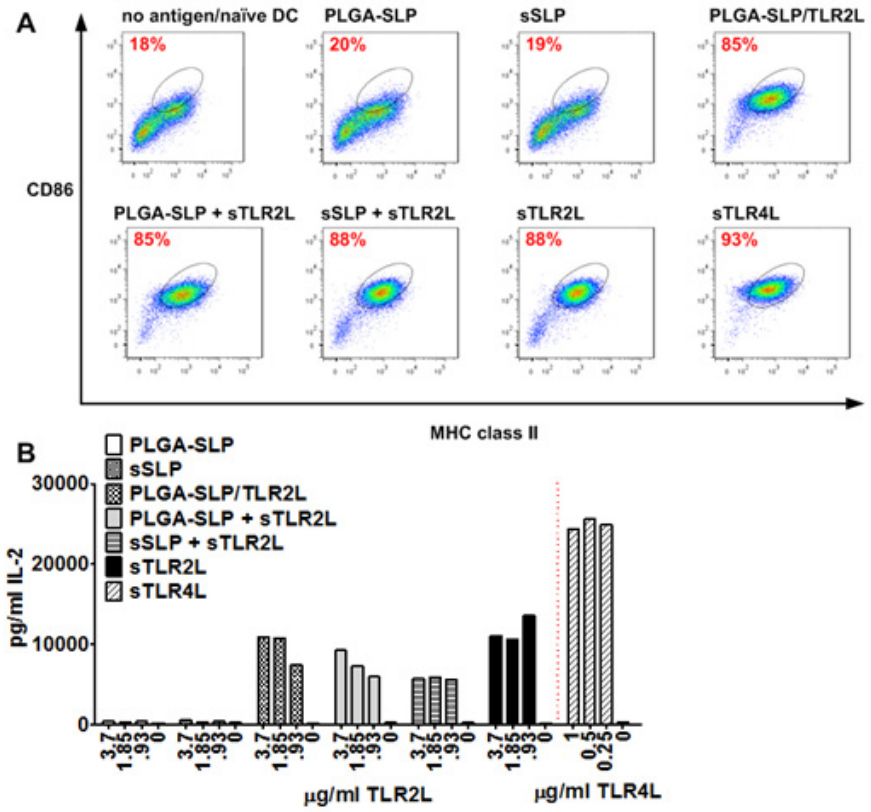


Figure 2. Addition of TLR2L to plain PLGA-SLP and sSLP improves adjuvanticity and results in DC maturation. D1 cells were incubated in the presence of titrated amounts of SLP formulations with or without Pam3CSK4 (Invivogen, tlrl-pms), and soluble LPS (TLR4L) (Sigma L4130, Escherichia coli 0111:B4). After 24 hr supernatants were collected, DC harvested and stained for MHC class II and CD86 and their expression analyzed by FACS **(A)**. Percentages indicate the numbers of double positive cells. Supernatants were analyzed via ELISA for IL-2 levels **(B)**. Data shown are representative of three independent experiments.

3.4. DC loaded with SLP encapsulated in NP are capable of sustained *in vivo* priming after adoptive transfer

The *in vitro* observations were confirmed *in vivo* by transferring DC which were loaded with Ag on t = -96 hr into recipient mice enriched with OT-I CD8⁺ T cells on day before adoptive transfer. The extent of CD8⁺ T cell expansion was compared to mice receiving DC loaded with Ag (-2.5 hr, "direct").

Only DC which were loaded with PLGA-SLP or PLGA-SLP/TLR2L induced significant OT-I CD8⁺ T expansion using freshly Ag-loaded DC (**Figure 3**). DC loaded with sSLP performed poorly as APC at the concentrations of sSLP tested. Poor priming of OT-I CD8⁺ T cells can be related to the lower MHC class I presentation of sSLP compared to PLGA-SLP but is most likely a result of sub-optimal activation of naive OT-I CD8⁺ T cells, which are co-stimulation dependent, by sSLP loaded DC which have a immature phenotype (**Figure 2**). PLGA-SLP and even PLGA-NP in general, do not mature DC. However NP-based Ag delivery is very efficient leading to high density of Ag-epitope loaded MHC class I molecules on the cell-surface leading to sufficient triggering of OT-I CD8⁺ T cells for them to proliferate *in vivo*. PLGA-SLP with co-encapsulated TLR2L results in significant OT-I expansion even after 96 hrs incubation indicating sustained antigen storage of the particle-delivered antigen by DC.

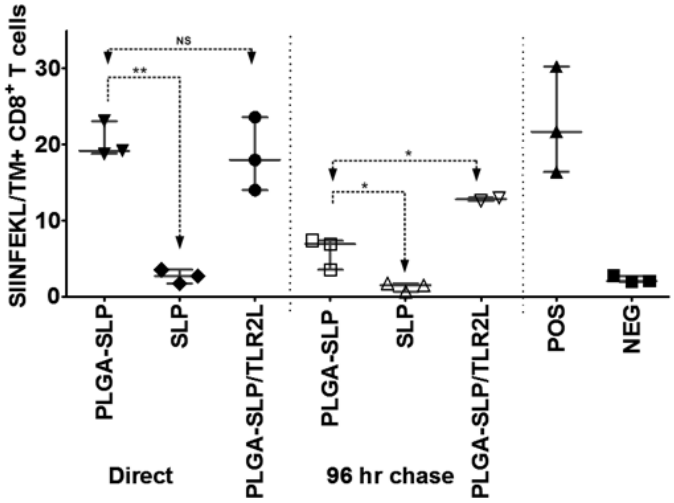


Figure 3. PLGA-SLP loaded DC possesses APC capacity and stimulated CD8⁺ T cells 96 hr after Ag-loading. Ag loaded BMDC (C57BL/6) were tested for their capacity to expand specific CD8⁺ T cells *in vivo*. WT C57BL/6 animals received 10*106 OVA-specific Thy1.1⁺ OT-I splenocytes i.v. on t = day -1. On t = 0, OVA-specific T cell enriched mice received i.v. 1*106 DC loaded with PLGA-SLP, PLGA-SLP/TLR2L and sSLP on t = -96 hr and t = -2.5 hr. Blood samples were taken on t = day 3 post-transfer of DC and analyzed for the percentages of Thy1.1⁺CD8⁺ T cells. DC loaded with SIINFEKL and 10 µg/ml LPS and antigen naïve DC were used as positive (pos) and negative (neg) control. Percentages determined for each individual mice are displayed and data shown are representative of one independent experiments. *P< 0.05 using a unpaired student t-test.

3.5. Re-routing and prolonged presence of SLP into the endosomes by encapsulation in PLGA-NP

We analyzed if the intracellular localisation of PLGA-SLP after uptake by DC might play a role in the observed MHC class I presentation. For this purpose DC were incubated with sSLP, PLGA-SLP and PLGA-SLP/TLR2L and analyzed directly by confocal microscope. D1 dendritic cells and BMDC were highly capable to internalizing Bp-labeled SLP in the tested formulations (green fluorescence, **Figures 4 & 5**).

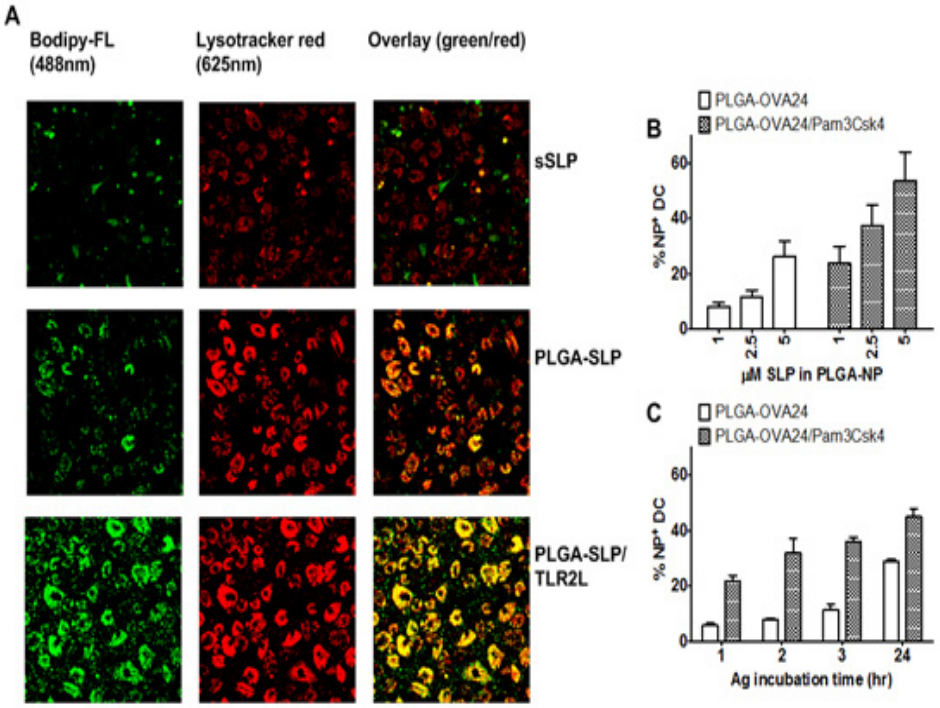


Figure 4. Rerouting of SLP into endo-lysosomal compartments upon encapsulation of in PLGA-NP. BMDC were incubated with 20 µM sSLP-Bp, 20 µM PLGA-SLP-Bp (10% SLP-Bodipy-FL) or 20 µM PLGA-SLP-Bp/TLR2L (10% SLP-Bodipy-FL) (excitation- 488nm, visualized as the green signal) for 2.5 hr and co-stained with Lysotracker red for visualization of the endo-lysosomes (red signal) and visualized by confocal microscopy. **(A)** 1st column shows images depicting green signal as the fluorescence of Bodipy-FL (488nm). 2nd column shows images depicting red signal as the fluoresence of the lysotracker red/endo-lysosomes (625nm). 3rd column depicts overlay images of red/green. Yellow-overlay signal marks co-localization. Images were analyzed using Leica software. BMDC were incubated at 4°C and 37°C with **(B)** titrated amounts of PLGA-SLP, PLGA-SLP/TLR2L and PLGA-SLP + sTLR2L (10% SLP-Bodipy-FL). (C) Alternatively, DC were incubated with with 2.5 µM of PLGA-SLP and PLGA-SLP/TLR2L (10% SLP-Bodipy-FL). Ag uptake was quantified by flow cytometry and data shown are absolute values (% NP^+ DC at 37°C - % NP^+ DC at 4°C) and represent Avg + SEM of 3 independent experiments (A) and Avg + SD of 2 independent experiments.

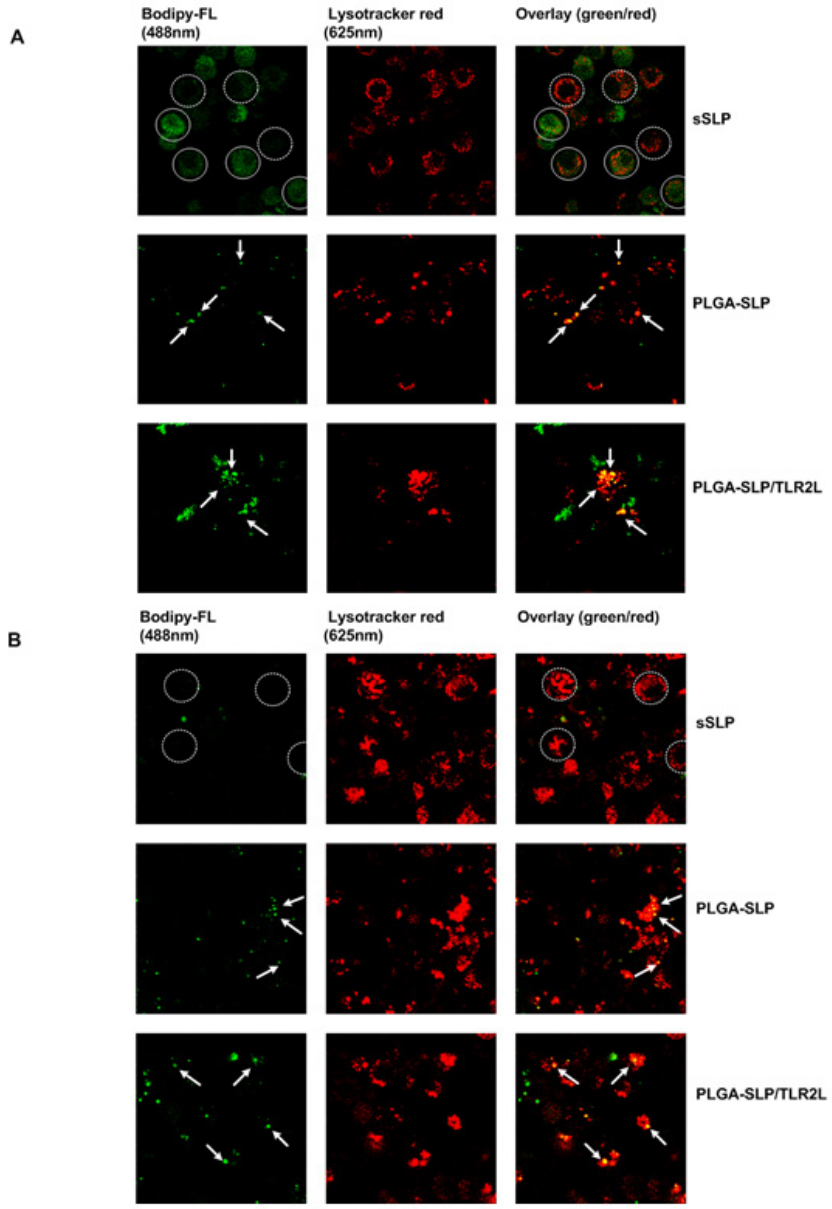


Figure 5. Sustained presence of PLGA-NP encapsulated SLP in endo-lysosomes. D1 cells were incubated with 20 µM sSLP-Bp, 20 µM PLGA-SLP-Bp (10% SLP-Bodipy-FL) or 20 µM PLGA-SLP-Bp/TLR2L (10% SLP-Bodipy-FL) (488nm) for 2.5 hr on t = - 2.5 h **(A)** and t = - 96 h **(B)** and co-stained with Lysotracker red for visualization of the endo-lysosomes on t = - 15 min. Live cell imaging were performed at t = 0. 1st column shows images depicting green signal as the fluorescence of Bodipy-FL (488nm). 2nd column shows images depicting red signal as the fluoresence of the lysotracker red/endo-lysosomes (625nm). 3rd column depicts overlay images of red/green. White arrow indicates hotspots (yellow) within a cell were green and red co-localize. Closed circles show cells containing green-flourescent signal (and red fluorescent signal). Dashed circles depict cells containing only red fluorescent signals. Images were analyzed using Leica software.

sSLP internalized by DC was present for a large part outside the endo-lysomes as we showed before [20] whereas PLGA-encapsulated SLP showed high co-localization, with endo-lysomes, suggested by the formation of bright yellow spots, marked in the **Figures 4 & 5** by the arrows. DC which took up NP tend to have larger endo-lysosomal compartments (bright red spots, **Figures 4 & 5**) compared to DC which internalized sSLP which might suggest formation of phagolysosomes. The results indicate that encapsulation of SLP inside PLGA-SLP modulates intracellular trafficking of SLP by keeping the Ag inside endosomal compartments, thereby directing it away from the cytosol.

Brighter yellow spots were observed when BMDC internalized PLGA-SLP in comparison to sSLP. This suggest that DC take up much higher amounts of SLP on a single cell basis when it is encapsulated in PLGA-NP, pointing to the more efficient uptake by DC of SLP when encapsulated (**Figure 4**). Interestingly, encapsulation of TLR2L in PLGA-NP (PLGA-SLP/TLR2L) even further enhanced the efficiency (**Figure 4B**) and rapidity of NP internalization (**Figure 4C**) by BMDC suggesting an additional role for TLR2 in the internalization of NP.

DC are known to preserve internalized PLGA-(micro)spheres inside endo-lysosomal compartments for up to 48 hr [25] which suggests that intracellular hydrolysis of PLGA particles to be a slow process. In our study, sustained MHC class I cross-presentation of PLGA-encapsulated SLP could be detected even after 96 hr. We therefore analyzed D1 cells loaded with sSLP, PLGA-SLP, PLGA-SLP/TLR2L directly after loading (**Figure 5A**) or after 96 hr rest (**Figure 5B**). sSLP-loaded D1 cells analyzed after 96 hr rest showed clear differences with DC analyzed directly after Ag-loading. Fluorescent signal of SLP was largely undetectable in DC cultured with sSLP whereas labeled SLP originated from the PLGA-SLP or PLGA-SLP/TLR2L could be clearly detected still inside endosomal compartments (**Figure 5**).

It is known that internalized soluble proteins are also routed into endosomal compartments [24-26]. However, endosomal presence of an Ag does not guarantee preservation as soluble protein was not detected inside DC after 96 hr (**Supplemental Figure 1**). This observation fits with our previous reports showing that soluble proteins do not lead to sustained Ag presentation [18].

3.6. Processing of PLGA-NP encapsulated SLP can be blocked by inhibitors of endo-lysosomal acidification, proteasome and TAP

Exogenous Ag routed towards the MHC class I cross-presentation pathway can follow two pathways I) the classical cytosolic pathway or II) the endosomal pathway [27-29]. The endosomal pathway is dependent on the pH inside the endo-lysosomes but the cytosolic pathway is (mostly) independent of the pH gradient inside endo-lysosomes [29]. We observed that $CD8^+$ T cell activation by DC loaded with PLGA-SLP and PLGA-SLP/TLR2L can be completely blocked (**Figure 6**) in a dose dependent manner

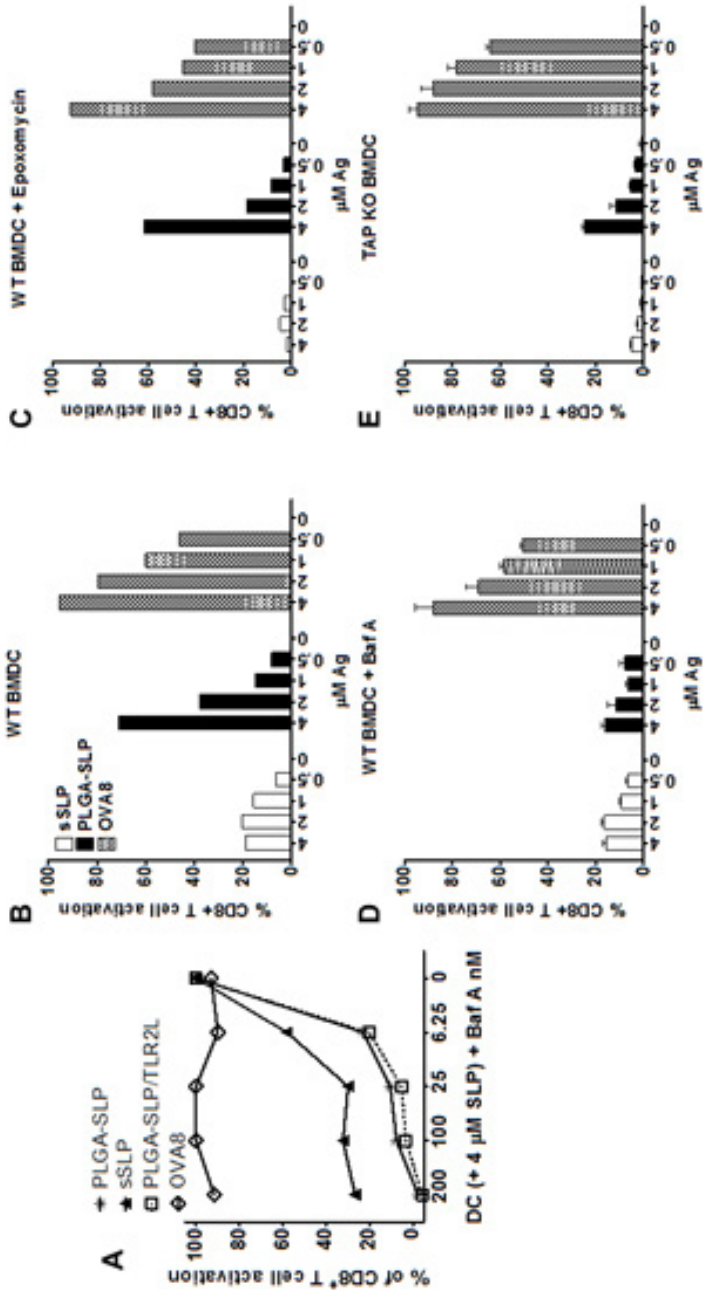


Figure 6. MHC class I cross-presentation PLGA-SLP and PLGA-SLP/TLR2L is impaired in the absence of functional proteasome, TAP and when endo-lysosomal acidification is inhibitedBafilomycin A). D1 cells were pre-incubated with indicated titrated concentrations of bafilomycin A1 (196000, Merck) followed by culture in the presence of 4 µM sSLP, PLGA-SLP, PLGA-SLP/TLR2L and 5 nM SSP-OVA8aa (SIINFEKL). Cells were washed 3x times with complete medium before the B3Z $CD8^+$T-cells were added followed by O/N incubation at 37°C. **(B)** WT BMDC were incubated with titrated amounts of sSLP or PLGA-SLP without additional treatments or in the presence of **(C)** epoxomycin or (E) Bafilomycin A. (D) TAP KO BMDC were incubated with titrated amounts of sSLP or PLGA-SLP.

using the lysotropic agent, bafilomycin A (Baf A). MHC class I cross-presentation of sSLP was slightly decreased as observed before [3] but $CD8^+$ T cell activation could still be detected at the highest concentration of the compound tested in this study. The presentation of the minimal MHC class I binding peptide (OVA8) was unaffected over the whole range of concentrations used. PLGA-SLP MHC class I cross-presentation was reduced in the presence of a proteasome inhibitor (**Figure 6B & C**). MHC class I processing of PLGA-SLP was significantly impaired in the absence of functional TAP (**Figure 6B & E**) or when when endo-lysozomal acidification is blocked (**Figure 6B & D**).

4. Discussion

In this study, we analyzed the effects on MHC class I presentation of co-encapsulating a TLR2L with SLP in PLGA-NP. SLP and PLGA-NP are inert synthetic materials with poor immunestimulating properties. The therapeutic effectivity of cancer vaccines is largely based on its potency to activate DCs, which have superiour capacity to induce robust anti-tumor T cell responses.

We previously reported the succesful formulation of PLGA-SLP using a novel double emulsion and solvent-evaporation technique. Applying PLGA-NP as a vaccine delivery system, the efficacy of MHC class I Ag presentation and subsequent $CD8^+$ T cell activation by DCs was significantly enhanced [10] compared to sSLP. We have also shown recently that plain PLGA-NP have poor DC activating properties compared to TLRL [12]. With the purpose of further increasing the vaccine potency of PLGA-SLP, we have included a TLR2L, Pam3CSK4; an adjuvant which has led to promising pre-clinical results when covalently coupled to SLP. To this end, TLR2L was co-encapsulated in PLGA-NP with the goal to achieve asubstantial DC activation leadingleads to better $CD8^+$ T cell activation in comparison to particles which are not adjuvanted.

In light of our recent findings showing that sSLP are efficiently cross-presented because of a rapid translocation into the cytosol facilatating proteasome dependent processing; the intracellular location of PLGA-SLP was studied to elucidate if PLGA-NP further enhances translocation of SLP, after release from the NP, to the cytosol or if different mechanisms are involved in the handling of PLGA-encapsulated SLP in comparison to the sSLP.

We show here that the addition of Pam3CSK4 to PLGA-NP encapsulating SLP strongly promotes DC maturation and $CD8^+$ T cell activation. The difference on Ag uptake and MHC class I cross-presentation of PLGA-SLP versus PLGA-SLP/TLR2L was was max 3-fold. Lower than we expected given our previous studies using SLP-TLR2L [14]. This moderate enhancement can be explained considering the already very efficient uptake, processing and presentation of plain PLGA-SLP (NP) by DC which perhaps is already close to maximum levels.

Moreover, Pam3CSK4 was not to required to be encapsulated inside particles for it to exert its positive effects on MHC class I presentation of SLP. Mixing sTLR2L with plain PLGA-SLP led to similar results as using PLGA-SLP/TLR2L. Thus Pam3CSK4 has a different effect on the potency of SLP when co-encapsulated in NP compared to covalently coupling the compound to a peptide, which results in very strong enhancement of $CD8^+$ T cell activation compared to mixtures of SLP and Pam3CSK4 [14].

Pam3CSK4 has a lipidic nature and contains positively charged lysine residues which direct adsorbtion to to the negatively charged PLGA-NP surface due to hydrophobic and (or) electrostatic interactions [3]. The adsorbtion of Pam3CSK4 to PLGA-SLP when mixed might lead to similar NP-characteristics as PLGA-SLP/TLR2L. This effect could explain why both the mixing or co-encapsulation will result in an similar participation of Pam3CSK4 in MHC class I presentation. In conclusion, we show that addition of a Pam3CSK4, whether co-encapsulated or not, further improves MHC class I cross-presentation of SLP and improves $CD8^+$ T cell activation compared to plain PLGA-SLP.

sSLP present in the cytosol are degraded via ubiquitin proteasome system (UPS) [30], whereas the Ag present inside endo-lysosomal compartments can be protected from rapid degradation by the UPS. Upon internalization of PLGA-NP by DC, a majority of the Ag could be detected inside the endo-lysosomes where hydrolysis of the polymer takes place releasing the encapsulated SLP [31]. Thus, the PLGA-NP encapsulated Ag inside endo-lysosomal compartments (**Figure 4**) serves as an intracellular reservoir which gradually releases the SLP for processing via the classical proteasome-TAP dependent MHC class I processing pathways. Baf A clearly interferes with MHC class I Ag presentation of PLGA-SLP. One possibility is that the compound blocks the transportation of SLP from the endo-lysosomes to the cytosol [32]. Another possibility is that Baf A modulates the activity of endo-lysosomal cathepsins [31]. Cathepsin S has been shown to have a role in MHC class I cross-presentation [27]. Using BMDC generated from Cathepsin S KO mice, we did not observe differences in MHC class I cross-presentation of PLGA-SLP compared to WT BMDC (data not shown). Taken our results using proteasome inhibitors and TAP-deficient BMDC we show that SLP encapsulated in PLGA-NP is cross-presented via the classical MHC class I processing pathway but we cannot exclude that the endo-lysosomal environment and other pH dependent proteases still play a role in the observed MHC class I Ag cross-presentation [28, 29].

The most important observation of this study was that encapsulation of sSLP in PLGA-NP results in sustained presence of the Ag inside DC upon internalization. More over, DC loaded with PLGA-SLP or PLGA-SLP/TLR2L showed prolonged MHC class I presentation in comparison to DC loaded with sSLP. Prolonged Ag presentation was not dependent on TLR2L stimulation but the addition of Pam3CSK4 does improve and sustain $CD8^+$ T cell activation over a longer time period *in vivo*. Others observed similar results using PLGA-particles encapsulating proteins [33]. However, in contrast to our results, it has been reported that PLGA-particles induce membrane rupture and rapid endo-lysosomal [34] followed by “leakage” of the PLGA-encapsulated Ag inside the cytosol; so called endosomal escape [33]. Membrane rupture by particulate Ag is

associated with inflammasome activation and secretion of IL-1β by APC [35]. In our system, however we could not detect IL-1β in culture supernatants using ELISA after 24 hr incubations of DC with PLGA-SLP nor PLGA-SLP/TLR2L (data not shown). As mentioned before, we could detect green-fluorescent signal of the SLP-OVA24-Bodipy-FL and $CD8^+$ T cell activation even after 96 hr indicating that in our study the majority of PLGA-encapsulated SLP was not directly tranported to the cytosol after DC internalized the NP. Thus, internalized PLGA-NP show functional similarities with intracullar storage compartments as reported for other targeted vaccine delivery systems [14, 18].

Therefore, we postulate that the efficient and prolonged MHC class I presentation observed using PLGA-SLP and PLGA-SLP/TLR2L is related to preservation of the Ag inside intact intracellular compartments. We show here based on the intracellular localization of internalized PLGA-NP and functional studies that these particles also end up in Ag storage compartments [18].

Finally, direct s.c. vaccinations with PLGA-SLP/TLR2L but not PLGA-SLP induces endogenous Ag-specific $CD8^+$ T cells capable of target cell lysis (**Supplemental Figure 2**). In conclusion, the study reported here supports a mechanism that $CD8^+$ T cell responses is enhanced when the Ag is cross-presented in MHC class I molecules in a sustained manner. We show that the co-encapsulation of a TLR2L further boosts these effects and thus supports the use of PLGA-NP co-encapsulating long peptide vaccines and adjuvants as an anti-cancer vaccine. Cancer cells are notorious for providing very few “danger signals”, which is one of the causes why the immune system sometimes fails to clear cancers. However, if one vaccinates with PLGA-SLP/TLR2L, encoding tumor associated Ag (TAA), for example the sustained release of Ag and adjuvant will lead to strong DC maturation, enhanced and prolonged MHC class I presentation and efficient priming of cytotoxic $CD8^+$ T cells. Indeed, vaccination with PLGA-NP based vaccines results in robust anti-tumor responses with the capacity to significantly control tumor out growth [36-39].

5. References

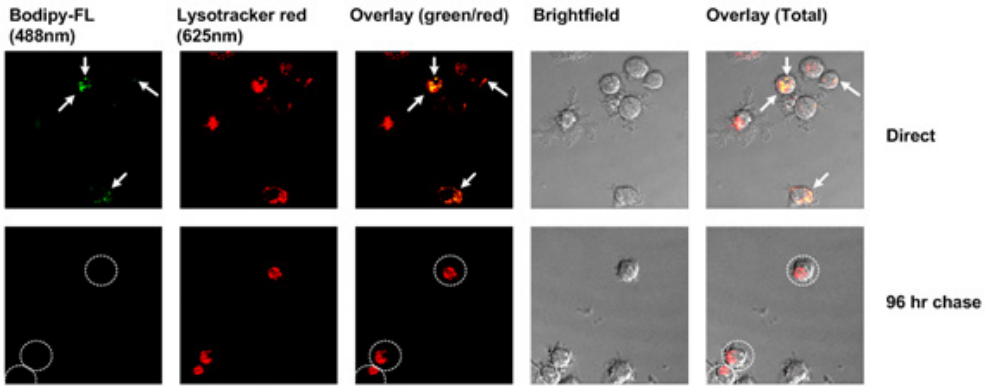
1. Flinsenberg, T.W., E.B. Compeer, J.J. Boelens, and M. Boes, Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention. Clin Exp Immunol, 2011. 165(1): p. 8-18.
2. Melief, C.J., Cancer immunotherapy by dendritic cells. Immunity, 2008. 29(3): p. 372-83.
3. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, A.L. Silva, W. Jiskoot, T. van Hall, P.A. van Veelen, G. Janssen, K. Franken, L.J. Cruz, A. Tromp, J. Oostendorp, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65.
4. Sabbatini, P., T. Tsuji, L. Ferran, E. Ritter, C. Sedrak, K. Tuballes, A.A. Jungbluth, G. Ritter, C. Aghajanian, K. Bell-McGuinn, M.L. Hensley, J. Konner, W. Tew, D.R. Spriggs, E.W. Hoffman, R. Venhaus, L. Pan, A.M. Salazar, C.M. Diefenbach, L.J. Old, and S. Gnjatic, Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clin Cancer Res, 2012. 18(23): p. 6497-508.
5. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, F. Essahsah, L.M. Fathers, R. Offringa, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, S.H. van der Burg, and C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. N Engl J Med, 2009. 361(19): p. 1838-47.
6. Park, H., L. Adamson, T. Ha, K. Mullen, S.I. Hagen, A. Nogueron, A.W. Sylwester, M.K. Axthelm, A. Legasse, M. Piatak, Jr., J.D. Lifson, J.M. McElrath, L.J. Picker, and R.A. Seder, Polyinosinic-polycytidylic acid is the most effective TLR adjuvant for SIV Gag protein-induced T cell responses in nonhuman primates. J Immunol, 2013. 190(8): p. 4103-15.
7. Jarvi, S.I., D. Hu, K. Misajon, B.A. Coller, T. Wong, and M.M. Lieberman, Vaccination of captive nene (Branta sandvicensis) against West Nile virus using a protein-based vaccine (WN-80E). J Wildl Dis, 2013. 49(1): p. 152-6.
8. Leenaars, P.P., M.A. Koedam, P.W. Wester, V. Baumans, E. Claassen, and C.F. Hendriksen, Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. Lab Anim, 1998. 32(4): p. 387-406.
9. Aucouturier, J., L. Dupuis, S. Deville, S. Ascarateil, and V. Ganne, Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert Rev Vaccines, 2002. 1(1): p. 111-8.
10. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient CD8(+) T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45.
11. Aucouturier, J., S. Ascarateil, and L. Dupuis, The use of oil adjuvants in

therapeutic vaccines. Vaccine, 2006. 24 Suppl 2: p. S2-44-5.
12. Rosalia, R.A., A.L. Silva, M. Camps, A. Allam, W. Jiskoot, S.H. van der Burg, F. Ossendorp, and J. Oostendorp, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer Immunol Immunother, 2013. 62(7): p. 1161-73.
13. Yoshida, M., J. Mata, and J.E. Babensee, Effect of poly(lactic-co-glycolic acid) contact on maturation of murine bone marrow-derived dendritic cells. J Biomed Mater Res A, 2007. 80(1): p. 7-12.
14. Khan, S., M.S. Bijker, J.J. Weterings, H.J. Tanke, G.J. Adema, T. van Hall, J.W. Drijfhout, C.J. Melief, H.S. Overkleeft, G.A. van der Marel, D.V. Filippov, S.H. van der Burg, and F. Ossendorp, Distinct uptake mechanisms but similar intracellular processing of two different toll-like receptor ligand-peptide conjugates in dendritic cells. J Biol Chem, 2007. 282(29): p. 21145-59.
15. Zom, G.G., S. Khan, D.V. Filippov, and F. Ossendorp, TLR ligand-peptide conjugate vaccines: toward clinical application. Adv Immunol, 2012. 114: p. 177-201.
16. van Montfoort, N., P.A. t Hoen, S.M. Mangsbo, M.G. Camps, P. Boross, C.J. Melief, F. Ossendorp, and J.S. Verbeek, Fcgamma receptor IIb strongly regulates Fcgamma receptor-facilitated T cell activation by dendritic cells. J Immunol, 2012. 189(1): p. 92-101.
17. Feltkamp, M.C., G.R. Vreugdenhil, M.P. Vierboom, E. Ras, S.H. van der Burg, J. ter Schegget, C.J. Melief, and W.M. Kast, Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. Eur J Immunol, 1995. 25(9): p. 2638-42.
18. van Montfoort, N., M.G. Camps, S. Khan, D.V. Filippov, J.J. Weterings, J.M. Griffith, H.J. Geuze, T. van Hall, J.S. Verbeek, C.J. Melief, and F. Ossendorp, Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. Proc Natl Acad Sci U S A, 2009. 106(16): p. 6730-5.
19. Schuurhuis, D.H., A. Ioan-Facsinay, B. Nagelkerken, J.J. van Schip, C. Sedlik, C.J. Melief, J.S. Verbeek, and F. Ossendorp, Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific $CD8^+$ CTL responses *in vivo*. J Immunol, 2002. 168(5): p. 2240-6.
20. Rogošiy, M., H.J. Mencer, and Z. Gomzi, Polydispersity index and molecular weight distributions of polymers. European Polymer Journal, 1996. 32(11): p. 1337–1344.
21. King, C.G., S. Koehli, B. Hausmann, M. Schmaler, D. Zehn, and E. Palmer, T cell affinity regulates asymmetric division, effector cell differentiation, and tissue pathology. Immunity, 2012. 37(4): p. 709-20.
22. Zehn, D., C. King, M.J. Bevan, and E. Palmer, TCR signaling requirements for activating T cells and for generating memory. Cell Mol Life Sci, 2012. 69(10): p. 1565-75.
23. Rudd, B.D., J.D. Brien, M.P. Davenport, and J. Nikolich-Zugich, Cutting edge: TLR ligands increase TCR triggering by slowing peptide-MHC class I decay rates. J Immunol, 2008. 181(8): p. 5199-203.
24. Chen, L. and M. Jondal, Alternative processing for MHC class I presentation

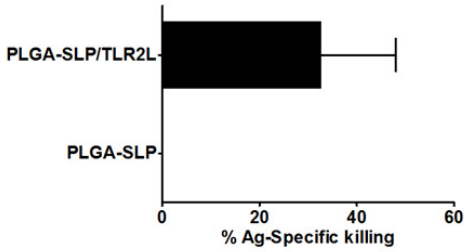
by immature and CpG-activated dendritic cells. Eur J Immunol, 2004. 34(4): p. 952-60.
25. Chen, L. and M. Jondal, Brefeldin A inhibits vesicular MHC class I processing in resting but not in CpG- and disruption-activated DC. Mol Immunol, 2008. 46(1): p. 158-65.
26. Chen, L. and M. Jondal, Endolysosomal processing of exogenous antigen into major histocompatibility complex class I-binding peptides. Scand J Immunol, 2004. 59(6): p. 545-52.
27. Rock, K.L., D.J. Farfan-Arribas, and L. Shen, Proteases in MHC class I presentation and cross-presentation. J Immunol, 2010. 184(1): p. 9-15.
28. Joffre, O.P., E. Segura, A. Savina, and S. Amigorena, Cross-presentation by dendritic cells. Nat Rev Immunol, 2012. 12(8): p. 557-69.
29. Compeer, E.B., T.W. Flinsenberg, S.G. van der Grein, and M. Boes, Antigen processing and remodeling of the endosomal pathway: requirements for antigen cross-presentation. Front Immunol, 2012. 3: p. 37.
30. Sijts, E.J. and P.M. Kloetzel, The role of the proteasome in the generation of MHC class I ligands and immune responses. Cell Mol Life Sci, 2011. 68(9): p. 1491-502.
31. Baltazar, G.C., S. Guha, W. Lu, J. Lim, K. Boesze-Battaglia, A.M. Laties, P. Tyagi, U.B. Kompella, and C.H. Mitchell, Acidic nanoparticles are trafficked to lysosomes and restore an acidic lysosomal pH and degradative function to compromised ARPE-19 cells. PLoS One, 2012. 7(12): p. e49635.
32. Horiguchi, M., M. Arita, D.E. Kaempf-Rotzoll, M. Tsujimoto, K. Inoue, and H. Arai, pH-dependent translocation of alpha-tocopherol transfer protein (alpha-TTP) between hepatic cytosol and late endosomes. Genes Cells, 2003. 8(10): p. 789-800.
33. Shen, H., A.L. Ackerman, V. Cody, A. Giodini, E.R. Hinson, P. Cresswell, R.L. Edelson, W.M. Saltzman, and D.J. Hanlon, Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology, 2006. 117(1): p. 78-88.
34. Demento, S.L., S.C. Eisenbarth, H.G. Foellmer, C. Platt, M.J. Caplan, W. Mark Saltzman, I. Mellman, M. Ledizet, E. Fikrig, R.A. Flavell, and T.M. Fahmy, Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy. Vaccine, 2009. 27(23): p. 3013-21.
35. Panyam, J., W.Z. Zhou, S. Prabha, S.K. Sahoo, and V. Labhasetwar, Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. FASEB J, 2002. 16(10): p. 1217-26.
36. Hamdy, S., O. Molavi, Z. Ma, A. Haddadi, A. Alshamsan, Z. Gobti, S. Elhasi, J. Samuel, and A. Lavasanifar, Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent $CD8^+$ T cell-mediated anti-tumor immunity. Vaccine, 2008. 26(39): p. 5046-57.
37. Mueller, M., W. Reichardt, J. Koerner, and M. Groettrup, Coencapsulation of tumor lysate and CpG-ODN in PLGA-microspheres enables successful immunotherapy of prostate carcinoma in TRAMP mice. J Control Release, 2012. 162(1): p. 159-66.
38. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to

incomplete Freund's adjuvant. Int J Cancer, 2011. 129(2): p. 407-16.
39. Zhang, Z., S. Tongchusak, Y. Mizukami, Y.J. Kang, T. Ioji, M. Touma, B. Reinhold, D.B. Keskin, E.L. Reinherz, and T. Sasada, Induction of anti-tumor *cytotoxic T cell responses through PLGA-nanoparticle mediated antigen delivery.* Biomaterials, 2011. **32**(14): p. 3666-78.

6. Supplemental Figures



Supplemental Figure 1. Endosomal localisation of whole protein after internalization does not lead to prolonged Ag presence. D1 cells were incubated with 20 µM ovalbumin-Alexa488 (excitation- 488nm, visualized as the green signal) for 2.5 hr and either directly analyzed (upper panels) or further cultured in the absence of additional Ag or stimuli for 96 hr (lower panels). Co-staining with Lysotracker red was performed for visualization of the endo-lysosomes (red signal) and visualized by confocal microscopy. 1st column shows images depicting green signal as the fluorescence of the dye (488nm). 2nd column shows images depicting red signal as the fluoresence of the lysotracker red/endo-lysosomes (625nm). 3rd column depicts overlay images of red/green. Yellow-overlay signal marks co-localization. Images were analyzed using Leica software.



Supplemental Figure 2. Vaccinations with PLGA-SLP/TLR2L but not PLGA-SLP induces CD8⁺ T cells with *in vivo* cytotoxic capacity. Priming efficacy of endogenous cytotoxic CD8⁺ T cells by PLGA-SLP formulations, mice were vaccinated with 20 nmol SLP encapsulated in PLGA with or without Pam3CSK4 co-encapsulated. On day 7 post-vaccination, SIINFEKL-loaded (OVA-specific) target and control-target cells were injected. To obtain OVA-specific target cells, splenocytes from naïve congeneic C57BL/6 Ly5.1 mice were pulsed for 1 h with 1 µM of SIINFEKL-peptide and co-stained with 10 µM CFSE (CFSE-high) (Molecular Probes, Eugene, OR). As a negative control, 1 µM of the immunodominant ASNENMETM-peptide derived from the influenza virus nucleoprotein co-stained with 0.5 µM CFSE (CFSE-low) was used. Specific and non-specific target cells were mixed 1:1 and injected intravenously (i.v.; 10 x 106 cells of each population). 18 hr after cells were transferred, mice were sacrificed and spleen cells were harvested to prepare single cell suspensions that were then subjected to flow cytometric analysis. Injected cells were distinguished by APC-conjugated rat anti-mouse CD45.1 mAb. The percentage specific killing was calculated as follow: 100 - ([(% SIINFEKL-peptide pulsed in treated/% ASNENMETM-pulsed in treated)/(% SIINFEKL-peptide pulsed in non-treated/% ASNENMETM-pulsed in non-treated)] x 100).

Chapter 7

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

Eleni Maria Varypataki*, Ana Luisa Silva*, Christophe Barnier-Quer, Nicolas Collin, Ferry Ossendorp, Wim Jiskoot

*contributed equally to this work

Manuscript in preparation for publication

Abstract

The potential of synthetic long peptide (SLP)-cancer vaccines, based on TLR ligand-adjuvanted liposomes and PLGA nanoparticles (NPs) to induce a cell-mediated immune response, as potential alternatives to clinically used Montanide ISA-51- and squalene-based emulsions is investigated in this study. The liposomal and PLGA NP formulations were successfully loaded with up to four different compounds and were able to enhance antigen uptake by DCs and subsequent activation of T cells *in vitro*. Subcutaneous vaccination of mice showed that the efficiency of the SLP-loaded liposomes and PLGA NPs to induce functional antigen-T cells *in vivo* was as good as or even better than that of the emulsions, with liposomes outperforming PLGA NPs. Moreover, after adoptive transfer of target cells in mice, liposomes showed the highest killing capacity. These findings, considering also the inadequate safety profile of the currently clinically used adjuvant Montanide ISA-51, make these two particulate delivery systems promising candidates as a delivery platform for SLP-based immunotherapy of cancer.

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

1. 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 tumor 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 Th epitopes of a TAA, has been applied in mouse models with superior efficacy to protein antigen [3] or minimal MHC class I restricted epitopes [4, 5]. 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 [5-7]. 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 [8]. Despite the efficacy of these emulsions as influenza vaccine adjuvants, and though some degree of Th1 responses have been observed, still they lack the ability to stimulate strong T cell responses [9]. 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 [10-16]. 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 [17, 18]. 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 [19-21]. Importantly, they can harbor 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 (TLRs), by incorporation of TLR ligands (TLRLs). 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 [22] and cationic liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [23] for the encapsulation of a 24-amino acid-long SLP (referred to as OVA24) harboring the CTL epitope SIINFEKL of ovalbumin (OVA). Encapsulation of SLP in PLGA NPs led to a significant enhancement of MHC class I Ag presentation and $CD8^+$ T cell activation compared to soluble SLP (sSLP) *in vitro* [24]. 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 soluble SLP. Furthermore, intradermal immunization of mice with 5 nmol SLP-liposomes combined with poly(I:C) led to a strong cytotoxic activity, in contrast to vaccination with a mixture of soluble SLP and poly(I:C) [23].

In this study, we investigated the potential of PLGA NPs and cationic liposomes as delivery systems for SLP-based vaccine candidates for the induction of a cell-mediated immunity. For that purpose, we studied the co-delivery of two SLPs containing the CTL (OVA24) and the T helper (Th, OVA17) epitopes of OVA together with poly(I:C) and Pam3CSK4, a TLR3 and TLR2/1 ligand, respectively, in comparison to the 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.

2. Materials and Methods

2.1. 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 [6]. The ovalbumin-derived SLP OVA17 [ISQAVHAAHAEINEAGR], including the helper Th-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 Pam3CSK4) with their labeled 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 Na2HPO4.2H2O and 2.3 mM NaH2PO4.2H2O, 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.

2.2. Mice

Female C57BL/6 (H-2b) 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.

2.3. Liposome preparation

Cationic liposomes loaded with SLPs were prepared by using the thin film dehydration-rehydration method, as described previously [23]. 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/H2O 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-labeled equivalent) in a total concentration of 200 µg/mL was added dropwisely to the dispersion, while for the Pam3CSK4-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 (Northen 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 [23].

2.4. PLGA NPs preparation

Nanoparticles loaded with OVA24 and/or OVA17 and/or TLRLs were prepared by using a double emulsion with solvent evaporation method [22]. In brief, 50 mg of PLGA dissolved in 1 ml of dichloromethane, with or without 0.25 mg Pam3CSK4 (and 0.1% Pam3CSK4 Rhodamine-labeled), 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-labeled ,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 -55oC in a Christ Alpha 1-2 freeze-dryer (Osterode am Harz, Germany) for 12 hours.

2.5. Liposome and PLGA NP characterization

Average diameter (Zave) 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 [23].

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 [24].

Loading efficiency of poly(I:C) and Pam3CSK4 was calculated by fluorescence detected with a Infinite® M 1000 Pro (Tecan, Switzerland) microplate reader (excitation/emission wavelengths: 492 nm/518 nm for fluorescein and 549 nm/566 nm for rhodamine).

2.6. Montanide ISA-51 and SWE emulsions preparation and characterization

Preparation of Montanide ISA-51 emulsion was performed by diluting the SLPs, Pam3CSK4 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 [25, 26]. 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 immunization.

2.7. *In vitro* MHC class I antigen presentation

Immature D1 cells were incubated in 96-well flat-bottomed plates at 37ºC in supplemented IMDM with SLP-loaded formulations or plain sSLP (with or without TLR-ligands) in PBS (sOVA24^{+}sOVA17) at different concentrations. After 2.5 hours T cell reporter hybridoma B3Z cells (50x105/well) were added and the mixture was incubated overnight at 37ºC. Chlorophenol red-β-galactopyranoside (CPRG) was used as lacZ substrate in cell lysates and color conversion was measured by detecting absorbance

at 590 nm.

2.8. Immunization of mice

Mice were immunized with SLP-loaded formulations or soluble peptides, sOVA24 and sOVA17 (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 immunizations were performed on day 0 (prime immunization) 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.

2.9. 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 labeled tetramer-OVA8 (TM-SIINFEKL) and fluorescently labeled antibodies specific for mouse CD3 (BD Biosciences), CD4, CD8 and the killer cell-lectin-like receptor G1 (KLRG1) (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 [23].

2.10. *In vivo* cytotoxicity assay

Splenocytes from naive congenic $CD45.1^+$ mice were lysed and split into two equal parts. Cells were labeled with CFSE and adoptively transferred intravenously in previously immunized recipient C57BL/6 mice in a volume of 200 µl in PBS as described [23]. Two days after the cell transfer (day 24), mice were sacrificed, spleens were isolated and single cell suspensions were analyzed by flow cytometry. Specific killing (SK) was calculated according to the following **equation 1**:

$$SK = \left\{ 1 - \frac{\left[\begin{matrix} \text{CFSE high} \\ \text{CFSE low} \end{matrix} \text{ vaccinated mice} \right]}{\left[\begin{matrix} \text{CFSE high} \\ \text{CFSE low} \end{matrix} \text{ naive mice} \right]} \right\} \text{x } 100\ \% \quad (1)$$

3. Results

3.1. Characterization of adjuvanted SLP-loaded liposomes and PLGA NPs

We have previously shown that effective tumor vaccines require the inclusion of both CTL and Th epitopes [6, 27]. 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 Pam3CSK4 (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 previously described [23].

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 Pam3CSK4 were larger and less monodisperse (PDI > 0.2). The positive zeta-potential was about 26 mV, independent of the formulation (**Table 1**).The loading efficiency (LE) 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 Pam3CSK4 (**Table 1**), 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. [22]. Irrespective of the type of the loading, PLGA NPs were negatively charged, with a zeta-potential ranging from -11 to -14 mV (**Table 1**), 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 Pam3CSK4 and 53 – 73% for poly(I:C). We have previously shown the importance of pH for the effective encapsulation of peptides in PLGA NPs and how crucial low burst release is in order to induce a cellular response [22]. Therefore, both OVA24 and OVA17 SLP were formulated at pH 8.0 for optimal encapsulation and showed a relatively low

burst release from NPs of circa 30% after 24 h (data not shown).

Table 1: Physicochemical properties of SLP (+TLR ligand)-loaded formulations

	Zave diameter (nm)	PDI	ZP (mV)	LE (%)			
				OVA24	OVA17	TLR-ligand	
						Pam3CSK4	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. Zave average: particle diameter; PDI: polydispersity index; ZP: zeta-potential; LE: loading efficiency; NA: not applicable

Altogether, the data presented in **Table 1** 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.

3.2. *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-harboring peptide (OVA24) to the $CD8^+$ antigen-specific 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 (sOVA24+sOVA17+Pam+poly(I:C)). Moreover, although PLGA NPs plots consistently show a larger dose-response effect, liposomes seem to be more effective in antigen presentation at lower concentrations (**Figure 1**).

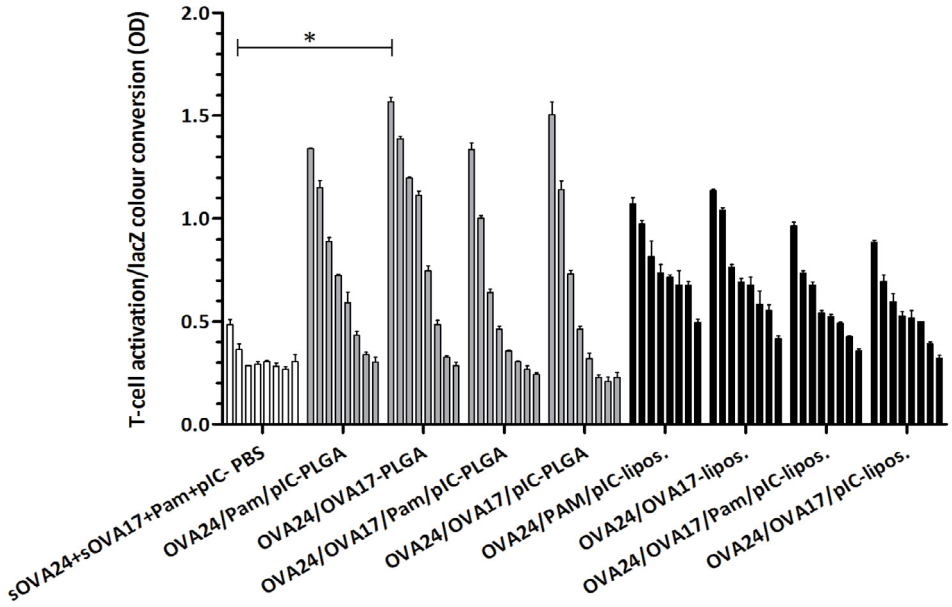


Figure 1: SIINFEKL-specific B3Z CD8+ T cell activation after overnight co-culture with DCs and titrated amounts (4.0-0.0625 μM, 2-fold dilution series) of SLPs/TLR ligands-loaded formulations. Graphs show T cell activation based on the optical density (OD) at 590 nm after color conversion of cell lysates 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 = Pam3CSK4; pIC = poly(I:C)

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 Pam3CSK4 did not significantly affect the *in vitro* T cell activation by the SLP-loaded particle formulations, as expected (**Figure 1**).

3.3. *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 tailbase 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.

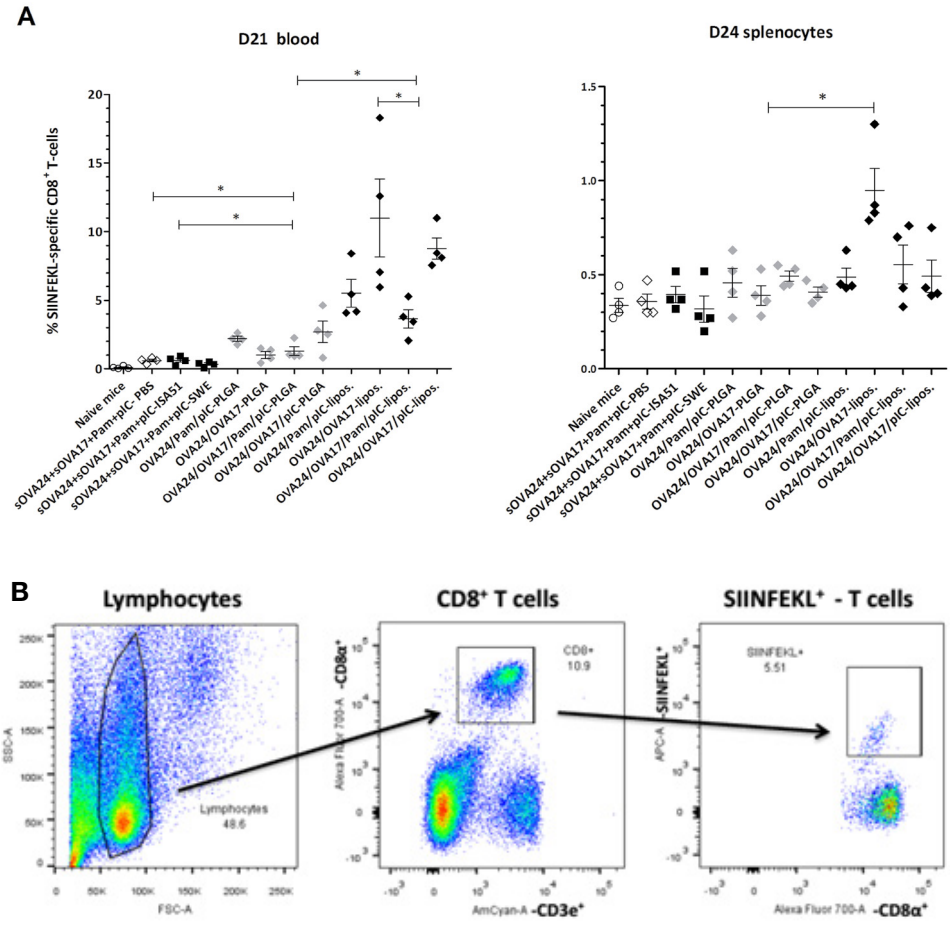


Figure 2: OVA24-specific CD8⁺ T cell responses in blood (Day 21) and in splenocytes (Day 24) following s.c. immunization with 1 nmol of SLPs on day 0 and 14 (A). Representative gating strategy for detection of SIINFEKL-specific CD8⁺ T cells by flow cytometry using specific MHC class I tetramers (B). * $p<0.05$, ** $p<0.01$ calculated with by one-tailed Mann-Whitney test. ISA51= Montanide; Pam = Pam3CSK4; pIC = poly(I:C).

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 Th 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.

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**). Interestingly, 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 Pam3CSK4 seemed to result in lower numbers of antigen-specific T cells. On day 24 in the analyzed 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 2A**).

3.4. *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 analyzed. 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, Pam3CSK4 and poly(I:C) (OVA24/Pam/polyI:C-PLGA) a higher percentage of cytokine-producing $CD8^+$ T cells was detected (0.7%) in comparison to the Montanide ISA-51 formulation (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 (~ 0.8 %) in comparison with the Montanide ISA-51 and SWE emulsions (~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 Pam3CSK4 into liposomes did not increase the number of induced T cells, although still much higher than the soluble SLP and TLR ligands. Although Pam3CK4 is known to improve SLP vaccination by itself [27], 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.

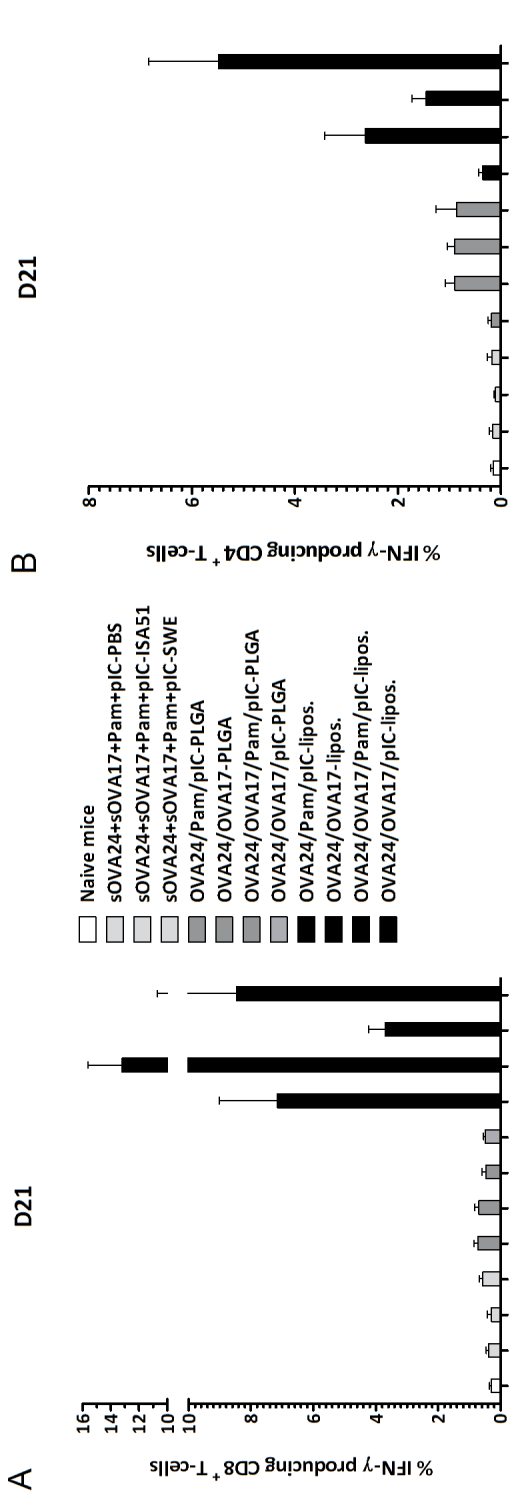


Figure 3: Intracellular cytokine analysis in blood of immunized mice at day 21 which was stimulated ex vivo overnight with the minimal SIINFEKL epitope and OVA17. Plots show percentages (+SEM) of $CD8^+$ (A) and $CD4^+$ (B) T cells producing interferon gamma (IFN-γ). ISA51 = Montanide; Pam = Pam3CSK4; pIC = poly(I:C)

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-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.

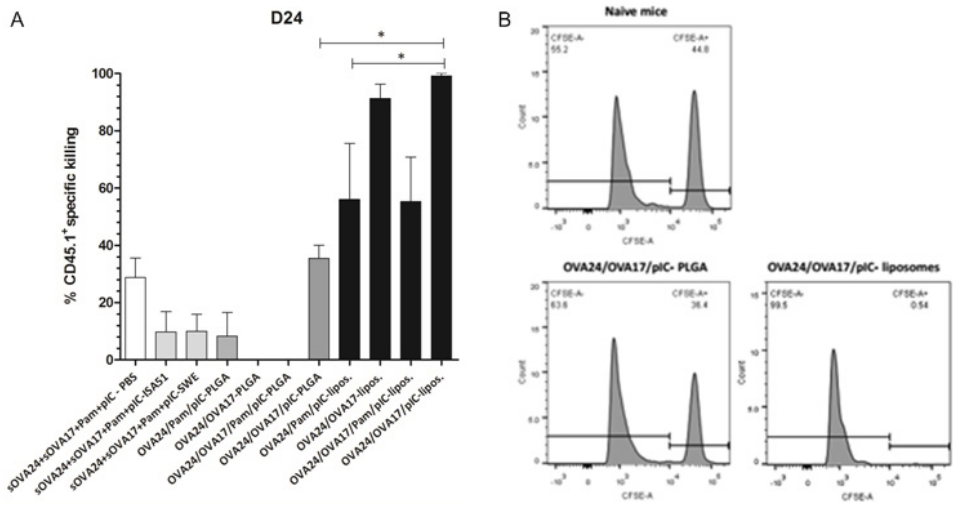


Figure 4: *In vivo* **cytotoxicity against SIINFEKL-presenting target cells.** The mean percentage of the killing activity of each SLP formulation is presented based on the frequency of the transferred $CD45.1^+$ cells that could be detected in splenocytes of mice immunized twice with the different formulations containing 1 nmol of OVA24. Bar graphs show the mean percentages (+SEM) of killed cells on day 24 (A).Representative histograms of CSFE-labeled positive target cells (right peak=SLP pulsed and left peak=negative control) (B). * $p<0.05$ calculated with by one-tailed Mann-Whitney test. ISA51= Montanide; Pam = Pam3CSK4; 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 immunized mice (**Figure 4**).

Incorporation of poly(I:C) into PLGA NPs including both SLPs (and not Pam3CSK4) 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 immunized 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 SLPs) is used like in the current study. Moreover, it appeared that the inclusion of an adjuvant (Pam3CSK4 or poly(I:C)) in the liposomal formulation might not be necessary for the priming of a T cell-based immune response, since solely the presence of the Th epitope seems to facilitate a Th1-based proinflammatory immune reaction essential for effective therapeutic vaccines, to maintain a robust and long-lasting anti-tumor $CD8^+$ T cell response.

4. Discussion

There is a growing interest in therapeutic vaccination against cancer. The identification of tumor associated antigens (TAAs) has allowed the development of novel therapeutic strategies resulting in tumor 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 Th1 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 Pam3CSK4) 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 their *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 Th 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 $CD4^+$ Th-based $CD8^+$ T cell priming [28, 29]. 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 [30], improved the survival of $CD4^+$ T cells and produced functional $CD8^+$ memory even in the absence of $CD4^+$ T cells [31]. 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 Pam3CSK4 in the formulations did not further improve the induced immune response. This may be due to its lipophilic nature, resulting in localization of the Pam3CSK4

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 [32, 33]. The formulation including both SLPs and poly(I:C), but not Pam3CSK4, was the most promising for the induction of functional and cytotoxic T cells. Although the effect of the Pam3CSK4 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 favor the induction of a T cell-based immune response.

In this study we showed that for SLPs-based vaccines, cationic liposomes appeared to be the most potent delivery system, followed by PLGA NPs. 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 [34] where they will interact with lymph node resident DCs [35]. 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 [36], which are more prone to be recognized by macrophages and other scavenger immune cells, leading to a poorer T cell activation capacity [33, 37]. 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 [38].

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. Subcutaneous vaccination at the tailbase 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 [2]. 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 Th1-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 Th1-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 favor. 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 [23]. 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, immunization protocols and antigen characteristics used in reported studies. The latter may result in a charge-dependent entrapment efficiency and antigen release pattern.

To sum up, 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, but is likely due to differences in size, zeta potential and/or surface chemistry. Further research is required to elucidate how these and other properties affect the functionality of particle types as vaccine delivery system, which should help to further improve their properties for effective immunotherapy of cancer.

5. 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 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 of inducing 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 unfavorable safety profile of the currently used adjuvant Montanide ISA-51, makes these particulate delivery systems attractive candidates as a delivery platform for SLP-based immunotherapy of cancer.

6. References

1. Black, M., A. Trent, M. Tirrell, and C. Olive, Advances in the design and delivery of peptide subunit vaccines with a focus on toll-like receptor agonists. Expert Rev Vaccines, 2010. 9(2): p. 157-73.
2. Silva, A.L., R.A. Rosalia, E. Varypataki, S. Sibuea, F. Ossendorp, and W. Jiskoot, Poly-(lactic-co-glycolic-acid)-based particulate vaccines: particle uptake by dendritic cells is a key parameter for immune activation. Vaccine, 2015. 33(7): p. 847-54.
3. Rosalia, R.A., E.D. Quakkelaar, A. Redeker, S. Khan, M. Camps, J.W. Drijfhout, A.L. Silva, W. Jiskoot, T. van Hall, P.A. van Veelen, G. Janssen, K. Franken, L.J. Cruz, A. Tromp, J. Oostendorp, S.H. van der Burg, F. Ossendorp, and C.J. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65.
4. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, S.H. van der Burg, and R. Offringa, Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation. Eur J Immunol, 2008. 38(4): p. 1033-42.
5. Zwaveling, S., S.C. Ferreira Mota, J. Nouta, M. Johnson, G.B. Lipford, R. Offringa, S.H. van der Burg, and C.J. Melief, Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol, 2002. 169(1): p. 350-8.
6. Ossendorp, F., E. Mengede, M. Camps, R. Filius, and C.J. Melief, Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med, 1998. 187(5): p. 693-702.
7. Bijker, M.S., S.J. van den Eeden, K.L. Franken, C.J. Melief, R. Offringa, and S.H. van der Burg, $CD8^+$ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. J Immunol, 2007. 179(8): p. 5033-40.
8. Salvador, A., M. Igartua, R.M. Hernandez, and J.L. Pedraz, An overview on the field of micro- and nanotechnologies for synthetic Peptide-based vaccines. J Drug Deliv, 2011. 2011: p. 181646.
9. Peek, L.J., C.R. Middaugh, and C. Berkland, Nanotechnology in vaccine delivery. Adv Drug Deliv Rev, 2008. 60(8): p. 915-28.
10. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, F. Essahsah, L.M. Fathers, R. Offringa, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, S.H. van der Burg, and C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
11. Melief, C.J., Synthetic vaccine for the treatment of lesions caused by high risk human papilloma virus. Cancer journal, 2011. 17(5): p. 300-1.
12. Melief, C.J., Treatment of established lesions caused by high-risk human papilloma virus using a synthetic vaccine. Journal of immunotherapy, 2012. 35(3): p. 215-6.

13. Chiang, C.L., L.E. Kandalaft, and G. Coukos, Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. International reviews of immunology, 2011. 30(2-3): p. 150-82.
14. van Driel, W.J., M.E. Ressing, G.G. Kenter, R.M. Brandt, E.J. Krul, A.B. van Rossum, E. Schuuring, R. Offringa, T. Bauknecht, A. Tamm-Hermelink, P.A. van Dam, G.J. Fleuren, W.M. Kast, C.J. Melief, and J.B. Trimbos, Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I-II trial. European journal of cancer, 1999. 35(6): p. 946-52.
15. Slingluff, C.L., Jr., G. Yamshchikov, P. Neese, H. Galavotti, S. Eastham, V.H. Engelhard, D. Kittlesen, D. Deacon, S. Hibbitts, W.W. Grosh, G. Petroni, R. Cohen, C. Wiernasz, J.W. Patterson, B.P. Conway, and W.G. Ross, Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. Clinical cancer research : an official journal of the American Association for Cancer Research, 2001. 7(10): p. 3012-24.
16. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, R. Offringa, S.H. van der Burg, and C.J. Melief, Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 169-77.
17. Aucouturier, J., S. Ascarateil, and L. Dupuis, The use of oil adjuvants in therapeutic vaccines. Vaccine, 2006. 24 Suppl 2: p. S2-44-5.
18. Leenaars, P.P., M.A. Koedam, P.W. Wester, V. Baumans, E. Claassen, and C.F. Hendriksen, Assessment of side effects induced by injection of different adjuvant/antigen combinations in rabbits and mice. Laboratory animals, 1998. 32(4): p. 387-406.
19. Elamanchili, P., C.M. Lutsiak, S. Hamdy, M. Diwan, and J. Samuel, “Pathogen-mimicking” nanoparticles for vaccine delivery to dendritic cells. J Immunother, 2007. 30(4): p. 378-95.
20. Joshi, M.D., W.J. Unger, G. Storm, Y. van Kooyk, and E. Mastrobattista, Targeting tumor antigens to dendritic cells using particulate carriers. J Control Release, 2012. 161(1): p. 25-37.
21. Reddy, S.T., M.A. Swartz, and J.A. Hubbell, Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol, 2006. 27(12): p. 573-9.
22. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient CD8(+) T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45.
23. Varypataki, E.M., K. van der Maaden, J. Bouwstra, F. Ossendorp, and W. Jiskoot, Cationic liposomes loaded with a synthetic long Peptide and poly(i:C): a defined adjuvanted vaccine for induction of antigen-specific T cell cytotoxicity. Aaps J, 2015. 17(1): p. 216-26.
24. Silva, A.L., R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J.

Oostendorp, and W. Jiskoot, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: Low-burst release is crucial for efficient CD8+ T cell activation. European Journal of Pharmaceutics and Biopharmaceutics, 2013. 83(3): p. 338-345.
25. Westdijk, J., P. Koedam, M. Barro, B.P. Steil, N. Collin, T.S. Vedvick, W.A. Bakker, P. van der Ley, and G. Kersten, Antigen sparing with adjuvanted inactivated polio vaccine based on Sabin strains. Vaccine, 2013. 31(9): p. 1298-304.
26. Ventura, R., L. Brunner, B. Heriyanto, O. de Boer, M. O'Hara, C. Huynh, M. Suhardono, and N. Collin, Technology transfer of an oil-in-water vaccine-adjuvant for strengthening pandemic influenza preparedness in Indonesia. Vaccine, 2013. 31(12): p. 1641-5.
27. Zom, G.G., S. Khan, C.M. Britten, V. Sommandas, M.G. Camps, N.M. Loof, C.F. Budden, N.J. Meeuwenoord, D.V. Filippov, G.A. van der Marel, H.S. Overkleeft, C.J. Melief, and F. Ossendorp, Efficient induction of antitumor immunity by synthetic toll-like receptor ligand-peptide conjugates. Cancer Immunol Res, 2014. 2(8): p. 756-64.
28. Ossendorp, F., R.E. Toes, R. Offringa, S.H. van der Burg, and C.J. Melief, Importance of CD4(+) T helper cell responses in tumor immunity. Immunology letters, 2000. 74(1): p. 75-9.
29. Castellino, F. and R.N. Germain, Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol, 2006. 24: p. 519-40.
30. Palucka, K. and J. Banchereau, Dendritic-cell-based therapeutic cancer vaccines. Immunity, 2013. 39(1): p. 38-48.
31. Hervas-Stubbs, S., A. Olivier, F. Boisgerault, N. Thieblemont, and C. Leclerc, TLR3 ligand stimulates fully functional memory CD8+ T cells in the absence of CD4+ T-cell help. Blood, 2007. 109(12): p. 5318-26.
32. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup, TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, 2008. 26(13): p. 1626-37.
33. Rosalia, R.A., L.J. Cruz, S. van Duikeren, A.T. Tromp, A.L. Silva, W. Jiskoot, T. de Gruijl, C. Lowik, J. Oostendorp, S.H. van der Burg, and F. Ossendorp, CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses. Biomaterials, 2015. 40: p. 88-97.
34. Swartz, M.A., The physiology of the lymphatic system. Adv Drug Deliv Rev, 2001. 50(1-2): p. 3-20.
35. Girard, J.P., C. Moussion, and R. Forster, HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. Nat Rev Immunol, 2012. 12(11): p. 762-73.
36. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
37. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. The AAPS journal, 2013. 15(1): p. 85-94.
38. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses.

Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
39. Ataman-Onal, Y., S. Munier, A. Ganee, C. Terrat, P.Y. Durand, N. Battail, F. Martinon, R. Le Grand, M.H. Charles, T. Delair, and B. Verrier, Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. Journal of controlled release : official journal of the Controlled Release Society, 2006. 112(2): p. 175-85.
40. Serda, R.E., Particle platforms for cancer immunotherapy. International journal of nanomedicine, 2013. 8: p. 1683-96.
41. Foged, C., C. Arigita, A. Sundblad, W. Jiskoot, G. Storm, and S. Frokjaer, Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. Vaccine, 2004. 22(15-16): p. 1903-13.
42. Chikh, G. and M.P. Schutze-Redelmeier, Liposomal delivery of CTL epitopes to dendritic cells. Biosci Rep, 2002. 22(2): p. 339-53.
43. Nakanishi, T., J. Kunisawa, A. Hayashi, Y. Tsutsumi, K. Kubo, S. Nakagawa, M. Nakanishi, K. Tanaka, and T. Mayumi, Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. J Control Release, 1999. 61(1-2): p. 233-40.
44. Chen, W., W. Yan, and L. Huang, A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. Cancer immunology, immunotherapy : CII, 2008. 57(4): p. 517-30.
45. Vangasseri, D.P., Z. Cui, W. Chen, D.A. Hokey, L.D. Falo, Jr., and L. Huang, Immunostimulation of dendritic cells by cationic liposomes. Molecular membrane biology, 2006. 23(5): p. 385-95.

Chapter 8

Summary, Discussion & Perspectives

1. Summary

In recent years, there has been a growing interest in therapeutic vaccination as a treatment modality against diseases such as cancer [1]. In particular, synthetic long peptides (SLPs) have been studied as well-defined antigens for immunotherapy of cancer. So far, SLPs have been formulated in Montanide ISA 51-based water-in-oil (w/o) emulsions in (pre-)clinical trials. However, the use of Montanide has some important limitations, such as suboptimal efficacy and side effects, so alternative formulations for peptide-based cancer vaccines are highly needed. The field of cancer immunotherapy, the current status of peptide-based cancer vaccines, their lack of efficacy and the need for new adjuvants are introduced in Chapter 1. Poly(lactic-co-glycolic acid) (PLGA) biodegradable particulate delivery systems are particularly interesting because they are biocompatible; can protect soluble antigens from degradation and rapid clearance once administered; allow for co-encapsulation of (multiple) antigens and adjuvants; and mimic the size and structure of a pathogen, being more efficiently taken up by DCs than soluble antigen [2, 3]. In Chapter 2 we provide a detailed overview of the use of PLGA particulate delivery systems for the delivery of protein- and peptide-based vaccines. This chapter discusses formulation parameters influencing the adjuvanticity of these systems, such as size, charge, antigen localization, release profile, and the co-delivery of immune modulators, such as Toll-like receptor ligands (TLRLs), and/or specific targeting molecules, such as antibodies. It further outlines how these characteristics affect uptake, processing and antigen presentation by dendritic cells (DCs) and the ensuing immune response. It also provides a summary of the PLGA formulations that have been studied for the delivery of synthetic peptide-based vaccines.

The principal aim of the research described in this thesis was to investigate how PLGA-based particulate systems can act as an adjuvant for SLP-based cancer vaccines in a pre-clinical setting in order to gain insight into how to improve the immunogenicity, clinical efficacy and safety of SLP-based vaccines for cancer immunotherapy, to be used as a safer and more effective alternative to Montanide. The main objectives of this research included:

- Determination of the best size range of PLGA particles for subcutaneous vaccine delivery
- Development of SLP-loaded PLGA NP formulations
- Development of formulations based on PLGA NPs co-encapsulating SLPs and TLRLs

The first objective was achieved in Chapter 3, where the role of particle size to induce an immune response was studied. For that purpose, we performed a comparative study between NPs versus MPs containing equivalent amounts of a model antigen, ovalbumin (OVA), and a TLRL, poly(I:C), with comparable release kinetics, and studied how their ability to be internalized by DCs affects MHC class I antigen presentation *in vitro* and the ensuing immune responses *in vivo*, in comparison to sustained release from a local subcutaneous depot. Particles were formulated to obtain NPs that could be efficiently internalized by DCs, forming intracellular depots, versus MPs with a size (>

20 µm) too large to be taken up, thus functioning exclusively as an extracellular depot, similarly to Montanide. We showed that efficient particle uptake is crucial to induce an immune response: NPs were efficiently taken up by DCs upon *in vitro* incubation, whereas MPs were not, resulting in increased MHC class I antigen presentation *in vitro* for NPs but not MPs. Moreover, upon subcutaneous vaccination in mice, significantly higher numbers of antigen-specific $CD8^+$ T were obtained with NPs compared to MPs or OVA emulsified in incomplete Freund's adjuvant (IFA). In addition, NP led to better antibody responses compared to MP, and induced a more balanced TH1/TH2-type antibody response than IFA. We concluded that particulate vaccines should be formulated in a nano-size range that allows efficient uptake, significant MHC class I cross-presentation and effective T and B cell responses.

Having determined the optimal size range of PLGA particles for protein-based vaccines, in Chapter 4 we describe the application of these PLGA NPs as a delivery vehicle for ex vivo loading of DCs, in order to stimulate antigen-specific $CD8^+$ T cells to be used for adoptive T cell immunotherapy. For that purpose, DCs previously incubated with PLGA NPs encapsulating model antigen OVA or the soluble protein were used to stimulate $CD8^+$ T cells, which were then transferred to mice. Ex vivo stimulation of CTLs by DCs incubated with PLGA NPs, as compared to soluble protein, resulted in a superior capacity to lyse target cells *in vivo* after adoptive transfer. Furthermore, administration of CTLs stimulated by PLGA NP-loaded DCs resulted in more efficient tumor control leading to prolonged survival of tumor bearing animals, showing that protein antigens benefit from encapsulation in PLGA-NPs, clearly enhancing MHC class I presentation and CTL activation.

As we showed that protein antigen delivery through encapsulation in PLGA NPs is an efficient way to stimulate potent anti-tumor T cells, in Chapter 5 we studied the feasibility of the application of encapsulation in PLGA NPs to SLPs, achieving the second objective. Using OVA24, a 24-residue long synthetic antigenic peptide covering a CTL epitope of OVA (SIINFEKL), as a model antigen, our aim was to define the formulation parameters required to successfully encapsulate an SLP in PLGA NPs, and to optimize PLGA NPs for SLP delivery with respect to encapsulation and release kinetics, to improve the efficacy of SLP cross-presentation by DCs. When using the standard "double emulsion with solvent evaporation" encapsulation techniques, we observed that either encapsulation was very low (< 30%), or burst release extremely high (> 70%). By adjusting formulation and process parameters, we uncovered that the pH of the first emulsion was critical to efficient encapsulation and sustained release. By using an alkaline inner aqueous phase rather than an acidic one, we were able not only to optimize the encapsulation of the SLP but also to reduce its burst release, finally obtaining stable NPs of approximately 330 nm, with an encapsulation efficiency of circa 40% and a burst release lower than 10%. Encapsulation of OVA24 in PLGA NP resulted in enhanced MHC class I restricted T cell activation *in vitro* when compared to high-burst releasing NP and soluble OVA24, revealing the importance of low burst release to induce a potent cellular immune response. This encapsulation method may be a promising approach for encapsulation of peptides with amphiphilic and/or hydrophilic properties, and has been successfully applied to other SLPs as well. This study underscores the importance of optimizing the encapsulation process for the

development of an effective and stable delivery system, and may be considered as a basis for the development of NP formulations for SLP-based immunotherapy of cancer.

Subsequently, the third objective was addressed in Chapter 6, where we studied the co-encapsulation of SLP OVA24 and TLR2L Pam3CSK4 in PLGA NPs, which were used to characterize the intracellular mechanisms via which DC process PLGA-SLP NPs and to determine the study the immunological effects the combination of SLPs with an adjuvant. We showed that TLR 2 stimulation enhanced MHC class I presentation of SLP by DCs *in vitro*. DCs loaded with PLGA-SLP(/TLR2L) NPs internalized them into endolysosomal compartments and not the cytosol as occurs with soluble SLP. Moreover, encapsulated SLP could be detected for long periods inside DCs endolysosomal compartments, resulting in prolonged MHC class I presentation for up to 96 h. PLGA-SLP NPs and especially PLGA-SLP/TLR2L NPs induced sustained $CD8^+$ T cell proliferation *in vivo* after adoptive transfer of PLGA-SLP(/TLR2L) NP-loaded DCs. These findings demonstrate that $CD8^+$ T cell response is enhanced when the antigen is cross-presented in MHC class I molecules in a sustained manner, and that co-encapsulation of a TLRL further boosts these effects, and thus supports the use of PLGA NPs co-encapsulating SLPs and TLRLs as anti-cancer vaccines.

Finally, in Chapter 7 we investigated the potential of PLGA NPs and cationic liposomes as delivery systems for SLP-based vaccines for the induction of cell-mediated immunity. For that purpose, we studied the co-delivery of two SLPs containing the CTL (OVA24) and the Th (OVA17) epitopes of OVA together with TLRLs Pam3CSK4 and/or poly(I:C) in a direct comparison to the clinically used adjuvants Montanide (a water-in-oil emulsion) and a squalene-based oil-in-water emulsion analog to MF59. The obtained formulations were assessed *in vitro* and *in vivo* for their potency to induce $CD8^+$ and $CD4^+$ T cell immune responses. The liposomal and PLGA NP formulations were able to enhance antigen uptake by DCs and subsequent activation of T cells *in vitro*. Subcutaneous vaccination of mice showed that the efficiency of the SLP-loaded liposomes and PLGA NPs to induce functional antigen-specific T cells *in vivo*, was at least as good (PLGA NPs) or better (cationic liposomes) than that of the emulsion-based formulations, while liposomes induced T cells with the highest killing capacity of transferred target cells in mice, outperforming PLGA NPs. Considering the questionable safety profile of the currently clinically used adjuvant Montanide, these findings indicate that both particulate systems are promising biodegradable delivery vehicles for clinical application of SLP-based cancer immunotherapy.

2. Discussion & Perspectives

At the Leiden University Medical Center (LUMC), several (pre-)clinical studies have been conducted, showing that SLP-based vaccines efficiently induce the immune system against cancer [4-6]. In a clinical setting, a vaccine consisting of 13 overlapping SLPs covering the entire sequence of the E6 and E7 oncogenic proteins of high-risk human papillomavirus 16 (HPV16) emulsified in Montanide ISA 51 was administered to women suffering from HPV16-induced (pre-)malignant vulvar intraepithelial neoplasia, resulting in robust immunogenicity in end-stage cervical cancer patients, and complete regression of pre-malignant lesions in 9 of 20 women [4-6]. However, Montanide, a clinical grade version of IFA, has some important limitations: it shows poor control of the antigen release rate, lacks specific DC-activating capacity, and the mineral oil component, which is non-biodegradable, has been associated with significant local side effects [4, 7, 8]. Moreover, though SLP-vaccines showed potent therapeutic efficacy against pre-malignant lesions, they failed to achieve durable clinical responses in late-stage cancer patients, underlining the necessity for better SLP-vaccine formulations that could enhance their therapeutic efficacy. This was the starting point for designing the PhD project described in this thesis, aimed at optimizing the efficacy of SLP vaccines via the encapsulation in poly-(lactic-co-glycolic acid) (PLGA) particles, while reducing the side effects associated with the current (pre-)clinical administration of SLP vaccines emulsified in water-in-oil preparations.

This thesis describes the research toward the optimization of PLGA particulate systems for the delivery of synthetic long peptide (SLP)-based vaccines for immunotherapy of cancer as an alternative to Montanide. For this purpose we explored the use of PLGA particles as delivery systems for SLPs from the pharmaceutical formulation to the immunological evaluation.

Two main approaches have been used to target DCs in cancer immunotherapy: administration of ex vivo TAA-loaded DCs and *in vivo* delivery of TAAs. In Chapter 4 we showed the superior ability of particulate delivery systems compared to soluble antigen to activate DCs *in vitro*, resulting in better $CD8^+$ T cell priming and increased tumor protection *in vivo*. However, though ex vivo-loaded DC-based cancer vaccines have been successfully used in therapeutic settings, each vaccine needs to be specifically prepared for each individual, requiring the harvest and *in vitro* culture of their own DCs, which is time-consuming and requires extensive logistics, thus making these vaccines extremely expensive [9]. By using *in vivo* delivery of tumor associated antigens (TAAs) and adjuvant together to DCs, cancer vaccines could be widely applied to cancer patients. Therefore, our aim was to study formulations to be applied *in vivo*.

Several aspects concerning particulate vaccine formulations have been studied in this thesis, in particular the characterization of the formulations and their immunological effects *in vitro* and *in vivo*. Depending on their physicochemical characteristics, delivery systems can modulate the immune response, mainly due to direct influence in the following mechanisms: facilitated uptake by DCs, regulation of the internalization and presentation pathways, and interaction with specific receptors that mediate the immune

response towards humoral or cellular bias.

One of the most important factors playing a crucial role in vaccine efficacy is size. While smaller particles tend to be more easily taken up by DCs, larger particles can form stable extracellular depots from where the antigen can be slowly released [1]. In Chapter 3, we studied the role of particle size to induce an immune response by comparing NPs that could be internalized by DCs with MPs that could not. Although previous studies had investigated the role of size in particle uptake and ensuing immune response, the extreme differences in antigen release kinetics observed between the formulations would most likely have affected the results [10]. By comparing NPs and MPs with similar sustained release profiles, we were able to get better insight on the actual differences between antigen release from intracellular versus extracellular depots at a similar rate. Indeed, NPs were effectively taken up by DCs, inducing more potent immune responses than MPs, and more importantly than IFA, pointing to the importance of efficient particle uptake by DCs.

While MPs and IFA allow the formation of a depot that can trigger inflammation and the local recruitment of immune cells to the site of injection, to which the antigen and TLRL can be delivered extracellularly in soluble form, the findings in Chapter 6 show that the mechanisms of uptake and processing soluble or particulate antigen by DCs do differ. DCs internalized NPs into the endolysosomal compartments and not the cytosol, as occurs with soluble SLPs. Encapsulated SLPs could be detected for long periods inside DCs' endolysosomal compartments, resulting in prolonged MHC class I presentation in comparison to soluble SLPs. Indeed, these observations concur with the results described in Chapter 4, where ex vivo PLGA NP-loaded DCs more effectively primed $CD8^+$ T cells to confer tumor protection after adoptive transfer *in vivo*. Moreover, they are also in line with the observations in Chapter 5, where we compared NP with similar physicochemical characteristics in terms of charge, size and antigen loading, but different release profiles, having determined that a low-burst release, with the majority of antigen being delivered to DCs still encapsulated in PLGA NPs. was crucial to enhance MHC class I presentation.

The importance of release kinetics and especially the burst release points to the importance of thorough characterization and optimization of SLPs encapsulation in PLGA NPs in order to obtain a successful vaccine formulation. In Chapter 5, we studied the encapsulation of OVA24 SLP in PLGA NP as function of formulation and process parameters. Whereas hydrophilic and hydrophobic peptides have been successfully encapsulated in PLGA NP and/or microparticles in the past [11-13], efficient entrapment of the moderately hydrophobic OVA24 proved to be challenging. The observed high burst release indicated that most of the OVA24 molecules might not be encapsulated in the NP's polymeric matrix, but instead were adsorbed to their surface, being quickly released when resuspended in physiological buffers. Efficient entrapment of OVA24 SLP in the polymeric matrix was obtained by exploring and fine-tuning of formulation and process parameters, in particular the composition of the inner aqueous phase, as encapsulation and release characteristics were strongly dependent on the pH of the first emulsion. Our novel PLGA NP formulation method allowed us to achieve up to 40% encapsulation efficiency of OVA24, exhibiting minimal burst release. Moreover,

this novel method was also successfully applied to encapsulate OVA17, encoding the T helper epitope of OVA, and gp100, encoding an immunodominant CTL epitope present in melanoma. These encouraging results illustrate the applicability of our encapsulation process to different SLPs, and the possibility to be applied in the future to encapsulate the 13 overlapping SLPs encoding the HPV16 E6 and E7 oncoproteins in PLGA NPs to be tested in pre-clinical models and eventually in the clinic.

The formulation method developed in Chapter 5 was also applied to co-encapsulate OVA24 SLP with TRL2L agonist Pam3CSK4 in the PLGA NPs studied in Chapter 6. The prolonged $CD8^+$ T cell activation observed indicates that the internalized NPs slowly release the encapsulated SLPs inside the endolysosomal compartments. The released antigen is then gradually processed in the cytosol, after escape from the endosome, where it is continuously transferred to the proteasome and degraded into smaller peptides to be loaded on MHC class I molecules, resulting in sustained MHC class I Ag presentation. The enhanced MHC class I presentation observed is likely related to the protection from the rapid degradation that occurs with soluble SLPs in the cytosol, which were barely detectable after 24 h, whereas encapsulated SLPs were detected up to 72 h after antigen loading. Co-encapsulation of Pam3CSK4 significantly enhanced the capacity for DCs to prolong MHC class I Ag presentation and $CD8^+$ T cell activation *in vivo*. TLR stimulation is critically involved in the uptake and processing of antigens by DCs, and can trigger cross-presentation [14, 15]. TLR activation induces the secretion of proinflammatory cytokines and type I interferon, and leads to upregulation of CD40, CD80 and CD86 costimulatory molecules on the surface of APCs, as well as release of Th1 cytokines leading to T cell activation [16]. Moreover it increases the half-life of MHC class I-peptide complexes on the cell surface [17], which together with the prolonged presence of encapsulated antigen inside endolysomal compartments can account for the sustained $CD8^+$ T cell activation we described using PLGA-SLP/TLR2L NPs.

For successful implementation of PLGA NPs as delivery systems for SLP-based vaccines in a clinical setting, PLGA NPs should be applicable to co-encapsulate multiple SLPs with different physicochemical properties, such as the 13 overlapping long peptides encoding the HPV16 E6 and E7 oncoproteins. Furthermore, as most pathogens present multiple TLR agonists to APCs, the combination of multiple TLRLs can result in a synergistic effect in order to induce strong immune responses [15]. In Chapter 7 we studied the potential of liposomes and PLGA NPs, co-encapsulating two SLPs containing the CTL (OVA24) and the Th (OVA17) epitopes of OVA together with TLRLs Pam3CSK4 and/or poly(I:C), as alternatives to clinically used Montanide- and squalene-based emulsions in a direct comparison. Both liposomal and PLGA NP formulations were able to enhance antigen uptake by DCs and subsequent activation of T cells *in vitro*, and induce functional antigen-specific T cells *in vivo*, with at least equivalent or even better efficacy than the emulsion-based formulations after subcutaneous vaccination in mice. Liposomes outperformed PLGA NPs, showing the highest killing capacity after transfer of target cells in mice. The differences observed between liposomes and PLGA NPs may be attributed to the combination of two main factors, particle size and surface charge. The size of particulate adjuvants is crucial for their adjuvant activity and the immunogenicity, as small particles (10-150 nm) can easily penetrate the extra-cellular matrix and be transported via the lymphatic

system into the lymph nodes where they come in contact with resident DCs [18], while particles smaller than 200 nm, such as the liposomes used in this study, but not the PLGA NPs, will likely be taken up by DCs more efficiently than bigger particles [19]. Furthermore, as immune cells are negatively charged, cationic particles are more efficiently uptaken due to electrostatic interactions [20], while polycations can also aid in phagosomal/endosomal escape after being internalized by APCs [2], potentially influencing the antigen presentation pathway and type of immune response. Therefore cationic liposomes are in advantage in comparison with PLGA NPs, which are both bigger and negatively charged. Making smaller PLGA NPs similar to liposomes and/or coating them with cationic surfactants may be a way to increase their adjuvanticity. Still, both liposomes and PLGA NPs were used successfully to co-encapsulate up to four components, showing their ability to deliver (multiple) SLPs and TLRLs together to DC, and outperforming both clinically used Montanide and squalene-based emulsions.

For successful implementation of PLGA NPs as a delivery vehicle for SLP-based vaccines to human use, the pharmaceutical formulation should be straightforward, reproducible, stable, and meet GMP quality requirements and regulations. Important factors influencing vaccine efficacy include size; release kinetics; surface characteristics; concomitant delivery of antigen and immunostimulants, allowing DCs to associate danger signals with the antigen, while co-encapsulation of multiple TLRLs may result in a synergistic effect; coating or coupling of DC-specific targeting moieties, increasing DC uptake and enhancing antigen presentation to T cells. Future developments in vaccine delivery will likely involve the combination of (multiple) TLRLs with delivery vehicles modified with DC-specific targeting ligands/antibodies to significantly enhance the delivery of SLP-vaccines to DCs.

Taking into consideration particle size, antigen release kinetics, adjuvanticity and *in vivo* uptake, the findings described in this thesis indicate that robust cellular immune responses can be obtained by using small NPs rather than MPs, with low-burst sustained release, positively charged and co-encapsulating (multiple) antigens and TLRLs (Chapters 3, 5, 6 and 7).

In conclusion, the results described in this thesis present evidence that PLGA NPs may be successfully used as a delivery system for SLP-vaccines as a suitable replacement for emulsions-based formulations for cancer immunotherapy.

3. References

1. Melief, C.J. and S.H. van der Burg, Immunotherapy of established (pre) malignant disease by synthetic long peptide vaccines. Nat Rev Cancer, 2008. 8(5): p. 351-60.
2. Leleux, J. and K. Roy, Micro and nanoparticle-based delivery systems for vaccine immunotherapy: an immunological and materials perspective. Adv Healthc Mater, 2013. 2(1): p. 72-94.
3. Elamanchili, P., C.M. Lutsiak, S. Hamdy, M. Diwan, and J. Samuel, "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. J Immunother, 2007. 30(4): p. 378-95.
4. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. The New England journal of medicine, 2009. 361(19): p. 1838-47.
5. Kenter, G.G., M.J. Welters, A.R. Valentijn, M.J. Lowik, D.M. Berends-van der Meer, A.P. Vloon, . . . C.J. Melief, Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 169-77.
6. Welters, M.J., G.G. Kenter, S.J. Piersma, A.P. Vloon, M.J. Lowik, D.M. Berends-van der Meer, . . . S.H. van der Burg, Induction of tumor-specific $CD4^+$ and $CD8^+$ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. 14(1): p. 178-87.
7. van den Hende, M., M.I. van Poelgeest, J.M. van der Hulst, J. de Jong, J.W. Drijfhout, G.J. Fleuren, . . . G.G. Kenter, Skin reactions to human papillomavirus (HPV) 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia. Int J Cancer, 2008. 123(1): p. 146-52.
8. Mueller, M., E. Schlosser, B. Gander, and M. Groettrup, Tumor eradication by immunotherapy with biodegradable PLGA microspheres--an alternative to incomplete Freund's adjuvant. Int J Cancer, 2011. 129(2): p. 407-16.
9. Hamdy, S., A. Haddadi, R.W. Hung, and A. Lavasanifar, Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. Adv Drug Deliv Rev, 2011. 63(10-11): p. 943-55.
10. Joshi, V.B., S.M. Geary, and A.K. Salem, Biodegradable particles as vaccine delivery systems: size matters. AAPS J, 2013. 15(1): p. 85-94.
11. Mata, E., M. Igartua, R.M. Hernandez, J.E. Rosas, M.E. Patarroyo, and J.L. Pedraz, Comparison of the adjuvanticity of two different delivery systems on the induction of humoral and cellular responses to synthetic peptides. Drug delivery, 2010. 17(7): p. 490-9.
12. Wieber, A., T. Selzer, and J. Kreuter, Characterisation and stability studies of a hydrophilic decapeptide in different adjuvant drug delivery systems: a comparative study of PLGA nanoparticles versus chitosan-dextran

sulphate microparticles versus DOTAP-liposomes. International Journal of Pharmaceutics, 2011. 421(1): p. 151-9.
13. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander, Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. J Drug Target, 2009. 17(8): p. 652-61.
14. Demento, S.L., A.L. Siefert, A. Bandyopadhyay, F.A. Sharp, and T.M. Fahmy, Pathogen-associated molecular patterns on biomaterials: a paradigm for engineering new vaccines. Trends Biotechnol, 2011. 29(6): p. 294-306.
15. Black, M., A. Trent, M. Tirrell, and C. Olive, Advances in the design and delivery of peptide subunit vaccines with a focus on toll-like receptor agonists. Expert Rev Vaccines, 2010. 9(2): p. 157-73.
16. Van Gool, S.W., P. Vandenberghe, M. de Boer, and J.L. Ceuppens, CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. Immunol Rev, 1996. 153: p. 47-83.
17. Rudd, B.D., J.D. Brien, M.P. Davenport, and J. Nikolich-Zugich, Cutting edge: TLR ligands increase TCR triggering by slowing peptide-MHC class I decay rates. J Immunol, 2008. 181(8): p. 5199-203.
18. Girard, J.P., C. Moussion, and R. Forster, HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. Nature reviews. Immunology, 2012. 12(11): p. 762-73.
19. Oyewumi, M.O., A. Kumar, and Z. Cui, Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines, 2010. 9(9): p. 1095-107.
20. Thomas, C., V. Gupta, and F. Ahsan, Influence of surface charge of PLGA particles of recombinant hepatitis B surface antigen in enhancing systemic and mucosal immune responses. Int J Pharm, 2009. 379(1): p. 41-50.

8

Chapter 9

Appendix

Nederlandse Samenvatting
List of Abbreviations
List of Publications
Curriculum Vitae

Nederlandse Samenvatting

In de laatste jaren is er een toenemende interesse in therapeutische vaccinatie als behandelingsmethode tegen ziektes zoals kanker [1]. In het bijzonder zijn synthetische lange peptiden (SLPs) bestudeerd als goed gedefinieerde antigenen voor kanker-immunotherapie. Tot dusver zijn in (pre-)klinische studies SLPs geformuleerd in Montanide ISA 51, een water-in-olie (w/o) emulsie. Het gebruik van Montanide heeft echter belangrijke beperkingen, zoals een suboptimale effectiviteit en bijwerkingen. Er is dus een grote behoefte aan alternatieve formuleringen voor SLP-vaccins tegen kanker.

Het domein van kanker-immunotherapie, de huidige status van peptidevaccins tegen kanker, hun gebrek aan voldoende effectiviteit en de behoefte aan nieuwe adjuvantia worden geïntroduceerd in Hoofdstuk 1. In het bijzonder zijn biologisch afbreekbare afgiftesystemen op basis van een polymeer van melk- en glycolzuur (poly(lactic-co-glycolic acid; PLGA)) interessant omdat ze biologisch compatibel zijn; vrije antigenen na toediening kunnen beschermen tegen afbraak en snelle uitscheiding; co-inkapseling van (meerdere) antigenen en adjuvantia toelaten; en de grootte en structuur van een pathogeen imiteren. Bovendien worden ze efficiënter opgenomen door dendritische cellen (DCs) dan vrije antigenen [2, 3].

In Hoofdstuk 2 wordt een gedetailleerd overzicht gegeven van het gebruik van PLGA-deeltjes voor de afgifte van eiwit- en peptidevaccins. Dit hoofdstuk bespreekt de formuleringsparameters die de adjuvanticiteit van deze systemen beïnvloeden, zoals grootte (nanodeeltjes (NDs) versus microdeeltjes (MDs), lading, antigeenlokalisatie, afgifteprofiel, en het includeren van immuunmodulatoren, zoals Toll-like receptor ligands (TLRLs), en/of moleculen met specifieke sturing, zoals antilichamen. Verder schetst dit hoofdstuk hoe de formuleringsparameters opname, verwerking en presentatie van antigenen door DCs en de daarop volgende immuunrespons beïnvloeden. Dit hoofdstuk geeft ook een samenvatting van PLGA-formuleringen die in de literatuur bestudeerd zijn voor de afgifte van synthetische peptidevaccins.

Het voornaamste doel van het in dit proefschrift beschreven onderzoek was het bestuderen hoe PLGA-deeltjes kunnen dienen als adjuvans in peptidevaccins tegen kanker, als een veiliger en effectiever alternatief voor Montanide. De belangrijkste doelstellingen van dit onderzoek waren:

- Het bepalen van de beste grootte van PLGA-deeltjes voor subcutaan toegediende vaccins;
- Ontwikkeling van PLGA-ND-formuleringen die beladen zijn met SLPs;
- Ontwikkeling van PLGA-NDs formuleringen, waarbij zowel SLPs als TLRLs in de deeltjes ingebouwd worden

Het eerste doel werd bereikt in Hoofdstuk 3, waarin de rol van de deeltjesgrootte bij het opwekken van een immuunrespons werd bestudeerd. Hiervoor voerden we een vergelijkende studie uit tussen NDs en MDs die equivalente hoeveelheden van het modelantigeen ovalbumine (OVA), en een TLRL, poly(I:C), met vergelijkbare afgiftekinetiek bevatten. We bestudeerden hoe de mate van internalisering door DCs de MHC-I klasse antigeenpresentatie *in vitro* beïnvloedt, en de daaropvolgende immuunrespons in vivo, vergeleken met afgifte van een lokaal subcutaan depot. Hiertoe werd enerzijds PLGA geformuleerd als NDs die efficiënt door DCs geïnternaliseerd konden worden, waarbij intracellulaire depots gevormd worden. Anderzijds werd PLGA verwerkt tot MDs die te groot (> 20 µm) zijn om opgenomen te worden, om zo enkel te functioneren als een extracellulair depot, vergelijkbaar met Montanide. We toonden aan dat efficiënte opname van deeltjes cruciaal is om een immuunrespons op te wekken. NDs werden efficiënt opgenomen door DCs na *in vitro* incubatie, terwijl dit bij MDs niet gebeurde. Dit resulteerde bij het gebruik van NDs, in tegenstelling tot MDs, in verhoogde MHC- I klasse antigeenpresentatie *in vitro*. Bovendien werd bij subcutane vaccinatie bij muizen een significant groter aantal antigeenspecifieke $CD8^+$ T-cellen verkregen met NDs in vergelijking met MDs of OVA geëmulgeerd in incomplete Freund's adjuvant (IFA). Bovendien leidde de ND-formulering tot een betere antilichaamrespons in vergelijking met de MD-formulering. Bovendien induceerde de ND-formulering een meer evenwichtige TH1/TH2-type antilichaamrespons dan de IFA-formulering. We concludeerden dat vaccins geformuleerd zouden moeten worden in nanodeeltjes ter bewerkstelliging van efficiënte opname en significante MHC-I klasse cross-presentatie van het antigeen, maar ook voor effectieve T- en B-celresponsen.

Na het bepalen van de optimale grootte van PLGA-deeltjes voor eiwitvaccins, beschrijven we in Hoofdstuk 4 de toepassing van deze PLGA-NDs als afgiftesysteem voor het beladen van DCs ex vivo, om antigeen-specifieke $CD8^+$ T-cellen te stimuleren om gebruikt te worden voor adoptieve T-celimmuuntherapie. Daarvoor werden DCs, die voorheen geïncubeerd werden met PLGA-NDs met daarin ingekapseld het modelantigeen OVA of met het vrije eiwit geformuleerd in fysiologische zoutoplosing, gebruikt om $CD8^+$ T-cellen te stimuleren, die vervolgens wereden toegediend aan muizen. DCs geïncubeerd met PLGA-NDs stimuleerden cytotoxische T-lymfocyten (CTLs) ex vivo efficiënter dan niet verpakt eiwit. Bovendien leidde het toedienen van CTLs gestimuleerd met PLGA-NDs beladen DCs tot een betere lyse van de doelcellen in vivo en een efficiëntere tumorcontrole, waardoor muizen met agressieve tumoren langer konden overleven. Met deze studie is aangetoond dat de effectiviteit van eiwitantigenen voor vaccinatie tegen kanker verbeterd kan worden door ze te verpakken in PLGA-NDs.

Aangezien uit bovenstaande studie gebleken was dat de afgifte van eiwitantigeen via inkapseling in PLGA-NDs een efficiënte manier is om krachtige anti-tumor T-cellen te induceren, bestudeerden we in Hoofdstuk 5 de toepassing van inkapseling van SLPs in PLGA-NDs, om zo het tweede doel te bereiken. Met gebruikmaking van het modelantigeen OVA24, een 24-residu lang synthetisch peptide met daarin een CTL-epitoop van OVA (SIINFEKL), was ons doel de formuleringsparameters te bepalen die nodig zijn om een SLP succesvol in PLGA-NDs in te kapselen. Daarnaast werden PLGA-NDs geoptimaliseerd wat betreft de SLP-inkapseling en -afgiftekinetiek, om

daarmee de efficiëntie van SLP cross-presentatie door DCs te verbeteren. Wanneer de standaard inkapselingstechnieken gebruikt werden (een dubbele emulsie, waarbij het oplosmiddel wordt verdampt), was de inkapseling erg laag (< 30%), of de directe afgifte (burst release) erg hoog (> 70%). Door de formulerings- en procesparameters aan te passen, ontdekten we dat de pH van de eerste emulsie van kritiek belang is voor efficiënte inkapseling en constante afgifte. Door in de binnenste fase een alkalische, in plaats van een zure, omgeving te creëren, waren we in staat om niet alleen de inkapseling van het SLP te optimaliseren, maar ook de burst release te reduceren, waarbij uiteindelijk stabiele NDs van ongeveer 330 nm werden verkregen met een inkapselingsefficiëntie van ongeveer 40% en een burst release van minder dan 10%. Inkapseling van OVA24 in deze PLGA-NDs gaf sterkere MHC-I klasse gemedieerde T-celactivatie *in vitro* ten opzichte van NDs met een hoge burst release en vrij (niet verpakt) OVA24. Dit toont aan dat een lage burst release van belang is voor het opwekken van een krachtige cellulaire immuunrespons. Deze inkapselingsmethode kan een veelbelovende aanpak zijn voor het formuleren van peptiden met amfifiele en/of hydrofiele eigenschappen, en is ook succesvol toegepast op andere SLPs. Deze studie onderschrijft het belang van het optimaliseren van het inkapselingsproces bij het ontwikkelen van een effectief en stabiel vaccinafgiftesysteem, en kan gezien worden als een basis voor de ontwikkeling van ND-formuleringen met SLPs voor kanker-immunotherapie.

Vervolgens werd de derde doelstelling onderzocht in Hoofdstuk 6, waarbij de inkapseling van een SLP (OVA24) samen met een TLRL (Pam3CSK4) in PLGA-NDs wordt beschreven. De verkregen formuleringen werden gebruikt om de intracellulaire mechanismen te karakteriseren waarmee DCs PLGA-NDs verwerken, en om de immunologische effecten van de combinatie van SLPs met een adjuvans te onderzoeken. We toonden aan dat TLR2-stimulatie de MHC-I klasse presentatie van SLPs door DCs *in vitro* versterkte. DCs die geïncubeerd werden met PLGA-SLP(/TLR2L)-NDs internaliseerden de NDs in endolysosomale compartimenten, wat zorgde voor een verlengde MHC-I klasse presentatie. PLGA-SLP-NDs en met name PLGA-SLP/TLR2L-NDs wekten constante $CD8^+$ T-celproliferatie in vivo op na adoptieve transfer van PLGA-SLP(/TLR2L)-ND-beladen DCs. Deze bevindingen tonen aan dat de $CD8^+$ T-celrespons versterkt wordt wanneer het antigeen langdurig cross-gepresenteerd is in MHC-I klasse moleculen, en dat mede-inkapseling van een TLRL deze effecten verder versterkt.

Ten slotte onderzochten we in Hoofdstuk 7 het het vermogen van PLGA-NDs en kationische liposomen als afgiftesystemen voor SLP-vaccins voor het opwekken van T-celgemedieerde immuniteit. Daarvoor bestudeerden we de inkapseling van twee SLPs die een CTL- en een Th-epitoop (respectievelijk OVA24 en OVA17) van OVA bevatten, samen met TLRLs PAM3CSK3 en/of poly(I:C) in één deeltje, in een directe vergelijking met twee klinisch gebruikte adjuvantia, Montanide (een water-in-olie emulsie) en een op squaleen gebaseerde (olie-in-water emulsie) analoog aan MF59. De verkregen formuleringen werden *in vitro* en in vivo beoordeeld op hun capaciteit om $CD8^+$ en $CD4^+$ T-celimmuunresponsen op te wekken. De liposomale en PLGA-ND-formuleringen waren in staat de antigeenopname door DCs en de daaropvolgende activatie van T-cellen *in vitro* te versterken. Subcutane vaccinatie van muizen toonde aan dat de efficiëntie van met SLP beladen liposomen en PLGA-NDs om functionele

antigeen-specifieke T-cellen in vivo op te wekken minstens zo goed (PLGA NDs) of beter (kationische liposomen) was dan SLPs geformuleerd in de emulsies. Hierbij waren liposomen het beste in staat om T-cellen te induceren met de hoogste capaciteit om overgebrachte doelcellen in muizen te doden. Uit deze bevindingen blijkt dat dat beide systemen, kationische liposomen en PLGA-NDs, veelbelovende afgiftesystemen zijn voor de klinische toepassing van SLP-vaccins voor kanker-immunotherapie.

List of Abbreviations

Ab	Antibody
ACN	Acetonitrile
AF488	Alexa-fluor 488
Ag	Antigen
ANOVA	Analysis of variance
APC	Antigen-presenting cell
B3Z	B3Z $CD8^+$ T-cell hybridoma cell line, specific for the CTL epitope of ovalbumin SIINFEKL
BCA	Bicinchoninic acid protein assay
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
$CD4^+$ T cell	T helper cell
$CD8^+$ T cell	Cytotoxic T lymphocyte
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptor
CpG ODN	Unmethylated cytosine-phosphodiester-guanine oligodeoxynucleotide motif
CTAB	Cetyltrimethylammonium
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DCM	Dichloromethane
DEAE	Diethylaminoethyl
DL	Drug loading
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPC	Dioleoyl- glycero-phosphatidylcholine
DOTAP	Dioleoyl-trimethylammonium-propane
dsRNA	Double-stranded RNA
DSS	Dioctylsulfosuccinate
EE	Encapsulation efficiency
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum

FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Good manufacturing practice
gp	Glycoprotein
HBcAg	Hepatitis B core antigen
HbsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
i.d.	Intradermal
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IgG1	Immunoglobulin G subtype 1
IgG2*a*/b	Immunoglobulin G subtype 2*a*/b
IL	Interleukin
INF-g	Interferon gamma
ISCOM	Immune stimulatory complex
LE	Loading efficiency
LO	Light obscuration
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
M-cell	Microfold cell
Men B	Neisseria meningitidis serotype B
MHC I/II	Major histocompatibility complex class I/II
MP	Microparticle
MPLA	Monophosphoryl lipid A
NOD	Nucleotide-binding oligomerization domain receptor
NP	Nanoparticle
o/w	Oil-in-water (emulsion)
OVA	Ovalbumin
OVA17	17-residue synthetic long peptide of ovalbumin (ISQAVHAAHAEINEAGR)

OVA24	24-residue synthetic long peptide of ovalbumin (DEVSGLEQLESIINFEKLAAAAAK)
Pam3CSK4	Synthetic triacylated lipopeptide
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PDI	Polydispersity index
PD-L1/2	Programmed cell death protein 1 ligand 1/2
PEG	Poly(ethylene glycol)
PEI	Poly(ethylene imine)
PLA	Poly-lactic acid
PLA2	Phospholipase A2
PLGA	Poly(lactic-co-glycolic acid)
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RGD	Arginine-glycine-aspartate
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high-pressure liquid chromatography
s.c	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SEM	Standard error of the mean
SLP	Synthetic long peptide
sSLP	Soluble synthetic long peptide
SSP	Synthetic short peptide
ssRNA	single-stranded RNA
SWE	Squalene oil-in-water emulsion
TAA	Tumor associated antigens
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TFA	Trifluoroacetic acid
Th	T helper
Th1	Type 1 T helper
Th2	Type 2 T helper
TLR	Toll-like receptor
TLRL	Toll-like receptor ligand
TMB	Tetramethylbenzidine
TMC	N-trimethyl chitosan

TNF	Tumor necrosis factor
TRP1/2	Tyrosinase-related protein 1/2
TT	Tetanus toxoid
w/o/w	Water-in-oil-in-water (emulsion)
Z-ave	Z-average diameter
ZP	Zeta potential

List of Publications

1. Varypataki EM*, **Silva AL***, Barnier-Quer C, Collin N, Ossendorp F, Jiskoot W, Synthetic long peptide-based vaccine formulations for induction of cell mediated immunity: a comparative study of cationic liposomes and PLGA nanoparticles, manuscript in preparation

2. Rosalia RA*, **Silva AL***, Stepanek I, van der Laan A, Oostendorp J, Jiskoot W, van der Burg SJ, Ossendorp F, Co-encapsulation of synthetic long peptide antigen and Toll like receptor 2 ligand in poly-(lactic-coglycolic-acid) particles results in sustained MHC class I cross-presentation by dendritic cells, manuscript in preparation

3. **Silva AL**, Soema P, Slütter BA, Ossendorp F, Jiskoot W, PLGA particulate delivery systems for subunit vaccines: linking particle properties to immunogenicity, accepted for publication at Human Vaccines & Immunotherapeutics

4. Rosalia RA, Cruz LJ, van Duikeren S,Tromp A, **Silva AL**, Jiskoot W, de Gruijl T, Löwik C, Oostendorp J, van der Burg SJ, Ossendorp F, CD40-targeted Dendritic Cell Delivery of PLGA-Nanoparticle vaccine induce potent anti-tumor responses, Biomaterials, 2015. 40: p. 88-97

5. **Silva AL***, Rosalia RA*, Varypataki E, Sibuea S, Ossendorp F, Jiskoot W, Poly-(lactic-co-glycolic-acid)-based particulate vaccines: nano-size is a key parameter for dendritic cell uptake and immune activation, Vaccine, 2014. 33(7): p. 847-54

6. **Silva AL**, Rosalia RA., Sazak A, Carstens M, Ossendorp F, Oostendorp J, Jiskoot W, Optimization of encapsulation of a synthetic long peptide in PLGA nanoparticles: low-burst release is crucial for efficient CD8(+) T cell activation. Eur J Pharm Biopharm, 2013. 83(3): p. 338-45

7. Rosalia RA, **Silva AL**, Camps M, Allam A, Jiskoot W, van der Burg SH, Ossendorp F, Oostendorp J, Efficient ex vivo induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles. Cancer Immunol Immunother, 2013. 62(7): p. 1161-73

8. Keijzer C, Spieringa R*, **Silva AL***, van Edena W, Jiskoot W, Verveldea L, Broerea F, PLGA nanoparticles enhance the expression of retinaldehyde dehydrogenase enzymes in dendritic cells and induce FoxP3(+) T-cells in vitro. J Control Release, 2013. 168(1): p. 35-40

9. Rosalia RA, Quakkelaar ED, Khan S, Redeker A, Drijfhout JW, **Silva AL**, Jiskoot W, van Hall T, van Veelen PA, Janssen G, Franken K, Cruz LJ, Tromp A, Oostendorp J, van der Burg SH, Ossendorp F, Melief CJM, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. Eur J Immunol, 2013. 43(10): p. 2554-65

*Authors contributed equally

Curriculum Vitae

Ana Luísa Silva was born on the tenth of February 1980 in Monterey, California, USA. In 1998 she finished high school at Escola Secundária Ibn Mucana in Alcabideche, Portugal. After graduating she studied Biological Engineering at Instituto Superior Técnico in Lisbon, Portugal. During this period she did an internship through the Erasmus program at the Universitá degli Studi di Milano, Italy, where she worked on the study of enantioselective hydrolysis of esters of 1,2-O-isopropylideneglycerol by enzymatic catalysis, before graduating in 2004. In 2005 she started her master's studies at Instituto Superior Técnico and performed her primary research project on enzymatic degradation of pDNA, and completed a master's thesis on the "Effect of chemical agents on plasmid resistance to nucleases" under the supervision of Prof. Dr. Gabriel Monteiro. She obtained a Master of Science degree in Biotechnology in 2007 and entered a Portuguese government program InovContacto, in which she received a grant to do an internship for 9 months at Genentech, Inc., in South San Francisco, California, USA.

In 2008 she started her PhD project on "PLGA-based particulate vaccine delivery systems for immunotherapy of cancer" under supervision of Prof. Dr. Wim Jiskoot at the Division of Drug Delivery Technology of the Leiden Academic Centre for Drug Research (LACDR) and Prof. Dr. Ferry Ossendorp at the Leiden University Medical Center (LUMC), both at Leiden University, the Netherlands. The research results of this project are described in this thesis.