Journal of Controlled Release 203 (2015) 16–22

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Polymeric nanoparticles for co-delivery of synthetic long peptide antigen and poly IC as therapeutic cancer vaccine formulation

Sima Rahimian [a], Marieke F. Fransen [b], Jan Willem Kleinovink [b], Jonatan Riis Christensen [a], Maryam Amidi [a], Wim E. Hennink [a], Ferry Ossendorp [b,*]

[a] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
[b] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO

Article history:
Received 29 November 2014
Received in revised form 30 January 2015
Accepted 4 February 2015
Available online 7 February 2015

Keywords:
Therapeutic cancer vaccine
Human papillomavirus (HPV)
Synthetic long peptide (SLP)
TLR3 ligand
Poly IC
Nanoparticles
pLHMGA
Incomplete Freund's adjuvant (IFA)

ABSTRACT

The aim of the current study was to develop a cancer vaccine formulation for treatment of human papillomavirus (HPV)-induced malignancies. Synthetic long peptides (SLPs) derived from HPV16 E6 and E7 oncoproteins have been used for therapeutic vaccination in clinical trials with promising results. In preclinical and clinical studies adjuvants based on mineral oils (such as incomplete Freund's adjuvant (IFA) and Montanide) are used to create a sustained release depot at the injection site. While the depot effect of mineral oils is important for induction of robust immune responses, their administration is accompanied with severe adverse and long lasting side effects. In order to develop an alternative for IFA family of adjuvants, polymeric nanoparticles (NPs) based on hydrophilic polyester (poly(D,L lactic-co-hydroxymethyl glycolic acid) (pLHMGA)) were prepared. These NPs were loaded with a synthetic long peptide (SLP) derived from HPV16 E7 oncoprotein and a toll like receptor 3 (TLR3) ligand (poly IC) by double emulsion solvent evaporation technique. The therapeutic efficacy of the nanoparticulate formulations was compared to that of HPV SLP + poly IC formulated in IFA. Encapsulation of HPV SLP antigen in NPs substantially enhanced the population of HPV-specific CD8+ T cells when combined with poly IC either co-encapsulated with the antigen or in its soluble form. The therapeutic efficacy of NPs containing poly IC in tumor eradication was equivalent to that of the IFA formulation. Importantly, administration of pLHMGA nanoparticles was not associated with adverse effects and therefore these biodegradable nanoparticles are excellent substitutes for IFA in cancer vaccines.

1. Introduction

Infection with human papillomavirus (HPV) is the main cause of cervical cancer and is associated with other anogenital malignancies such as vaginal and anal cancer [1]. More than 99% of cervical cancer cases – the second most common cancer in women worldwide [2] – are attributed to infection with high risk oncogenic HPV types [3]. The predominant high risk type is HPV16 [4] which accounts for approximately 50% of the cervical tumors [5]. Known etiology of the disease provides an appealing opportunity to develop vaccines against the high risk HPV types. The registered prophylactic vaccines are highly successful in prevention of cancer [6] but have no therapeutic efficacy [7]. Therefore there is great need for therapeutic vaccines to treat HPV-induced malignancies. HPV16 encodes two major oncoproteins, namely E6 and E7 which are expressed in all HPV16-induced cervical cancer cells [5] and are responsible for interference in the cell cycle [8]. Several clinical studies have used E6 and E7 proteins as targets in immunotherapy of HPV-induced cancer using different strategies such as peptide and protein based vaccines, virus like particles and DC-based vaccines [9–12]. Although these studies showed activation of CD8+ T cells – the immune cells capable of killing tumor cells – the majority exhibited limited tumor regression and therapeutic efficacy [13–15]. Vaccination with minimal peptide epitopes of oncoproteins has been a popular approach in several studies for treatment of HPV-induced malignancies [16–18]. Nevertheless, it has not been very successful due to several reasons, including direct binding of the peptide epitope to MHC molecules on the surface of cells which results in tolerization of T cells [15,18] and failure to develop a long-term memory [19]. Increasing the length of the peptide antigens has substantially improved the efficacy of the peptide vaccines [19,20]. As synthetic long peptides (SLPs) are too large to be able to directly bind to MHC molecules on the surface of cells, their epitope can only be presented by professional antigen presenting cells (APCs) which are able to take up and process the peptide for subsequent presentation to T cells. This restriction to professional APCs is essential as only these cells can provide the co-stimulatory signals necessary for adequate T cell activation. Dendritic cells (DCs) are the most efficient professional APCs. These cells process SLPs more efficiently than whole proteins [21] and provide long term antigen presentation in the draining lymph nodes which is crucial for T cell activation and expansion [22].

* Corresponding author at: Albinusdreef 2, 2333 ZA Leiden, The Netherlands.
E-mail address: F.A.Ossendorp@lumc.nl (F. Ossendorp).

http://dx.doi.org/10.1016/j.jconrel.2015.02.006
0168-3659/

Vaccination with SLPs overlapping the whole sequence of HPV16 E6 and E7 oncoproteins formulated in Montanide ISA 51 has proven the immunogenicity of the vaccine in clinical trials [23–26]. Incomplete Freund's adjuvant (IFA) and similar adjuvants such as Montanide ISA 51 act as depots that deliver the peptide antigen in water-in-oil emulsions [27,28], however, their adjuvanticity is not sufficient for initiation of a strong T cell response [19,24,25]. An effective cancer vaccine is capable of activating the adaptive as well as the innate immune system which results in efficient targeting and elimination of cancer cells by eliciting a strong T cell-mediated immune response [29]. IFA formulations fail to cause strong DC maturation and, as a result, are not capable of inducing a strong CD8+ T cell response [30,31].

DC maturation bridges between innate and adaptive immune system and can be mediated by triggering a family of receptors on DCs (as well as other cells), called toll-like receptors (TLRs) [32,33]. Some TLR ligands such as TLR3 ligands bind to their receptors in the endosome, where the antigens are processed [34]. An advantage of using TLR3 ligands such as poly IC in cancer vaccines is that upon triggering, these ligands are able to enhance antigen cross-presentation to CD8+ T cells, therefore expanding the cytotoxic T cell population to induce cellular immune response [35]. However poly IC – a double stranded RNA construct, is degraded rapidly by nucleases in the body. This demands a high administered dose which consequently might result in toxicity and autoimmune responses [36]. Studies have demonstrated that the co-delivery of TLR ligands and peptide antigens in carrier systems can considerably enhance T cell response [37–40] while decreasing the risk of toxicity [36].

Another drawback of IFA other than its weak immunogenicity is adverse effects at the injection site such as painful granulomas, inflammation and swelling, sterile abscesses and cysts [41,42]. Hence, development of a delivery system to replace IFA in cancer vaccines is of great interest. The present study, aimed to 1) to increase the efficacy and safety of the HPV SLP cancer vaccine by designing a particulate cancer vaccine, and 2) to study the effect of co-delivery of HPV SLP and TLR3 ligand (poly IC) in nanoparticles (NPs). We have developed a nanoparticulate cancer vaccine based on a biodegradable polymer, poly(D,L lactic-co-hydroxymethyl glycolic acid) (pLHMGA) [43,44] and loaded with a 27 amino acid synthetic long peptide and poly IC (TLR3 ligand). This SLP contains both a CD8+ epitope (RAHYNIVTF) and a CD4+ epitope (DRAHYNI) of the HPV16 E7 protein for BL/6 mice. Previous studies have shown the advantages of pLHMGA over the well-known and frequently investigated pLGA, such as better compatibility with proteins and peptides [45,46]. In a recent study, ovalbumin-loaded pLHMGA NPs were used as a model vaccine. These particles showed prolonged presence in the lymph nodes upon subcutaneous administration and excellent *in vivo* T cell expansion which proved their potential as antigen delivery systems [47]. pLHMGA particles have shown good cytocompatibility *in vitro* [47,48] and are biocompatible *in vivo* after subcutaneous administration [48]. In the current study, we examined the therapeutic efficacy of HPV SLP-loaded pLHMGA NPs in a prime-boost vaccination regimen in TC-1 tumor-bearing mice and compared it with IFA formulation.

2. Materials and methods

2.1. Materials

Poly(D,L lactic-co-hydroxymethyl glycolic acid) (pLHMGA) with initial monomer ratios of 65/35 (d,l lactide/benzyloxy methyl methylglycolide (BMMG)) was synthesized and characterized as described before [43, 47] (Fig. S1-details are provided in the supporting information). Polyvinyl alcohol (PVA; Mw 30,000–70,000; 88% hydrolyzed), dimethyl sulfoxide (DMSO), tetrafluoroacetic acid (TFA), poly IC (sodium salt, gamma irradiated) and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES 1 M) were obtained from Sigma-Aldrich, USA. Dichloromethane (DCM) and acetonitrile (ACN) were obtained from Biosolve, The Netherlands. Sodium hydroxide (NaOH) and sodium dodecyl sulfate 20% (SDS) were purchased from Fluka, The Netherlands. Pyrogen-free water was obtained from Carl Roth, Germany. Phosphate buffered saline (1.8 mM NaH_2PO_4, 8.7 mM Na_2HPO_4, 163.9 mM Na^+, 140.3 mM Cl^-, pH 7.4) (PBS) was obtained from B Braun, Germany. IFA was purchased from Difco (USA). Synthetic long peptide 27-mer HPV SLP (GQAEPDRAHYNIVTF(Abu)(Abu)K(Abu)DSTLRL(Abu)V), containing the cytotoxic T lymphocyte (CTL) epitope RAHYNIVTF of HPV E7 oncoprotein ($E7_{43-69}$) was synthesized in interdepartmental GMP facility of the Department of Clinical Pharmacy and Toxicology of Leiden University Medical Center. In this SLP, cysteines were replaced by the unnatural amino acid, aminobutyric acid (Abu). Chemicals were used as received without further purification, unless otherwise stated.

2.2. Preparation of HPV SLP loaded nanoparticles with/without poly IC

HPV SLP-loaded pLHMGA NPs with/without poly IC (HPV SLP ± poly IC NPs) were prepared using a double emulsion solvent evaporation technique essentially as described before [49] with a few adjustments. In brief, 200 μL of 10 mg/mL HPV SLP (100 μL of ACN and 100 μL of TFA 0.1% in pyrogen-free water) ± 50 μL of 20 mg/mL poly IC in pyrogen-free water were emulsified by sonication (30 s, 20% amplitude-ultrasonic homogenizer (Labsonic P, B. Braun Biotech, Germany)) in 50 mg of pLHMGA dissolved in 1 mL of DCM to prepare a water-in-oil emulsion (W_1/O). Next 2 mL of an aqueous PVA 1% w/v solution (filtered through 0.2 μm cellulose acetate sterile filter) was added to this first emulsion and the mixture was emulsified again by sonication forming the double emulsion ($W_1/O/W_2$). This double emulsion was then added drop-wise to 25 mL of PVA 0.3% w/v at 40 °C while stirring for rapid removal of DCM. After 1 h, the particles were harvested by centrifugation for 30 min at 20,000 *g*, washed with pyrogen-free water, resuspended in pyrogen-free water and freeze-dried overnight.

2.3. Nanoparticle characterization

2.3.1. Size, zeta-potential and morphology of the NPs

2.3.1.1. Dynamic light scattering. The size of NPs was measured with dynamic light scattering (DLS) on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He–Ne laser operating at 632.8 nm, an optical fiber-based detector, and a digital LV/LSE-5003 correlator. Freeze dried NPs were suspended in deionized water (RI = 1.332 and viscosity of 0.8898 cP) and measurements were done at 25 °C at an angle of 90°.

2.3.1.2. Transmission electron microscopy (TEM). The size and morphology of NPs were analyzed using transmission electron microscopy (TEM, Philips-FEI Tecnai T10, USA). Twenty microliter of particle suspension in water was placed on parafilm. A glow discharged Formvar carbon film on copper grid (Agar scientific, UK) was placed on the particle suspension to absorb the NPs. After 2 min the excess liquid was removed using a filter paper. The sample was stained with 20 μL of 2% uranyl acetate in water for 1 min and consecutively dried with filter paper and was left for 5 min to dry completely. NPs were visualized with 7–73 k fold magnification and analyzed by Olympus MeasureIT software.

2.3.1.3. Zeta potential. The zeta-potential (ζ) of the NPs was measured using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) with disposable folded capillary cells. Nanoparticles were dispersed in 10 mM HEPES pH 7.0 and zeta potential was measured at 25 °C and analyzed using DTS Nano 4.20 software.

2.3.2. Loading efficiency measurements

2.3.2.1. HPV SLP content. The HPV SLP loading efficiency of the NPs was determined by measuring the peptide content of digested NPs as previously described [61]. Approximately 5 mg of freeze-dried NPs was carefully weighed and dissolved in 0.25 mL of DMSO. After complete dissolution of NPs (60 °C, 1 h) 375 μL of ACN and 375 μL of TFA 0.1% in water was added to precipitate the polymer. After 2 h at 60 °C, samples were centrifuged at 14,000 *g* for 15 min and 100 μL of the supernatant was injected into an HPLC system equipped with a C18 column (Dr. Maisch Reprosil-Pur C18-AQ, 3 μm, 150 × 4.6 mm) and an ultraviolet detector (Waters 2487). Mobile phases were 5% ACN in water with 0.1% TFA (solvent A), and 95% ACN in water with 0.1% TFA (solvent B). HPV SLP was separated by applying a linear gradient from 0% to 100% solvent B over 15 min, at a flow rate of 1 mL/min, and peptide detection was at 210 nm. Calibration was done using 100 μL of HPV SLP (1.5–200 μg/mL).

2.3.2.2. Poly IC content. The amount of poly IC encapsulated in the NPs was determined as previously described [50] with some modifications. In detail, approximately 5 mg of freeze-dried NPs was accurately weighed and dissolved in 1 mL of DCM. Then 3 mL of Tris EDTA (TE-provided with the Quantifluor kit, (Promega Corporation, USA)) buffer was added and the tube was vortexed for 30 s. The two immiscible layers were separated by centrifugation at 5000 *g* for 5 min. Next, 2.5 mL of the upper layer containing poly IC in TE buffer was removed and replaced by 2.5 mL of fresh TE buffer and the extraction was repeated 4–5 times until the amount of poly IC extracted was less than 1% of the initial loading. The amount of poly IC in the extraction medium was measured by using Quantifluor RNA quantification assay kit and calibration was done using poly IC in TE buffer (4.8–2500 ng/mL). HPV SLP NPs and empty NPs as well as empty NPs plus known amount of poly IC were used as controls. These samples were treated similar to (HPV SLP + poly IC) NPs.

Loading efficiency (LE%) is reported as the amount of HPV SLP/poly IC encapsulated in the NPs divided by the amount of HPV SLP/poly IC added × 100%. Loading percentage (L%) is reported as the amount of HPV SLP/poly IC entrapped in the NPs per total dry mass of the NPs × 100%.

2.4. Mice and cells

Female C57BL/6 (H-2b) mice were purchased from Charles River laboratories (France) and kept in specified pathogen-free facility. The experiments were approved by the Animal Experimental Committee of the Leiden University Medical Center. TC-1 cells (growth factor independent and highly oncogenic in immunocompetent mice) are primary lung epithelial cells of C57BL/6 mice transformed with HPV16 E6 and E7 and c-Ha-Ras oncogenes [51]. TC-1 cells were cultured at 37 °C with 5% CO_2 in IMDM containing 8% FCS (Greiner), 2 mM glutamine, and 100 IU/mL penicillin, 400 μg/mL geneticin (G418; Life Technologies), non-essential amino acids (Life Technologies), and 1 mM sodium pyruvate (Life Technologies).

2.5. Therapeutic vaccination

2.5.1. Vaccination regimen

TC-1 tumor cells (10^5) dispersed in 200 μL of PBS were injected subcutaneously (s.c.) in the right flank of mice (age 8–10 weeks). Tumor size was measured with calipers two times per week in three dimensions and the tumor volume was calculated using this formula: tumor length × width × height. Only mice with palpable tumors (0.5–3 mm^3) were enrolled in the experiment. Therapeutic vaccination was given in a prime-boost fashion; when the tumors were palpable, on day 8 after tumor inoculation the prime dose was injected and the boost was given on day 22 after tumor inoculation. Mice (5–10 in each group) were vaccinated s.c. in the left flank with 100 μg of HPV SLP NPs with or without 50 μg of poly IC either co-encapsulated in NPs or in soluble form. IFA formulation containing HPV SLP and poly IC was used as control. This IFA formulation was prepared on the vaccination day by mixing 100 μg HPV SLP and 50 μg poly IC in PBS/IFA with a volume ratio of 1:1 and vortexing for 30 min [52]. As we aimed to compare the effect of NPs to IFA formulations used previously, the doses of HPV SLP and poly IC were chosen according to the previous treatments which showed good T cell response and tumor regression [52]. Empty pLHMGA NPs were excluded from this experiment. Earlier studies showed that empty pLHMGA NPs do not elicit a T cell response [47]. Furthermore, empty pLHMGA microparticles have been used as control in tumor therapy (unpublished data) where no delay in tumor growth and survival of mice was observed. Mice were sacrificed for ethical reasons when tumors exceeded 1000 mm^3 or in case of tumor ulceration. The experiment was ended when none of the mice had a palpable tumor. Tumor growth for each group is presented as relative tumor volume (RTV) calculated using this formula: RTV_t = Tumor volume at time t/initial tumor volume. Data were fitted using non-linear regression and survival of mice is presented in Kaplan–Meier plot.

2.5.2. Detection of HPV16 E7-specific CD8+ T cells in peripheral blood of tumor-bearing mice by tetramer staining

Peripheral blood was collected from the tail-vein of the mice 9 days after the prime dose (17 days after tumor inoculation). After lysis of erythrocytes, the blood samples were stained for cell surface markers CD3ε, CD8α and the allophycocyanine (APC)-conjugated H-2Db $E7_{49–57}$ tetramer which binds to the T cell receptor recognizing RAHYNIVTF epitope [53].

2.6. Statistical analyses

All data were analyzed using GraphPad Prism 5.02 software. For tumor experiments Kaplan–Meier survival curves were applied and the differences between survival curves were analyzed by log-rank test ($p < 0.05$ was considered statistically significant). Expansion of HPV-specific CD8+ T cells in the blood of tumor-bearing mice 9 days after the prime vaccination in mice treated with HPV SLP and poly IC formulations was compared to untreated mice by Dunn's multiple comparisons test. Statistical significance of total experiment ($p < 0.001$) was calculated by Kruskal–Wallis test.

3. Results and discussion

3.1. Preparation and characterization of (HPV SLP ± poly IC) NPs

HPV SLP loaded NPs and (HPV SLP + poly IC) loaded NPs were prepared by a double emulsion solvent evaporation technique. This method has been extensively used for encapsulation of peptides and nucleic acids as well as other biomacromolecules in nano/microparticles based on aliphatic polyesters [54–58]. The obtained NP formulations showed comparable characteristics in terms of size and morphology and HPV SLP loading efficiency. The mean hydrodynamic diameter of the NPs measured by DLS ranged between 400 and 500 nm with a PDI of approximately 0.20–0.29. Analysis of freeze-dried samples with TEM showed spherical NPs with a smooth surface and a particle size of approximately 100–200 nm (Fig. 1). The difference between the observations from TEM and results of DLS measurements can be explained by the algorithms used for calculation of hydrodynamic diameter of the NPs by DLS which results in overestimation of the average population [59]. The HPV SLP loaded NP exhibited negative zeta potential (approximately −14 mV). The negative zeta potential of the HPV loaded NPs could be ascribed to the carboxylic (COOH) end groups present in the pLHMGA polymer (Fig. 1—Supporting information). These COOH groups are deprotonated in HEPES buffer and therefore causing a negative zeta potential. The isoelectric point (pI) of HPV SLP is 7.7 and

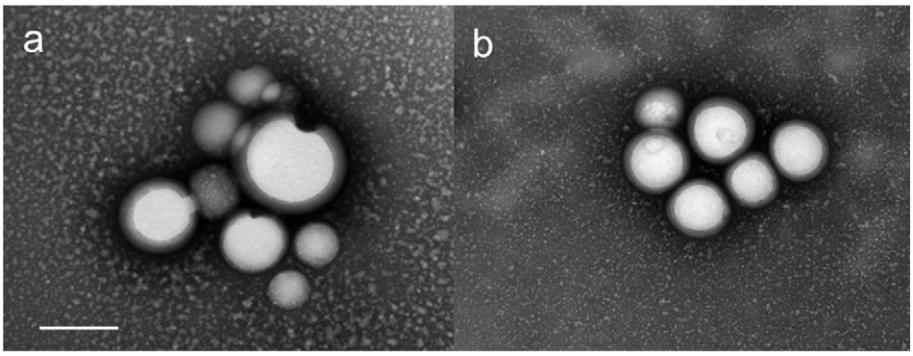

Fig. 1. TEM photographs of a) (HPV SLP + poly IC) NPs and b) HPV SLP NPs. Scale bar represents 200 nm.

therefore it bears very low charge in HEPES buffer pH 7.0 (supporting information) which is unlikely to contribute to the negative zeta potential values. The zeta potential values of (HPV SLP + poly IC) NPs were approximately −25 mV which can be attributed to negatively-charged poly IC molecules that are associated with/close to the surface of the NPs. The loading efficiency of HPV SLP for both NP formulations was approximately 60% and independent of presence of poly IC in the formulations. The loading efficiency of poly IC in (HPV SLP + poly IC) NPs was approximately 65% and the ratio between HPV SLP and poly IC was close to the target ratio (2 to 1 (w/w)). For the ease of comparison, the ratio between HPV SLP and poly IC was chosen according to the previous studies in which HPV SLP was used in soluble form or emulsified in Montanide [52]. Analysis of HPV SLP NPs and empty NPs using Quantifluor kit showed the baseline signal for empty and HPV SLP NPs formulations. Additionally poly IC was completely recovered when added to the empty NPs, Therefore it was concluded that neither HPV SLP in the particles nor the particles as such affect the quantification of poly IC.

The HPV SLP has a poor solubility in aqueous solutions. As it has been studied by Silva et al., the *in vitro* release of long peptides from pLGA NPs could not be accurately followed for more than 24 h, likely due to the precipitation of peptide during release. However in the abovementioned study, pLGA NPs with low burst release showed superior T cell activation *in vitro*, when compared to pLGA NPs with high burst release [49]. The *in vitro* release of HPV SLP from pLHMGA NPs in PBS was followed up to 48 h and not more than 5–7% of the encapsulated peptide was detected. Moreover, there is no good model system to investigate the release of peptides from carriers in DCs. The characteristics of the NPs are summarized in Table 1.

3.2. Tumor growth following treatment with NP and IFA formulations

To assess the anti-tumor efficacy of nanoparticulate cancer vaccines in comparison to commonly used IFA formulations in a mouse model of HPV-induced cervical cancer, mice were inoculated subcutaneously with TC-1 tumor cells expressing HPV16 oncogenes E6 and E7. When the tumors were palpable, at day 8 after tumor inoculation, the mice were s.c. vaccinated with various formulations and received the boost dose at day 21 after tumor inoculation. In order to evaluate the necessity of using poly IC in co-encapsulated form with HPV SLP, one group of mice was vaccinated with HPV SLP NPs and poly IC in PBS. Control mice were either treated with poly IC in PBS or left untreated. Tumor growth curves in the different groups of mice are presented in Figs. 2a and S2 (Supporting information). All untreated mice developed tumors larger than 1000 mm^3 and were euthanized no later than 28 days post inoculation. In the groups of mice which received poly IC in soluble form, tumors grew out similarly to untreated mice. This confirms previous observations showing that a TLR ligand *per se* does not cause regression in pre-established tumors [52].

HPV SLP NPs increased the overall survival of mice but their inhibitory effect was limited, in agreement with previous studies in which the administration of the HPV SLP in IFA was able to delay the tumor growth, but did not completely eradicate tumors [20]. In contrast, NPs loaded with HPV SLP and poly IC strongly delayed the tumor growth with approximately 20 days, comparable to the effect after administration of HPV SLP NPs in combination with soluble poly IC. Treatment with HPV and poly IC in IFA emulsion showed similar tumor regression to nanoparticle formulations administered with poly IC (either encapsulated or in soluble form).

The anti-tumor efficacy of the various HPV SLP formulations in tumor-bearing mice is presented in the Kaplan–Meier plot (Fig. 2b). Neither Poly IC in soluble form (median survival: 22 days) nor HPV SLP NPs (median survival: 25 days) were able to significantly prolong the survival of mice as compared to untreated mice (median survival: 22 days), whereas vaccination with nanoparticle formulations containing HPV SLP in combination with a TLR3 ligand (poly IC) significantly increased the survival time of mice ($p < 0.001$) (median survival 43 days). There were no differences in survival proportion and median survival of mice which received (HPV SLP + poly IC) NPs and the group which received HPV SLP NP co-administered with poly IC in soluble form. Although previous studies suggested that the co-encapsulation of the antigen and the adjuvant substantially enhances the T cell response, our data did not support this notion [36]. This can likely be ascribed to the relatively high dose of soluble poly-IC that was administered. It can be expected that at lower doses, co-encapsulation will have a beneficial effect. The favorable aspect of co-encapsulation over soluble administration is the lower risk of systemic immune activation by spread of the soluble TLR-ligand. Upon vaccination of mice with HPV SLP + poly IC in IFA, the vaccine formed a lump at the injection site which persisted until the end of the experiment, whereas in mice treated with NPs, no residue (remainder) of NPs was observed. This is in line with previous observations where upon local administration of pLHMGA NPs of similar size in mice, NPs were cleared from the injection site in a sustained manner [47]. Moreover pLHMGA particles when

Table 1
Characteristics of NPs. Results are representative of two independent formulations.

Nanoparticle	Theoretical loading % HPV SLP/poly IC	Size (nm)	PDI	Zeta potential (mV)	HPV SLP loading efficiency (%)	Poly IC loading efficiency (%)	μg HPV SLP/μg poly IC in 1 mg of nanoparticle formulation
HPV SLP	4/0	423 ± 49	0.21 ± 0.03	−14.9 ± 0.3	58.3 ± 9.9	–	–
HPV SLP + poly IC	4/2	491 ± 16	0.29 ± 0.08	−25.0 ± 1.3	58.8 ± 5.9	63.0 ± 10.0	1.92 ± 0.06

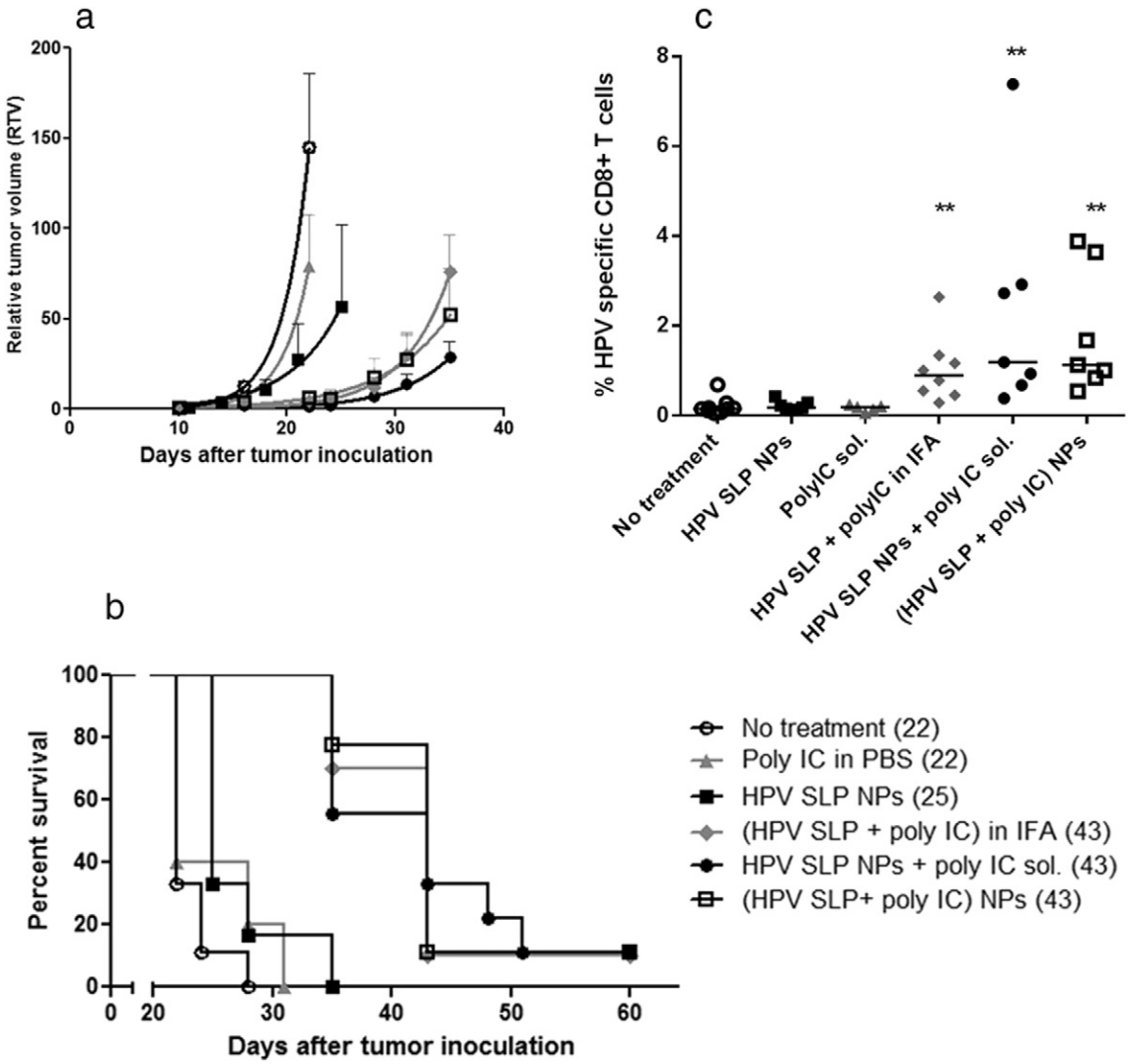

Fig. 2. a) Relative tumor growth in tumor bearing mice. Seven days after TC-1 tumor inoculation in wild-type C57BL/6 mice, and when tumors were palpable, mice were either left untreated or were s.c. vaccinated in the opposite flank with formulations consisting of HPV SLP with or without poly IC formulated in NPs or in IFA followed by a boost injection 14 days after the prime dose. The mice were sacrificed when the tumor volume reached 1000 mm^3 or were ulcerated. The relative TC-1 tumor volume is plotted against time and fitted using non-linear regression. For each group, line is only plotted until the first mouse is sacrificed. 5–10 mice per group were used. Data is representative of two independent experiments. b) Survival of mice per group presented in Kaplan–Meier plot. The differences between the groups were calculated using log-rank (Mantel–Cox) test. The survival of mice vaccinated with HPV SLP NPs with poly IC (either co-encapsulated or in soluble form) and HPV SLP + poly IC in IFA was significantly longer compared to the non-treated mice ($p < 0.001$). Numbers in the legends represent median survival in days. Data is representative of two independent experiments. c) Expansion of HPV-specific CD8+ T cells in the blood of tumor-bearing mice 9 days after the prime vaccination. Each dot represents one mouse. HPV-specific CD8+ T cell expansion in tumor-bearing mice 9 days after the prime vaccination in mice treated with HPV SLP and poly IC formulations is compared to untreated mice by Dunn's multiple comparisons test. Statistical significance of total experiment ($p < 0.001$) is calculated by Kruskal–Wallis test. Statistical significance between groups is shown by asterisks, ** = $p < 0.01$.

injected subcutaneously have shown biocompatibility with no toxicity [48]. In an earlier study, a postmortem examination of the injection site 60 days after administration of both pLHMGA microparticles and IFA formulation showed that the IFA depot was still present while no remainders of pLHMGA particles were observed (unpublished data) which confirms previous observations on *in vitro* degradation of pLHMGA particles in 30–50 days [45].

3.3. HPV-specific T cell expansion in peripheral blood of tumor-bearing mice after treatment with NP and IFA formulations

Earlier studies have shown that the ability of a peptide vaccine to induce a systemic CD8+ T cell response predicts the anti-tumor efficacy of the vaccine [20,52]. Therefore, the population of HPV-specific CD8+ T cells in blood was measured by tetramer staining 9 days after the prime vaccination. Mice treated with poly IC containing NP formulations and HPV SLP + poly IC in IFA showed high and comparable frequencies of HPV-specific CD8+ T cells whereas this effect was not observed for mice treated with the HPV SLP NP formulation (Fig. 2c). In agreement with previous observations [60–62], TLR3 ligand alone (poly IC in PBS) did not induce any HPV-specific T cell expansion. Importantly, the height of the HPV-specific CD8+ T cell response in blood correlated with the delay in tumor growth, suggesting an important role for CD8+ T cells in the anti-tumor effect of the vaccines.

4. Conclusion

This study highlights the potential of pLHMGA nanoparticles as substitute for IFA-based cancer vaccines. Vaccination with NPs comprising a synthetic long peptide derived from HPV E7 oncoprotein and poly IC as a TLR3 ligand substantially prolonged the survival of mice (three weeks) in a therapeutic tumor setting. There was no difference between the effects of using poly IC in encapsulated NP form or in a soluble form when the HPV SLP was encapsulated in pLHMGA NPs. Importantly, the anti-

tumor effect of these nanoparticle formulations was comparable to that of IFA, while as opposed to IFA, subcutaneous administration of pLHMGA nanoparticles was not associated with local adverse effects. Moreover, the HPV-specific CD8 + T cell expansion following administration of nanoparticles could predict their therapeutic efficacy in tumor-bearing mice. Although the efficacies of the studied vaccine formulations; HPV SLP + poly IC in IFA, (HPV SLP + poly IC) NPs and HPV SLP NPs + poly IC were similar, safety concerns are considerably distinct for each formulation. Encapsulation of poly IC prevents autoimmunity caused by high systemic concentrations of poly IC. Additionally NPs do not exhibit the local adverse effects associated with administration of IFA. In conclusion, the (HPV SLP + poly IC) NPs provide equal efficacy and are superior in terms of safety and therefore the preferred formulation in cancer immunotherapy as alternatives for IFA in immunotherapy of cancer.

Acknowledgments

This research was performed within the framework of Cancer Vaccine Tracking project (#03O-302), center for translational molecular medicine (CTMM).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.02.006.

References

[1] F. Bosch, A. Lorincz, N. Munoz, C. Meijer, K. Shah, The causal relation between human papillomavirus and cervical cancer, J. Clin. Pathol. 55 (2002) 244–265.
[2] H. zur Hausen, Papillomaviruses and cancer: from basic studies to clinical application, Nat. Rev. Cancer 2 (2002) 342–350.
[3] J.M.M. Walboomers, M.V. Jacobs, M.M. Manos, F.X. Bosch, J.A. Kummer, K.V. Shah, P.J.F. Snijders, J. Peto, C.J.L.M. Meijer, N. Munoz, Human papillomavirus is a necessary cause of invasive cervical cancer worldwide, J. Pathol. 189 (1999) 12–19.
[4] N. Munoz, F. Bosch, X. Castellsague, M. Diaz, S. De Sanjose, D. Hammouda, K. Shah, C. Meijer, Against which human papillomavirus types shall we vaccinate and screen? The international perspective, Int. J. Cancer 111 (2004) 278–285.
[5] N. Munoz, F.X. Bosch, S. de Sanjose, R. Herrero, X. Castellsague, K.V. Shah, P.J.F. Snijders, C.J.L.M. Meijer, Epidemiologic classification of human papillomavirus types associated with cervical cancer, N. Engl. J. Med. 348 (2003) 518–527.
[6] C.M. Wheeler, Advances in primary and secondary interventions for cervical cancer: human papillomavirus prophylactic vaccines and testing, Nat. Clin. Pract. Oncol. 4 (2007) 224–235.
[7] A. Hildesheim, R. Herrero, S. Wacholder, A.C. Rodriguez, D. Solomon, M.C. Bratti, J.T. Schiller, P. Gonzalez, G. Dubin, C. Porras, S.E. Jimenez, D.R. Lowy, Effect of human papillomavirus 16/18 L1 virus-like particle vaccine among young women with preexisting infection — a randomized trial, JAMA 298 (2007) 743–753.
[8] S. Tyring, Human papillomavirus infections: epidemiology, pathogenesis, and host immune response, J. Am. Acad. Dermatol. 43 (2000) S18–S26.
[9] N.P. Tran, C. Hung, R. Roden, T.C. Wu, Control of HPV infection and related cancer through vaccination, Recent Results Cancer Res. 193 (2014) 149–171.
[10] B. Ma, B. Maraj, Nam Phuong Tran, J. Knoff, A. Chen, R.D. Alvarez, C. Hung, T. Wu, Emerging human papillomavirus vaccines, Expert Opin. Emerg. Drugs 17 (2012) 469–492.
[11] T. Liu, W.M. Hussein, I. Toth, M. Skwarczynski, Advances in peptide-based human papillomavirus therapeutic vaccines, Curr. Top. Med. Chem. 12 (2012) 1581–1592.
[12] A. Bolhassani, E. Mohit, S. Rafati, Different spectra of therapeutic vaccine development against HPV infections, Hum. Vaccin. 5 (2009) 671–689.
[13] M. Ressing, W. van Driel, R. Brandt, G. Kenter, J. de Jong, T. Bauknecht, G. Fleuren, P. Hoogerhout, R. Offringa, A. Sette, E. Celis, H. Grey, B. Trimbos, W. Kast, C. Melief, Detection of T helper responses, but not of human papillomavirus-specific cytotoxic T lymphocyte responses, after peptide vaccination of patients with cervical carcinoma, J. Immunother. 23 (2000) 255–266.
[14] L. Muderspach, S. Wilczynski, L. Roman, L. Bade, J. Felix, L. Small, W. Kast, G. Fascio, V. Marty, J. Weber, A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive, Clin. Cancer Res. 6 (2000) 3406–3416.
[15] R.E.M. Toes, R. Offringa, R.J.J. Blom, C.J.M. Melief, W.M. Kast, Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7855–7860.
[16] S.H. van der Burg, M.S. Bijker, M.J.P. Welters, R. Offringa, C.J.M. Melief, Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy, Adv. Drug Deliv. Rev. 58 (2006) 916–930.
[17] M.S. Bijker, C.J. Melief, R. Offringa, S.H. van der Burg, Design and development of synthetic peptide vaccines: past, present and future, Expert Rev. Vaccines 6 (2007) 591–603.
[18] R.E.M. Toes, R.J.J. Blom, R. Offringa, W.M. Kast, C.J.M. 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. 156 (1996) 3911–3918.
[19] M.S. Bijker, S.J.F. van den Eeden, K.L. Franken, C.J.M. Melief, R. Offringa, 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. 179 (2007) 5033–5040.
[20] S. Zwaveling, S. Mota, J. Nouta, M. Johnson, G. Lipford, R. Offringa, S. van der Burg, C. Melief, Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides, J. Immunol. 169 (2002) 350–358.
[21] R.A. Rosalia, 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 derBurg, F. Ossendorp, C.J.M. Melief, Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation, Eur. J. Immunol. 43 (2013) 2554–2565.
[22] M.S. Bijker, S.J.E. van den Eeden, K.L. Franken, C.J.M. Melief, S.H. van der Burg, R. Offringa, Superior induction of anti-tumor CTL immunity by extended peptide vaccines involves prolonged, DC-focused antigen presentation, Eur. J. Immunol. 38 (2008) 1033–1042.
[23] C.J.M. Melief, S.H. van der Burg, Immunotherapy of established (pre) malignant disease by synthetic long peptide vaccines, Nat. Rev. Cancer 8 (2008) 351–360.
[24] M.J.P. Welters, G.G. Kenter, S.J. Piersma, A.P.G. Vloon, M.J.G. Lowik, D.M.A. Berends-van der Meer, J.W. Drijfhout, A.R.P.M. Valentijn, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, R. Offringa, C.J.M. Melief, 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, Clin. Cancer Res. 14 (2008) 178–187.
[25] G.G. Kenter, M. Welters, M. Lowik, J. Drijfhout, R. Valentijn, J. Ostendorp, F. Gertjan, S. Van de Burg, C. Melief, Therapeutic HPV 16 vaccination with long E6 and E7 peptides shows immunological and clinical efficacy, Gynecol. Oncol. 108 (2008) S19-S19.
[26] G.G. Kenter, M.J.P. Welters, A.R.P.M. Valentijn, M.J.G. Lowik, D.M.A. Berends-van der Meer, A.P.G. Vloon, F. Essahsah, L.M. Fathers, R. Offringa, J.W. Drijfhout, A.R. Wafelman, J. Oostendorp, G.J. Fleuren, S.H. van der Burg, C.J.M. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia, N. Engl. J. Med. 361 (2009) 1838–1847.
[27] J. Aucouturier, L. Dupuis, S. Deville, S. Ascarateil, V. Ganne, Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines, Expert Rev. Vaccines 1 (2002) 111–118.
[28] J. Aucouturier, S. Ascarateil, L. Dupuis, The use of oil adjuvants in therapeutic vaccines, Vaccine 24 (Suppl. 2) (2006) S2-44-5.
[29] J.M. Silva, M. Videira, R. Gaspar, V. Preat, H.F. Florindo, Immune system targeting by biodegradable nanoparticles for cancer vaccines, J. Control. Release 168 (2013) 179–199.
[30] D.E. Speiser, D. Lienard, N. Rufer, V. Rubio-Godoy, D. Rimoldi, F. Lejeune, A.M. Krieg, J.C. Cerottini, P. Romero, Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909, J. Clin. Invest. 115 (2005) 739–746.
[31] A. Shibaki, S. Katz, Induction of skewed Th1/Th2 T-cell differentiation via subcutaneous immunization with Freund's adjuvant, Exp. Dermatol. 11 (2002) 126–134.
[32] S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, Nat. Immunol. 2 (2001) 675–680.
[33] C. Melief, S. van der Burg, R. Toes, F. Ossendorp, R. Offringa, Effective therapeutic anticancer vaccines based on precision guiding of cytolytic T lymphocytes, Immunol. Rev. 188 (2002) 177–182.
[34] B. Salaun, S. Lebecque, S. Matikainen, D. Rimoldi, P. Romero, Toll-like receptor 3 expressed by melanoma cells as a target for therapy? Clin. Cancer Res. 13 (2007) 4565–4574.
[35] F. Steinhagen, T. Kinjo, C. Bode, D.M. Klinman, TLR-based immune adjuvants, Vaccine 29 (2011) 3341–3355.
[36] A.M. Hafner, B. Corthesy, H.P. Merkle, Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant, Adv. Drug Deliv. Rev. 64 (2013) 1386–1399.
[37] E. Schlosser, M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, 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 26 (2008) 1626–1637.
[38] S. Hamdy, O. Molavi, Z. Ma, A. Haddadi, A. Alshamsan, Z. Gobti, S. Elhasi, J. Samuel, 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 26 (2008) 5046–5057.
[39] C. Wischke, J. Zimmermann, B. Wessinger, A. Schendler, H. Borchert, J.H. Peters, T. Nesselhut, D.R. Lorenzen, Poly(I:C) coated PLGA microparticles induce dendritic cell maturation, Int. J. Pharm. 365 (2009) 61–68.
[40] S. De Koker, B.N. Lambrecht, M.A. Willart, Y. van Kooyk, J. Grooten, C. Vervaet, J.P. Remon, B.G. De Geest, Designing polymeric particles for antigen delivery, Chem. Soc. Rev. 40 (2011) 320–339.
[41] H. Toledo, A. Baly, O. Castro, S. Resik, J. Laferte, F. Rolo, L. Navea, L. Lobaina, O. Cruz, J. Miguez, T. Serrano, B. Sierra, L. Perez, M.E. Ricardo, M. Dubed, A.L. Lubian, M. Blanco, J.C. Millan, A. Ortega, E. Iglesias, E. Penton, Z. Martin, J. Perez, M. Diaz, C.A. Duarte, A phase I clinical trial of a mufti-epitope polypeptide TAB9 combined with Montanide ISA 720 adjuvant in non-HIV-1 infected human volunteers, Vaccine 19 (2001) 4328–4336.
[42] R. Gupta, E. Relyveld, E. Lindblad, B. Bizzini, S. Benefraim, C. Gupta, Adjuvants — a balance between toxicity and adjuvanticity, Vaccine 11 (1993) 293–306.

[43] M. Leemhuis, C. van Nostrum, J. Kruijtzer, Z. Zhong, M. ten Breteler, P. Dijkstra, J. Feijen, W. Hennink, Functionalized poly(alpha-hydroxy acid)s via ring-opening polymerization: toward hydrophilic polyesters with pendant hydroxyl groups, Macromolecules 39 (2006) 3500–3508.
[44] M. Leemhuis, J.A.W. Kruijtzer, C.F. van Nostrurn, W.E. Hennink, In vitro hydrolytic degradation of hydroxyl-functionalized poly(alpha-hydroxy acid)s, Biomacromolecules 8 (2007) 2943–2949.
[45] A.H. Ghassemi, M.J. van Steenbergen, A. Barendregt, H. Talsma, R.J. Kok, C.F. van Nostrum, D.J.A. Crommelin, 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. 29 (2012) 110–120.
[46] Y. Liu, A.H. Ghassemi, W.E. Hennink, S.P. Schwendeman, The microclimate pH in poly(D, L-lactide-co-hydroxymethyl glycolide) microspheres during biodegradation, Biomaterials 33 (2012) 7584–7593.
[47] S. Rahimian, J.W. Kleinovink, M.F. Fransen, L. Mezzanotte, H. Gold, P. Wisse, H. Overkleeft, M. Amidi, W. Jiskoot, C.W. Löwik, F. Ossendorp, W.E. Hennink, Near-infrared labeled, ovalbumin loaded polymeric nanoparticles based on a hydrophilic polyester as model vaccine: in vivo tracking and evaluation of antigen-specific CD8+ T cell immune response, Biomaterials 37 (2015) 469–477.
[48] F. Kazazi-Hyseni, J. Zandstra, E. Popa, R. Goldschmeding, A. Lathuile, G. Veldhuis, C. Van Nostrum, W. Hennink, R. Kok, Biocompatibility of poly(D, L-lactic-co-hydroxymethyl glycolic acid) microspheres after subcutaneous and subcapsular renal injection, Int. J. Pharm. (2015). http://dx.doi.org/10.1016/j.ijpharm.2014.12.014 (in press).
[49] A.L. Silva, R.A. Rosalia, A. Sazak, M.G. Carstens, F. Ossendorp, J. Oostendorp, 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. 83 (2013) 338–345.
[50] Y. Krishnamachari, PLGA Microparticle Based Vaccine Carriers for an Improved and Efficacious Tumor TherapyPhD thesis University of Iowa, 2011. (http://ir.uiowa.edu/etd/2922).
[51] K. Lin, F. Guarnieri, K. StaveleyOCarroll, H. Levitsky, J. August, D. Pardoll, T. Wu, Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen, Cancer Res. 56 (1996) 21–26.
[52] S. van Duikeren, M.F. Fransen, A. Redeker, B. Wieles, G. Platenburg, W. Krebber, F. Ossendorp, C.J.M. Melief, R. Arens, Vaccine-induced effector-memory cd8(+) t cell responses predict therapeutic efficacy against tumors, J. Immunol. 189 (2012) 3397–3403.
[53] J. Altman, P. Moss, P. Goulder, D. Barouch, M. McHeyzerWilliams, J. Bell, A. McMichael, M. Davis, Phenotypic analysis of antigen-specific T lymphocytes, Science 274 (1996) 94–96.
[54] R.C. Mundargi, V.R. Babu, V. Rangaswamy, P. Patel, T.M. Aminabhavi, Nano/micro technologies for delivering macromolecular therapeutics using poly(D, L-lactide-co-glycolide) and its derivatives, J. Control. Release 125 (2008) 193–209.
[55] P. Couvreur, F. Puisieux, Nanoparticles and microparticles for the delivery of polypeptides and proteins, Adv. Drug Deliv. Rev. 10 (1993) 141–162.
[56] W. Ma, M. Chen, S. Kaushal, M. McElroy, Y. Zhang, C. Ozkan, M. Bouvet, C. Kruse, D. Grotjahn, T. Ichim, B. Minev, PLGA nanoparticle-mediated delivery of tumor antigenic peptides elicits effective immune responses, Int. J. Nanomedicine 7 (2012) 1475–1487.
[57] Z. Zhang, S. Tongchusak, Y. Mizukami, Y.J. Kang, T. Ioji, M. Touma, B. Reinhold, D.B. Keskin, E.L. Reinherz, T. Sasada, Induction of anti-tumor cytotoxic T cell responses through PLGA-nanoparticle mediated antigen delivery, Biomaterials 32 (2011) 3666–3678.
[58] A.M. Tinsley-Bown, R. Fretwell, A.B. Dowsett, S.L. Davis, G.H. Farrar, Formulation of poly(D, L-lactic-co-glycolic acid) microparticles for rapid plasmid DNA delivery, J. Control. Release 66 (2000) 229–241.
[59] V. Filipe, A. Hawe, W. Jiskoot, Critical evaluation of nanoparticle tracking analysis (nta) by nanosight for the measurement of nanoparticles and protein aggregates, Pharm. Res. 27 (2010) 796–810.
[60] D.A. Wick, J.R. Webb, A novel, broad spectrum therapeutic HPV vaccine targeting the E7 proteins of HPV16, 18, 31, 45 and 52 that elicits potent E7-specific CD8T cell immunity and regression of large, established, E7-expressing TC-1 tumors, Vaccine 29 (2011) 7857–7866.
[61] S. Chen, R. Ou, J. Tang, X. Deng, Y. Wu, J.C. van Velkinburgh, B. Ni, Y. Xu, Enhanced anti-tumor effects of HPV16E7(49–57)-based vaccine by combined immunization with poly(I:C) and oxygen-regulated protein 150, Cancer Epidemiol. 37 (2013) 172–178.
[62] Z.R. Cui, F. Qiu, Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model, Cancer Immunol. Immunother. 55 (2006) 1267–1279.