Journal of Controlled Release 269 (2018) 347–354

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Hollow microneedle-mediated micro-injections of a liposomal HPV $E7_{43–63}$ synthetic long peptide vaccine for efficient induction of cytotoxic and T-helper responses

Koen van der Maaden[a,*], Jeroen Heuts[a,b], Marcel Camps[b], Maria Pontier[a], Anton Terwisscha van Scheltinga[c], Wim Jiskoot[a], Ferry Ossendorp[b], Joke Bouwstra[a]

[a] Division of Drug Delivery Technology, Cluster BioTherapeutics, Leiden Academic Centre for Drug Research (LACDR), Leiden University, 2300, RA, Leiden, The Netherlands
[b] Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre, P.O. Box 9600, 2300 RC, Leiden, The Netherlands
[c] Department of Clinical Pharmacy and Toxicology, Leiden University Medical Centre, Leiden, The Netherlands

ARTICLE INFO

Keywords:
Micro-injections
Hollow microneedles
Intradermal vaccination
Liposomes
Therapeutic cancer vaccine

ABSTRACT

Recent studies have shown that intradermal vaccination has great potential for T cell-mediated cancer immunotherapy. However, classical intradermal immunization with a hypodermic needle and syringe has several drawbacks. Therefore, in the present study a digitally controlled hollow microneedle injection system (DC-hMN-iSystem) with an ultra-low dead volume was developed to perform micro-injections (0.25–10 μL) into skin in an automated manner. A synthetic long peptide derived from human papilloma virus formulated in cationic liposomes, which was used as a therapeutic cancer vaccine, was administered intradermally by using the DC-hMN-iSystem. Fused silica hollow microneedles with an inner diameter of 50 μm and a bevel length of 66 ± 26 μm were successfully fabricated via hydrofluoric acid etching. Upon piercing these microneedles into the skin using a protrusion length of 400 μm, microneedles were inserted at a depth of 350 ± 55 μm. Micro-injections of 1–10 μL had an accuracy between 97 and 113% with a relative standard deviation (RSD) of 9%, and lower volumes (0.25 and 0.5 μL) had an accuracy of 86–103% with a RSD of 29% in ex vivo human skin. Intradermal administration of the therapeutic cancer vaccine via micro-injections induced strong functional cytotoxic and T-helper responses in mice, while requiring much lower volumes as compared to classical intradermal immunization. In conclusion, by using the newly developed DC-hMN-iSystem, very low vaccine volumes can be precisely injected into skin in an automated manner. Thereby, this system shows potential for minimally-invasive and potentially pain-free therapeutic cancer vaccination.

1. Introduction

The activation of the T-cells is necessary to elicit tumor destruction in therapeutic cancer vaccination [1–4]. Therapeutic cancer vaccination based on tumor specific peptides holds the potential for tailor-made immunotherapy [1,2]. The sequences of these peptides are based on tumor antigens and contain, ideally both, a cytotoxic ($CD8^+$) and a helper ($CD4^+$) T-cell epitope. The incorporation of both sequences into a synthetic long peptide (SLP) is a proven strategy to trigger a cellular immune response specifically directed towards the tumor. Dendritic cells are the main key in processing and presenting antigens to T-cells to provoke T-cell immune responses [5–7]. After in vivo administration of an SLP vaccine dendritic cells can internalize the SLPs, and subsequently process and present the cytotoxic and helper T-cell epitopes through major histocompatibility complexes (MHC) class I and class II molecules, respectively [1,2].

Intradermal vaccination has great potential to induce potent T-cell responses, because the skin contains large numbers of dendritic cells [5–7]. However, the standard intradermal immunization method, performed with a classical hypodermic needle and syringe (often referred to as the Mantoux method), is known for several impracticalities [8,9]. For example, due to the shallow injection depth (< 1.2 mm [10]) and low injection volumes (0.1 mL [10]), those injections are relatively difficult to perform and require specially trained medical personnel. Moreover, the accuracy and precision of these injections is low, because a vaccine formulation is often delivered too deep or leaks out of the skin [9]. A method to overcome these hurdles is by making use of a hollow microneedle system that is capable of accurately controlling the

* Corresponding author.
E-mail address: k.van.der.maaden@lacdr.leidenuniv.nl (K. van der Maaden).

https://doi.org/10.1016/j.jconrel.2017.11.035
Received 26 September 2017; Received in revised form 20 November 2017; Accepted 21 November 2017
Available online 22 November 2017

injection procedure (i.e., injection depth, injection volume, infusion speed, etc.) [11,12]. Microneedles are needle-like structures with dimensions of < 1 mm that are used to overcome the skin's physical barrier, the stratum corneum, in a minimally invasive and potentially pain-free manner [8,11–14].

In human papilloma virus (HPV) positive cancers, the oncogenic proteins, HPV16 E6 and E7, are targets for the T-cell repertoire. Hence, immunodominant sequences of these proteins can be synthesized in the form of SLPs and used as a therapeutic cancer vaccine. The administration of a mix of such SLPs derived from the E6 and E7 protein to patients with high-grade vulvar intraepithelial neoplasia resulted in complete regression of premalignant lesions in 47% of the patients [15]. Nevertheless, the SLP vaccine was not able to provoke a sufficient immune response to control end-stage HPV16 positive tumors [16]. Although the preferred route of T cell vaccination is intradermal, the mixes of 13 different SLPs emulsified in relative large volumes of Montanide (2.8 mL) were for practical reasons injected subcutaneously [15,16] (a standard intradermal injection is 0.1 mL [10]). Therefore, more effective vaccine formulations may be applied in smaller volumes via the intradermal route, which can result in further improved immunogenicity and effectiveness of tumor vaccines.

Another promising strategy to improve the immunogenicity of tumor antigens, and thereby improve tumor control, is to formulate them in liposomes [3,4,17–19]. Recently, it was shown that HPV $E7_{43-63}$ SLP (containing both the cytotoxic and helper T-cell epitope) formulated into cationic liposomes resulted in superior immune responses and tumor control as compared to the clinical “gold standard” vaccine formulation (SLP emulsified in Montanide ISA-51) in mice [3]. Moreover, it was shown that changing the administration route from subcutaneous to intradermal resulted in a complete regression of the tumor (100% of the mice were tumor-free), as compared to 38% tumor-free mice upon subcutaneous immunization [3]. Due to the improved therapeutic effect in combination with the impracticalities associated with intradermal injections, a hollow microneedle system to deliver our effective liposomal cancer vaccine formulations in low microliter volumes in an automated manner could be beneficial in therapeutic cancer vaccination.

Therefore, in this study a cationic liposomal HPV $E7_{43-63}$ SLP vaccine [3] was combined with a newly-developed digitally controlled hollow microneedle injection system (DC-hMN-iSystem). We show the accuracy and precision of micro-injections and the potential of a DC-hMN-iSystem to effectively induce antigen-specific T-cells upon delivery of a therapeutic cancer vaccine.

2. Materials and methods

2.1. Materials

For the preparation of buffers and solutions, Milli-Q (MQ) water with a resistivity of 18 MΩ cm was produced by using a Millipore water purification system. Polyimide-coated fused silica capillaries (375 μm outer diameter, 50 μm inner diameter) were obtained from Polymicro, Phoenix AZ, USA, and CapTite™ connectors were obtained from Labsmith, USA. Ammonium hydroxide was from Brocacef BV (Maarssen, the Netherlands) and Klinipath paraffin wax was purchased from VWR (Amsterdam, the Netherlands). Sulfuric acid 95–98% (w/v), hydrofluoric acid 49% (w/w), trifluoroacetic acid (TFA), lipopolysaccharide (LPS), and 96% ethanol were from Sigma-Aldrich (Zwijndrecht, the Netherlands). Chloroform, methanol, acetonitrile, and acetone were bought from Biosolve (Valkenswaard, the Netherlands). Sodium fluorescein was acquired from Merck Millipore (Amsterdam, the Netherlands). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, USA) and Vivaspin 2 membrane concentrators were acquired from Sartorius Stedim Biotech GmbH (Göttingen, Germany). IRDye 800CW-carboxylate was purchased from Li-cor (Lincoln, NE, USA). 10 mM phosphate buffer, pH 7.4 (PB) was composed of 7.7 mM Na_2HPO_4 (Honeywell Fluka) and 2.3 mM NaH_2PO_4 (Sigma-Aldrich) in MQ water which was sterile filtrated by using a 0.22-μm Millex GP PES-filter (Merck Millipore). Antibodies were purchased from eBiosciences (Landsmeer, the Netherlands) and BD biosciences (Breda, the Netherlands). Perm/Wash solution and brefeldin A were from BD biosciences. Bovine serum albumin (BSA) was obtained from Roche diagnostics (Almere, the Netherlands). HPV $E7_{43-63}$ SLP of HPV-16 protein (GQAEPDRAHYNIVTFCCKCDS), minimal HPV $E7_{49-57}$ peptide (RAHYNIVTF) ($H-2D^b$), and an influenza peptide (ASNENMDAM) ($H-2D^b$) were produced at Leiden University Medical Center (LUMC). IL-2 (Proleukin®) was from Novartis Pharma BV (LUMC pharmacy). Ketamine solution (10% w/v), xylazine solution (2% w/v), atropine solution (1% w/v), ficoll midotrizoate, and 1% sodium azide solution were obtained from the LUMC pharmacy. Iscove's modified Dulbecco's medium (IMDM) was obtained from Lonza Verniers (Verviers, Belgium), which was supplemented with 8% (v/v) fetal calf serum (FCS) (Greiner Bioscience (Alphen a/d Rijn, the Netherlands)), 50 μM 2-mercaptoethanol (Sigma-Aldrich), penicillin (100 IU/mL), and 2 mM glutamine from Life Technologies (Bleiswijk, the Netherlands). Phosphate buffered saline, pH 7.4 (PBS; composed of 140 mM NaCl, 8.7 mM Na_2HPO_4, 1.8 mM NaH_2HPO_4), was bought from B·Braun (Meslungen, Germany).

2.2. Mice

Female C57BL/6 ($H\text{-}2^b$) mice were obtained from Charles River (L'Arbresle, France) and were maintained under standardized conditions in the animal facility of LUMC. At the start of the immunization study mice were eight weeks old. The study was carried out under the guidelines as complied by the animal ethic committee of the Netherlands, and was approved by the “Dierexperimentencommissie LUMC (ADEC)” under number 1409.

2.3. Fabrication of hollow microneedles

Single hollow microneedles were fabricated from fused silica capillaries with an inner diameter of 50 μm, via a slightly different procedure as previously described [11,12]. Fused silica capillaries were first cut with a diamond cutter in pieces with a length of 80 cm (inner volume of 1.57 μL). To protect the inner lumen of the capillaries from the etchant, paraffin wax was degassed for two hours in a vacuum oven at 80 °C and was subsequently loaded into the first 2–4 cm (40–80 nL) of the lumen of the capillaries. After etching the capillaries into microneedles in 49% hydrofluoric acid for four hours, the polyimide coating at the microneedle tips was removed by incubating the first 2–3 mm of the etched capillaries in 98% sulfuric acid at 250 °C for one minute. To remove the paraffin from the lumen, the hollow microneedles were placed in a water bath at 80 °C while flushing them sequentially with 250 μL acetone and 250 μL ethanol. The fabricated hollow microneedles were imaged with a stereo microscope (Zeiss Stemi 2000-c) to investigate the reproducibility of fabrication (n = 8). The dimensions of the bevel (hypotenuse and length), the inner lumen, and the lumen diameter at the microneedle tip were determined by using the Zen 2 software (Carl Zeiss Microscopy GmbH).

2.4. Digitally controlled hollow microneedle injection system

To accurately perform micro-injections into the skin on an automated manner, a new DC-hMN-iSystem was designed (see Fig. 1). This system has two fewer connectors as compared to our previous system [12], resulting in an ultra-low dead volume of 1.6 μL from the syringe connector to the tip of the microneedle (instead of 16 μL). Furthermore, removal of the microneedle-capillary connector on the applicator circumvents the need to flush the system with 1–2 mL of formulation to

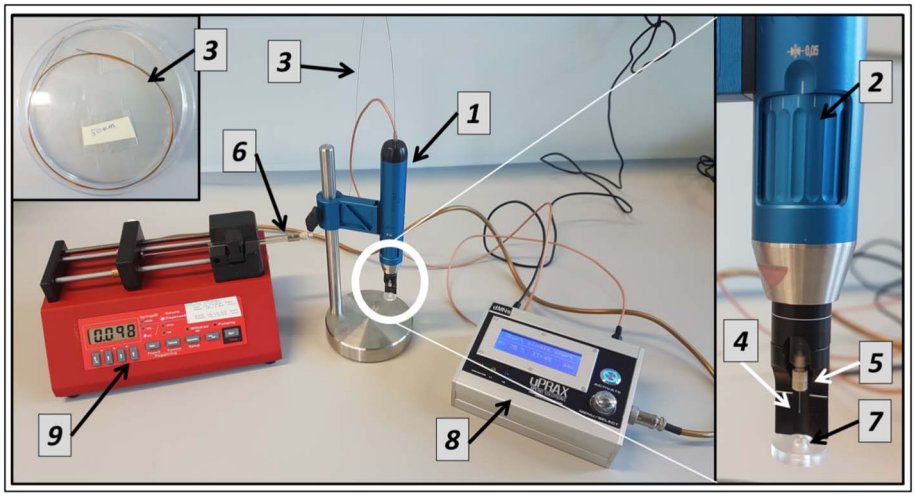

Fig. 1. Digitally controlled hollow microneedle injection system to perform automated micro-injections into skin at a predetermined depth. Microneedle applicator (1); micrometer actuator (2); capillary (3); hollow microneedle (4); CapTite™ connectors (5, 6); supporting plateau (7); applicator controller unit (8); syringe pump (9). See main text for explanation.

get the system air-free prior to use.

The DC-hMN-iSystem is composed of a microneedle applicator (Figs. 1) (1) that has a micrometer actuator to control the microneedle protrusion length in steps of 50 μm (2). In the applicator a capillary (3), from which one side is etched into a hollow microneedle (4), is locked by using a CapTite™ connector (5). The non-etched side of the capillary is connected to a 100 μL Hamilton syringe via CapTite™ connectors (6). When the applicator's solenoid is activated, the microneedle tip (4) will protrude through the supporting plateau (7). Furthermore, the system contains an applicator controller unit (8) (uPRAX, the Hague, the Netherlands) to simultaneously control the microneedle applicator and a syringe pomp (NE-300, Pump Systems Inc.). The electronics of the syringe pump were modified (9) to digitally control the micro-injections through the hollow microneedle via the applicator controller unit.

2.5. Digitally controlled micro-injections into skin

2.5.1. Microneedle insertion and micro-injections

To dispense a liquid drug formulation into the skin, a hollow microneedle was first pierced into the skin via impact-insertion. The elapsed time between the activation of the applicator's solenoid and the moment that the microneedle protruded through the supporting plateau ($t_{elapsed}$) was measured by using a microcontroller (Arduino Nano 3) and a piezoelectric sensor (Conrad Electronics). The average velocity by which a microneedle was pierced into the skin, was calculated by dividing the microneedle displacement by $t_{elapsed}$. The micrometer actuator was set at a protrusion length of 400 μm and the depth of microneedle insertion was determined by using fluorescein-labeled ex vivo human skin as previously described (n = 5) [11]. 150 milliseconds after the hollow microneedle was pierced into the skin, a controlled volume of drug formulation was injected into the skin through the bore of the microneedle. The volume was controlled by the flow rate (that was installed on the modified syringe pump, see Section 2.4) and the time that the syringe pump was pumping (which is controlled by the applicator controller unit). The microneedle was retained in the skin for 30 s after pumping, after which the microneedle was automatically retracted back into the applicator.

2.5.2. Validation of digitally controlled micro-injections

To evaluate the repeatability (precision) and accuracy of the digitally controlled micro-injections, volumes of 0.25, 0.5, 1, 5, and 10 μL of a solution of 1 mg/mL sodium fluorescein in PBS were dispensed into 500 μL PBS 10 times. All volumes were dispensed at a flow rate of 30 μL/min, except volumes of 0.25 μL, which were dispensed at a flow rate of 15 μL/min. The fluorescence of these samples as well as the fluorescence of a range of serially diluted standard solutions, containing 0.156–20 μg/mL sodium fluorescein in PBS, was measured on a Tecan Infinite M1000 plate reader ($\lambda_{exitation}$ = 494 nm; $\lambda_{emission}$ = 512 nm). The accuracy of the digitally controlled micro-injections was calculated as the relative deviation (expressed as percentage) from the corresponding amount of fluorescein from the calibration curve. Precision was defined as the relative standard deviation (%) of the average amount of sodium fluorescein of the repeated micro-injections (n = 10).

2.5.3. Micro-injections into ex vivo human skin

The repeatability and accuracy of the digitally controlled micro-injections was evaluated in full thickness abdomen ex vivo human skin (obtained from local hospitals after informed consent from the donors and handled according to the Declaration of Helsinki Principles). Micro-injections of 0.25, 0.5, 1, 5, and 10 μL of a 0.1 μg/μL IRDye 800CW-carboxylate solution in PBS were performed, whereby each volume was injected 10 times. As a control, 15 intradermal injections with 1 μg IRDye 800CW-carboxylate in 50 μL PBS were performed by using a conventional 30G needle and syringe. Furthermore, for each volume that was injected via the DC-hMN-iSystem, the corresponding amount of IRDye 800CW-carboxylate was injected by using a 30G hypodermic needle in a volume of 50 μL PBS as a control. The fluorescence of the skin was measured by using an IVIS® lumina II by using an ICG excitation filter (wavelength of 710–760 nm) and an ICG emission filter (wavelength of 810–875 nm) with an exposure time of 1 s. The fluorescence data was analyzed by using Living Image® software (version 4.3.1.0.15880). The fluorescence intensity (*Total Radiant Efficiency*: ([photons/s] / [μW/cm^2])) of the intradermal injections (either via the DC-hMN-iSystem or hypodermic needles) was normalized, as shown in equation 1.

$$\text{Normalized fluorescence} = (Fluo_{Skin} - Fluo_{Bcg})/(Fluo_{ID\text{-}1\mu g} - Fluo_{Bcg}) * 100\% \quad \text{(Eq. 1)}$$

Where $Fluo_{Skin}$ is the fluorescence of micro-injected skin, $Fluo_{Bcg}$ is the background fluorescence of non-injected skin, and $Fluo_{ID\text{-}1\mu g}$ is the fluorescence intensity of skin injected with 1 μg IRDye 800CW-carboxylate in 50 μL by using a conventional 30G needle.

2.6. Preparation of HPV-peptide-containing liposomes

HPV $E7_{43–63}$ SLP-containing liposomes were prepared by using a thin film dehydration-rehydration method followed by extrusion [3,4]. First, a lipid film was prepared in a round bottomed flask from a DOTAP:DOPC mixture (1:1 M ratio) in chloroform by rotary evaporation. To hydrate the lipid film, 1 mg/mL HPV $E7_{43–63}$ SLP in 0.04% (w/v) ammonium hydroxide was added to the round bottomed flask until a lipid concentration of 10 mg/mL was reached. Next, the hydrated lipid

film was snap-frozen in liquid nitrogen and freeze-dried overnight in a Christ alpha 1–2 freeze-dryer (Osterode, Germany). The resulting lipid cake was rehydrated stepwise with PB to reach a lipid concentration of 10 mg/mL as follows: two times 25% of the end volume PB were added successively to the lipid cake (with 30 min intervals) after which the resulting 50% of PB was added (the formulation was then stabilized for 1 h). After each rehydration step the mixture was vortexed well.

The liposomes were down-sized by applying high-pressure extrusion (Lipex extruder) through polycarbonate filters (Nucleopore Milipore, Kent, UK) with pore sizes of 400 and 200 nm (four extrusion cycles through each filter).

To remove non-encapsulated HPV $E7_{43–63}$ SLP from the liposomal formulation, Vivaspin 2 centrifugation concentrators (molecular-weight-cut-off: 300 kDa) were used to concentrate the liposomal formulation (3–4 fold) by centrifugation at 931 G (2000 RPM) for one hour and subsequently dilute the formulation with PB to reach the initial volume. The concentration and dilution steps were performed twice, which was followed by a final concentration step to ca. 3–4 fold.

2.7. Characterization of the liposomal formulation

2.7.1. Dynamic light scattering

The particle size and size distribution (polydispersity index (PDI)) of the liposomal formulation was determined by using dynamic light scattering and the ζ-potential by using laser Doppler electrophoresis on a Zetasizer Nano (Malvern Instruments). For these measurements 2.5 μL of the liposomal formulation was diluted 400 fold in PB.

2.7.2. Extraction of HPV $E7_{43–63}$ SLP from liposomes

In order to determine the concentration of HPV $E7_{43–63}$ SLP in the liposomal formulation, the SLP was first extracted from the liposomes by using a modified Bligh and Dyer extraction, as described below. Three μL of the liposomal formulation was diluted in 100 μL MQ water. Subsequently, 250 μL methanol and 125 μL chloroform were added and the mixture was vortexed. To this mixture 250 μL 0.1 M HCl and 125 μL chloroform were added after which it was vortexed again. Finally, the mixture was centrifuged at 233 G (1000 RPM) for 5 min and the upper phase was collected for quantification of the HPV $E7_{43–63}$ SLP.

2.7.3. Reversed-phase UPLC analysis

For the quantification of HPV $E7_{43–63}$ SLP, a UPLC system (Waters Acquity UPLC® with a Waters C18–1.7 μm (2.1 × 50 mm) column) was set at a flow rate of 0.370 mL/min, with initially 95% solvent A (ACN with 0.1% TFA) and 5% solvent B (MQ with 0.1% TFA), going to 79% solvent B in 3.97 min and back to 5% solvent B after 3.99 min. Serially diluted standards of HPV $E7_{43–63}$ SLP, ranging from 1.56 μg/mL − 50 μg/mL, and HPV $E7_{43–63}$ SLP extracted from liposomes (5 μL/sample) were injected into the UPLC system, after which the absorbance was measured for 6.00 min at a wavelength of 214 nm. Next, the area under the curve of the standards and extracts of the HPV $E7_{43–63}$ SLP-containing samples, having a retention time of 2.4 min, were integrated by using MassLynx (Waters, software version 4.1).

2.8. Immunizations

Prior to each microneedle-based immunization, mice were anesthetized with 100 mg/kg ketamine, 10 mg/kg xylazine, and 0.2 mg/kg atropine. The skin of anesthetized mice on the lower back at the tail base was shaved. Mice (5 per group) were immunized twice with intervals of two weeks (prime: day 1; boost: day 15) either by intradermal injection using a 30G hypodermic needle or by using the DC-hMN-iSystem. Mice that were immunized by using a 30G hypodermic needle either received 30 μL PB (mock treated) or 1 nmol (2.3 μg) HPV $E7_{43–63}$ SLP in liposomes in a volume of 30 μL. Mice that were immunized by using the DC-hMN-iSystem received 1 nmol HPV $E7_{43–63}$ SLP in liposomes via a single micro-injection of 5 μL. Blood samples were obtained from the tail vein at day 10 and at day 22. Mice were sacrificed at day 24 by CO_2 gas after which the spleens and inguinal lymph nodes were collected.

2.9. Analysis of antigen-specific T-cell responses by flow cytometry

2.9.1. Antigen-specific $CD8^+$ responses

The presence of E7-specific $CD8^+$ T-cells was assessed in blood after prime- and boost immunization, and was determined in spleens and inguinal lymph nodes. To analyze HPV E7-specific $CD8^+$ T-cells in blood, red blood cells were removed by lysis prior to staining. Blood cells, splenocytes and cells isolated from inguinal lymph nodes were stained with fluorescently-labeled (APC) MHC class I-specific tetramer $H{-}2D^b$ $E7_{49–57}$ (TM-RAHYNIVTF) for 30 min in FACS buffer (PBS + 0.1% (w/v) BSA + 0.05% (w/v) sodium azide). Next, cells were incubated with anti CD8α-PE antibody for 20 min after which the cells were washed twice with FACS buffer. Data acquisition was done with a BD LSR II flow cytometer and the data was analyzed with Flowjo software V10 (Tree star).

2.9.2. Restimulation of splenocytes to analyze antigen-specific $CD4^+$ and $CD8^+$ responses

The induction of antigen-specific $CD4^+$ and $CD8^+$ T-cell responses was assessed after the mice were sacrificed. To this end, splenocytes were incubated with dendritic cells (D1 cells) supplemented with HPV $E7_{43–63}$ SLP. Next, the splenocytes were harvested and separated on a ficoll midotrizoate gradient (the living splenocytes were collected from the interphase). Subsequently, the splenocytes were split over 3 wells and were incubated with IL-2. Next, the lymphocytes were restimulated by LPS-matured autologous dendritic cells loaded with specific HPV E7, or control (influenza NP) or minimum (MHC class I) HPV $E7_{49–57}$ peptide. Finally, the cells were incubated for 5 h with brefeldin A (2 μg/mL) and were stained for intracellular cytokines and extracellular proteins. During the whole procedure, cells were incubated in supplemented IMDM in an incubator at 37 °C and 5% CO_2.

2.9.3. Intracellular staining

The antigen-specific $CD4^+$ and $CD8^+$ T-cell responses were determined by using an intracellular cytokine staining (ICS) on the restimulated splenocytes. Cell surfaces were stained with fluorescently labeled antibodies CD3-*Pe-Cy7*, CD4-*Qdot605*, and CD8α-*Alexa Fluor® 700* in FACS buffer. Next, cells were fixed in 1% paraformaldehyde (w/v) for 30 min and permeabilized with Perm/Wash solution. ICS was performed with TNFα-*FITC*, IFNγ-*APC* and IL-2-*PE* antibodies for 30 min at 4 °C. Finally, data was acquired by using a BD LSR II flow cytometer and analyzed in Flowjo V10.

2.10. Statistical analysis

Statistical analysis was performed by using GraphPad Prism 7 for Windows. Data is represented as mean ± SD unless stated otherwise. Statistical significance of antigen-specific cytotoxic T-cells was determined by using an ordinary two-way ANOVA with a Tukey's multiple comparisons test (blood) and a one-way ANOVA with a Dunn's multiple comparisons test (spleen and lymph nodes). The statistical significance of antigen-specific T-cells (restimulated splenocytes) was determined by using a two-way repeated measurements ANOVA with a Tukey's multiple comparisons test. Statistical significance is presented as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3. Results

3.1. Characterization of the hollow microneedles

After fabricating hollow microneedles from fused silica capillaries with an inner lumen diameter of 50 μm, the microneedle dimensions of

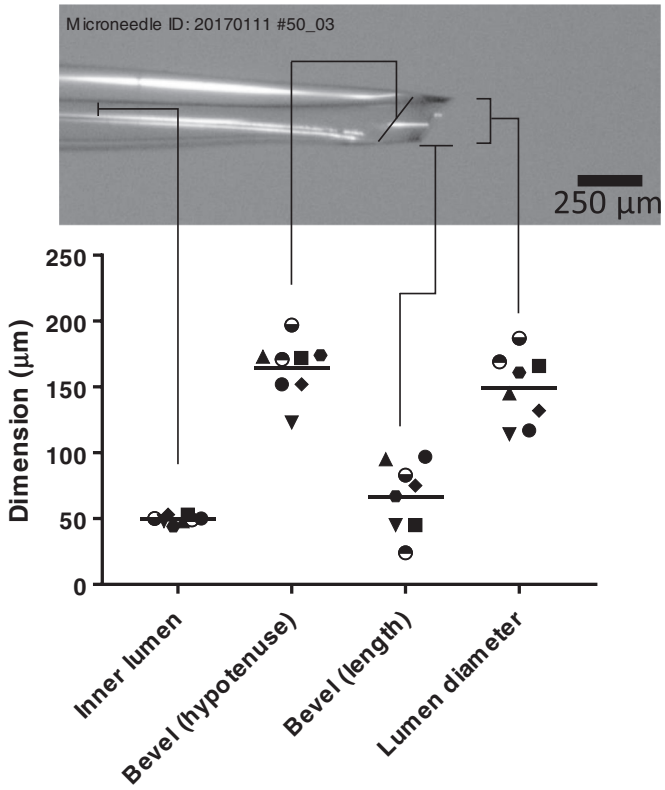

Fig. 2. Representative image of a hollow microneedle produced from a fused silica capillary with an inner lumen diameter of 50 µm (upper image). Dimensions of geometry parameters of 8 individual hollow microneedles, represented by a unique symbol (lower image). The bar represents the geometric mean.

each individual microneedle were measured, as shown in Fig. 2. The inner lumen was 49 ± 3 µm (n = 8), the lumen diameter at the microneedle tip was 149 ± 26 µm (n = 8), and the hypotenuse of the microneedle bevel was 164 ± 22 µm (n = 8). The bevel length of the fabricated hollow microneedles was 66 ± 26 µm (n = 8) and ranged from 24 to 97 µm.

3.2. Validation of digitally controlled micro-injections

The accuracy and repeatability of the digitally controlled micro-injections was determined by injecting a fluorescein solution into PBS. As shown in Fig. 3A, the fluorescence of the digitally controlled micro-injections was very comparable to that of the standard solutions at the corresponding concentration and exhibited a low relative standard deviation (RSD = 1.68%). Fig. 3B shows that the accuracy of digitally controlled micro-injections of 1–10 µL is between 90 and 95% with a RSD of approximately 5%. Volumes of 0.5 and 0.25 µL had a lower accuracy of 86% and 74%, respectively, with a higher RSD (15% and 20%, respectively).

3.3. Digitally controlled micro-injections into human skin

Single hollow microneedles were inserted into the skin by impact insertion at an average velocity of 35 ± 1 cm/s (n = 10) and a depth of 350 ± 55 µm (ranging from 257 to 392 µm). After performing micro-injections no visible breakage or alteration of the geometry of the hollow microneedles was observed. To reduce the chance of leakage while performing micro-injections into skin, the applicator controller unit was programmed in such a way that a micro-injection started 150 milliseconds after a hollow microneedle pierced into the skin. Additionally, to reduce the chance of leakage from the site of injection, the applicator controller unit was programmed to retain the hollow microneedle in the skin for 30 s after a micro-injection was performed.

To determine the repeatability of the digitally controlled micro-injections into skin, volumes ranging from 0.25 µL to 10 µL of a 0.1 µg/mL IRDye 800CW carboxylate solution were injected into ex vivo human skin via the DC-hMN-iSystem. As shown in Fig. 4, an injection of 1 µg IRDye 800CW carboxylate into ex vivo human skin in a volume of 50 µL by using a classical 30G hypodermic needle, or in a volume of 10 µL by using the DC-hMN-iSystem resulted in a similar relative fluorescence and standard deviation (100 ± 10.2% (n = 15) and 97 ± 9.8% (n = 10), respectively). Moreover, intradermal injections of lower volumes than 50 µL using a 30G needle and syringe had a decreased accuracy and repeatability as compared to micro-injections of 1 µL and higher. Furthermore, the normalized fluorescence of skin injected with 25 ng – 1 µg of IRDye 800CW carboxylate via a micro-injection of 0.25–10 µL was comparable to that of skin injected with 50 µL containing the same amount of dye by using a 30G hypodermic needle. This data shows that the intradermally delivered (drug) dose injected via the DC-hMN-iSystem or via a classical 30G hypodermic needle and syringe is similarly controllable, but much lower volumes were used in case of the micro-injections performed using the DC-hMN-iSystem.

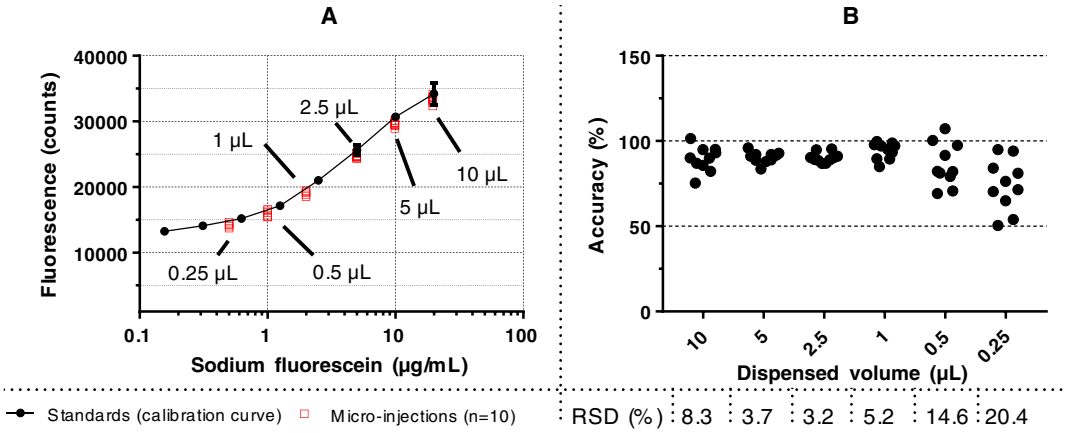

Fig. 3. Digitally controlled micro-injections. Different digitally controlled volumes of a 1 mg/mL sodium fluorescein solution, ranging from 0.25–10 µL, were injected into 500 µL PBS and the fluorescence of the resulting solution was plotted against the corresponding fluorescein concentration. As a control, the fluorescence of fluorescein solutions with a known concentration was determined (n = 2) (A). The accuracy and precision of different digitally controlled micro-injections (B). Each digitally controlled volume was injected 10 times in 500 µL PBS. Abbreviation: relative standard deviation (RSD).

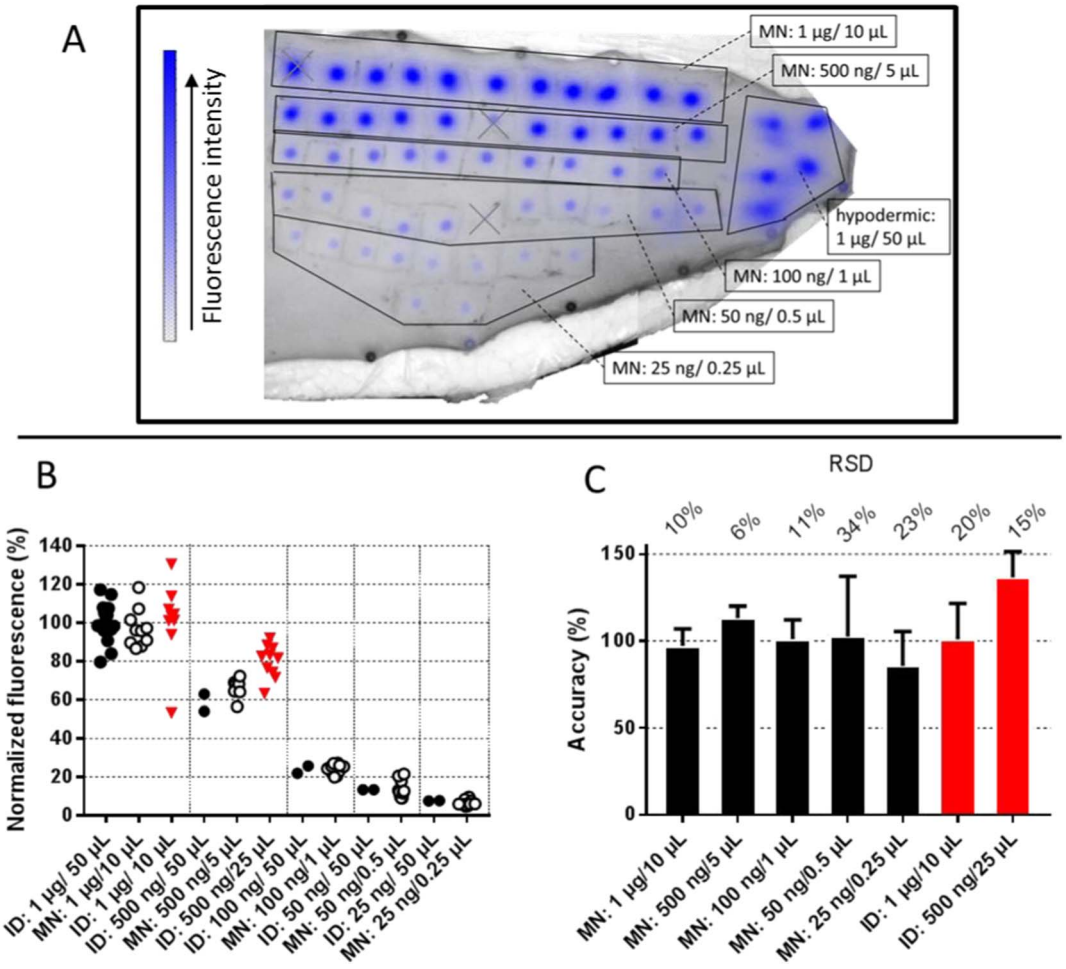

Fig. 4. Fluorescence imaging of ex vivo human skin into which a near infrared fluorescent dye (IRDye 800CW carboxylate) was delivered by performing micro-injections (0.25–10 µL) via the digitally controlled hollow microneedle injection system (MN) or injecting 50 µL using a classical 30G hypodermic needle and syringe (ID) (A). Normalized fluorescence of MN (open circles), and ID delivery of different amounts of IRdye800 (closed circles) and different volumes (triangles) (B). The accuracy and corresponding relative standard deviation (RSD) of micro-injections (black) and ID injections (red) (n = 10) into skin (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Characterization of the liposomal formulation

As a therapeutic cancer vaccine HPV $E7_{43–63}$ SLP was formulated into cationic liposomes. These liposomes had a hydrodynamic diameter of 191 ± 3 nm (n = 3), a PDI of 0.22 ± 0.01 (n = 3) with a ζ-potential of 26.7 ± 1.3 mV (n = 3). The HPV $E7_{43–63}$ SLP encapsulation efficiency was 40% and the formulation contained 2.3 µg (1 nmol) $E7_{43–63}$ SLP/µL liposome dispersion after the non-encapsulated peptide was removed. For the immunization of mice, the liposomal formulation was diluted 5-fold with PB to deliver 1 nmol $E7_{43–63}$ SLP in 5 µL via a micro-injection using the DC-hMN-iSystem and 30-fold to deliver the same amount in 30 µL PB by using a classical hypodermic needle and syringe.

3.5. Immunization study

The potential of the DC-hMN-iSystem for minimally-invasive and potentially pain-free vaccination was assessed in mice. As shown in Fig. 5, mock immunization with PB did not lead to the induction of antigen-specific cytotoxic T-cells in blood and spleen. In contrast, intradermally administering HPV $E7_{43–63}$ SLP liposomes led to high and comparable induction of antigen-specific cytotoxic T-cells in blood (Fig. 5A). Immunization by performing micro-injections with the DC-hMN-iSystem resulted in 3/5 mice having higher percentages of antigen-specific cytotoxic T-cells in spleens as compared to the highest responding mouse in the group immunized via a hypodermic needle and syringe (Fig. 5B). Albeit micro-injections did not result in statistically significant higher antigen-specific cytotoxic T-cells, delivery via the DC-hMN-iSystem led to strong antigen-specific cytotoxic T-cells in spleens which were at least similar to the ones obtained by intradermal injections using a classical needle and syringe.

Additionally, antigen-specific T-cell responses were determined by quantifying $IFN\gamma^+TNF\alpha^+$-producing $CD4^+$ and $CD8^+$ (restimulated) T-cells derived from the spleens of immunized mice. Similar to the splenic cytotoxic T-cell responses measured ex vivo (Fig. 5), higher (but not statistically significant) antigen-specific ($IFN\gamma^+TNF\alpha^+$) $CD8^+$ T-cell responses were observed when the liposomes were administered via a micro-injection as compared to a hypodermic needle (data not shown). As shown in Fig. 5C, restimulation with the $E7_{43–63}$ SLP (containing both a cytotoxic T-cell ($CD8^+$) sequence (RAHYNIVTF) and a T helper ($CD4^+$) sequence (DRAHYNIVTF) [3]), revealed that immunization via a micro-injection using the DC-hMN-iSystem led to significantly higher percentages of antigen-specific $IFN\gamma^+TNF\alpha^+$-producing $CD4^+$ T-cells as compared to intradermal immunization by using a conventional needle and syringe (3-fold higher). No $IFN\gamma^+TNF\alpha^+$-producing T-cells were observed when splenocytes were restimulated with the cytotoxic T-cell sequence or control peptide, nor when splenocytes were derived from mock immunized mice. Overall, these data show that microneedle-based immunization leads to effective cytotoxic T-cell and helper T-cell response induction.

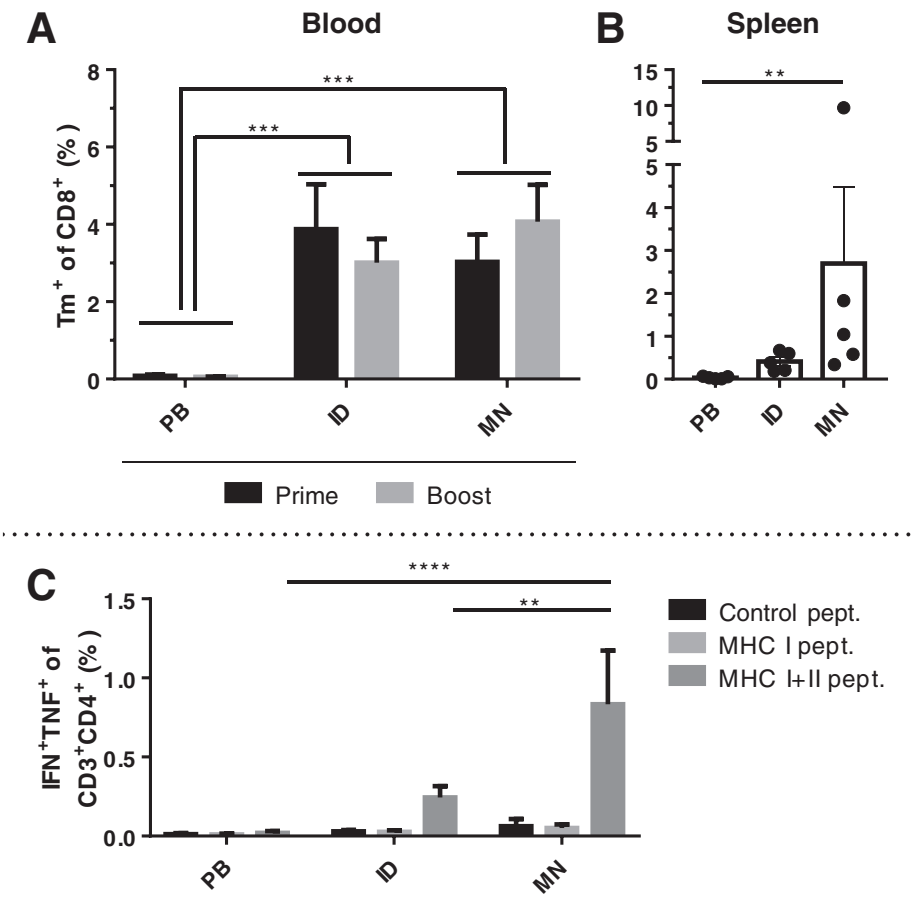

Fig. 5. Ex vivo HPV E7-specific $CD8^+$ T-cell responses in blood (after prime (day 10) and boost (day 22) immunization) (A) and in spleens (day 24) (B) and, HPV E7-specific $CD4^+$ T cell responses in spleens (C). Mice were intradermally injected with 30 μL phosphate buffer (PB), or with 1 nmol HPV $E7_{43–63}$ SLP in liposomes (in 30 μL PB) via a 30G hypodermic needle (ID) or with the same dose (in 5 μL PB) by using the digitally controlled hollow microneedle injection system (MN). RAHYNIVTF-specific $CD8^+$ T-cells were detected by using flow cytometry and using E7-specific MHC class I tetramers (Tm^+). Antigen-specific $CD3^+CD4^+$ T-cells responses were determined upon restimulating splenocytes with a control peptide, a MHC-I binding minimal peptide sequence and with a synthetic long peptide (SLP) harboring a cytotoxic and T-helper sequence. Interferon-γ (IFN) and tumor necrosis factor-α (TNF) positive restimulated $CD3^+CD4^+$ splenocytes are shown. Data is represented as mean + SEM, n = 5.

4. Discussion

In this study a new DC-hMN-iSystem was developed to accurately and precisely deliver vaccines, in particular a liposomal therapeutic cancer vaccine, into the skin. The new DC-hMN-iSystem was designed to minimize the loss of formulation while performing micro-injections in an automated manner.

Fabricating hollow microneedles from fused silica capillaries by hydrofluoric acid etching is an easy, cheap and scalable method to obtain hollow microneedles [11]. The length of the microneedle bevel is an important parameter, since it determines the minimal length that a hollow microneedle needs to be inserted into the skin without leakage. The maximum bevel length of the fabricated hollow microneedles was 97 μm, which indicates that the minimum insertion depth of these hollow microneedles to achieve a leakage-free injection is 100 μm. In a previous study it was shown that the depth of intradermal vaccination (ranging from 50 to 550 μm) with an inactivated polio vaccine (particles with a diameter of 30–35 nm) had no effect on the immune response in rats [12]. In this study a protrusion length of 400 μm was chosen, because using this length results in hollow microneedles to pierce at least 260 μm into the skin. The latter in combination with the maximum bevel length of 100 μm should lead to leakage-free injections and performing injections at a depth of least 160 μm.

By using the applicator controller unit, digitally controlled volumes as low as 1 μL were shown to be accurately and precisely injected by the DC-hMN-iSystem. The accuracy and repeatability of the micro-injections of ultra-low volumes (0.25 μL and 0.5 μL) was decreased as compared to the higher volumes (1 μL–10 μL). This may be caused by the stepper motor driver/controller in the syringe pump that regulates the acceleration and deceleration that is required to start and stop the stepper motor in a smooth way [20]. The acceleration and deceleration may have a large influence at short pumping times, such as the 1.00 s that was used to dispense volumes of 0.25 μL and 0.5 μL. Using a lower flow rate in combination with a longer pumping time may help to increase the precision and accuracy of these ultra-low volume digitally controlled micro-injections.

Our study shows that the (vaccine) dose that is intradermally delivered via micro-injections of 1–10 μL is similarly controllable as the delivery via a classical hypodermic needle and syringe (in 50 μL). However, the DC-hMN-iSystem has the advantage that very low volumes can be injected into the skin at low infusion rates, resulting in less pain during administration as compared to intradermal injections via a classical hypodermic needle and syringe [8]. Furthermore, the depth of micro-injections is more controlled [11,12] as compared to intradermal injections via a classical hypodermic needle and syringe. Besides, in previous studies no visual disruption of skin was observed when micro-injections were performed using similar injection depths, volumes and infusion rates [11,12].

Recently, it was shown that HPV $E7_{43–63}$ SLP formulated into cationic liposomes were able to induce high cellular (CD4 and CD8) immune responses at doses as low as 1 nmol (2.3 μg). In contrast, a 65 fold higher dose of the SLP (150 μg) emulsified in Montanide ISA-51 was required to obtain a similar delay on the tumor outgrowth in mice using the same administration route (subcutaneous at the tail-base). Moreover, it was shown that changing the administration route from subcutaneous to intradermal resulted in a superior tumor control [3]. Such low vaccine doses open possibilities to deliver SLP-based therapeutic cancer vaccine via microneedles.

Although relatively little is known about the intradermal administration of liposomal vaccines via microneedles, a few studies have shown that the delivery of liposomal and other nanoparticulate vaccines by means of microneedles can result in an efficient induction of

immune responses [21–29]. In these studies the liposomal vaccine was delivered by using dissolving microneedles (hepatitis B surface antigen (HBsAg) [21] and ovalbumin [22]), liposome-coated microneedles (ovalbumin [25]), and skin pretreatment via microneedles (HBsAg [23], ovalbumin [26,27], and diphtheria toxoid [28]). Besides, very recently a study was published wherein different protein-containing nanoparticles, including liposomes, were administrated by using hollow microneedles [29]. However, to our knowledge this is the first study reporting the microneedle-mediated delivery of a peptide-based liposomal therapeutic cancer vaccine.

In this study, a cancer-specific peptide was formulated in cationic liposomes with characteristics very similar to those of the previously described ones [3]. Upon intradermal administration of the liposomal formulation via micro-injections, high antigen-specific T-cell responses were induced in blood and spleen, which were in some cases higher than the responses obtained by using a classical hypodermic needle and syringe. This may be the result of more superficial delivery by micro-injections or having locally higher vaccine concentrations as compared to the injections performed by using a classical hypodermic needle and syringe.

Summarizing, our studies demonstrate that automated intradermal micro-injections can be easily performed with the newly developed DC-hMN-iSystem. Besides an accurate and precise dosing, the depth of vaccine delivery is controlled. Moreover, by using the DC-hMN-iSystem to inject very low volumes of a liposomal therapeutic cancer vaccine leads to strong functional T-cell (both CD4 and CD8) responses in mice. Therefore, this study shows the potential of a DC-hMN-iSystem for minimally invasive and potentially pain-free induction of cytotoxic and helper T-cells for therapeutic cancer vaccination.

5. Conclusion

In this study we presented a newly developed DC-hMN-iSystem to accurately and precisely perform micro-injections through a hollow microneedle into the skin in an automated manner. This system was designed to minimize the loss of expensive formulations, such as vaccines, by designing the fluidics to have an ultra-low dead volume, and preventing leakage by delaying the micro-injection after microneedle insertion and retaining the microneedle in the skin after a micro-injection has been performed. Moreover, using this system to intradermally deliver a liposomal HPV tumor vaccine led to strong functional cytotoxic and helper T-cell responses in mice, but in much lower volumes as compared to intradermal vaccination using a classical needle and syringe. In conclusion, this study shows the potential of a DC-hMN-iSystem for easy-to-perform, minimally-invasive and potentially pain-free cancer vaccination.

Funding

JH was funded by the Molecule to Patient program of the Translational Drug Discovery and Development (TDDD) profiling area of University Leiden, The Netherlands.

Conflict of interest

KvdM is co-founder of uPRAX Microsolutions. Other authors declare that they have no conflict of interest.

References

[1] C.J. Melief, S.H. van der Burg, Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines, Nat. Rev. Cancer 8 (2008) 351–360.
[2] S.H. van der Burg, R. Arens, F. Ossendorp, T. van Hall, C.J. Melief, Vaccines for established cancer: overcoming the challenges posed by immune evasion, Nat. Rev. Cancer 16 (2016) 219–233.
[3] E.M. Varypataki, N. Benne, J. Bouwstra, W. Jiskoot, F. Ossendorp, Efficient eradication of established tumors in mice with cationic liposome-based synthetic long-peptide vaccines, Cancer Immunol. Res. 5 (2017) 222–233.
[4] E.M. Varypataki, K. van der Maaden, J. Bouwstra, F. Ossendorp, 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. 17 (2015) 216–226.
[5] N. Romani, V. Flacher, C.H. Tripp, F. Sparber, S. Ebner, P. Stoitzner, Targeting skin dendritic cells to improve intradermal vaccination, Curr. Top. Microbiol. Immunol. 351 (2012) 113–138.
[6] C. Levin, H. Perrin, B. Combadiere, Tailored immunity by skin antigen-presenting cells, Hum. Vaccin. Immunother. 11 (2015) 27–36.
[7] B. Combadiere, C. Liard, Transcutaneous and intradermal vaccination, Hum. Vaccin. 7 (2011) 811–827.
[8] J. Gupta, S.S. Park, B. Bondy, E.I. Felner, M.R. Prausnitz, Infusion pressure and pain during microneedle injection into skin of human subjects, Biomaterials 32 (2011) 6823–6831.
[9] Y. Levin, E. Kochba, I. Hung, R. Kenney, Intradermal vaccination using the novel microneedle device MicronJet600: past, present, and future, Hum. Vaccin. Immunother. 11 (2015) 991–997.
[10] Skin Testing for Tuberculosis, in: C.C. Dacso, H.K. Walker, W.D. Hall, J.W. Hurst (Eds.), Clinical Methods: The History, Physical, and Laboratory Examinations, 1990 (Boston).
[11] K. van der Maaden, B. Trietsch, H. Kraan, E.M. Varypataki, S. Romeijn, R. Zwier, H. van der Linden, G. Kersten, T. Hankemeier, W. Jiskoot, J. Bouwstra, Novel hollow microneedle technology for depth controlled microinjection-mediated dermal vaccination: a study with polio vaccine in rats, Pharm. Res. 31 (2014) 1846–1854.
[12] P. Schipper, K. van der Maaden, S. Romeijn, C. Oomens, G. Kersten, W. Jiskoot, J. Bouwstra, Determination of depth-dependent intradermal immunogenicity of Adjuvanted inactivated polio vaccine delivered by microinjections via hollow microneedles, Pharm. Res. 33 (2016) 2269–2279.
[13] K. van der Maaden, W. Jiskoot, J. Bouwstra, Microneedle technologies for (trans) dermal drug and vaccine delivery, J. Control. Release 161 (2012) 645–655.
[14] H.S. Gill, D.D. Denson, B.A. Burris, M.R. Prausnitz, Effect of microneedle design on pain in human volunteers, Clin. J. Pain 24 (2008) 585–594.
[15] G.G. Kenter, 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, C.J. Melief, Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia, N. Engl. J. Med. 361 (2009) 1838–1847.
[16] G.G. Kenter, 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, 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, Clin. Cancer Res. 14 (2008) 169–177.
[17] J. Hansen, T. Lindenstrom, J. Lindberg-Levin, C. Aagaard, P. Andersen, E.M. Agger, CAF05: cationic liposomes that incorporate synthetic cord factor and poly(I:C) induce CTL immunity and reduce tumor burden in mice, Cancer Immunol. Immunother. 61 (2012) 893–903.
[18] K.S. Korsholm, J. Hansen, K. Karlsen, J. Filskov, M. Mikkelsen, T. Lindenstrom, S.T. Schmidt, P. Andersen, D. Christensen, Induction of CD8 + T-cell responses against subunit antigens by the novel cationic liposomal CAF09 adjuvant, Vaccine 32 (2014) 3927–3935.
[19] K. Zaks, M. Jordan, A. Guth, K. Sellins, R. Kedl, A. Izzo, C. Bosio, S. Dow, Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes, J. Immunol. 176 (2006) 7335–7345.
[20] ATMEL, AVR446: Linear Speed Control of Stepper Motor, (2006), pp. 1–15.
[21] T. Wang, Y. Zhen, X. Ma, B. Wei, S. Li, N. Wang, Mannosylated and lipid A-incorporating cationic liposomes constituting microneedle arrays as an effective oral mucosal HBV vaccine applicable in the controlled temperature chain, Colloids Surf. B: Biointerfaces 126 (2015) 520–530.
[22] L. Guo, J. Chen, Y. Qiu, S. Zhang, B. Xu, Y. Gao, Enhanced transcutaneous immunization via dissolving microneedle array loaded with liposome encapsulated antigen and adjuvant, Int. J. Pharm. 447 (2013) 22–30.
[23] H. Hirschberg, S. van Kuijk, J. Loch, W. Jiskoot, J. Bouwstra, G. Kersten, J.P. Amorij, A combined approach of vesicle formulations and microneedle arrays for transcutaneous immunization against hepatitis B virus, European journal of pharmaceutical sciences: official journal of the European Federation for Pharmaceutical, Science 46 (2012) 1–7.
[24] J. Tu, G. Du, M. Reza Nejadnik, J. Monkare, K. van der Maaden, P.H.H. Bomans, N. Sommerdijk, B. Slutter, W. Jiskoot, J.A. Bouwstra, A. Kros, Mesoporous silica nanoparticle-coated microneedle arrays for intradermal antigen delivery, Pharm. Res. 34 (2017) 1693–1706.
[25] P.C. DeMuth, J.J. Moon, H. Suh, P.T. Hammond, D.J. Irvine, Releasable layer-by-layer assembly of stabilized lipid nanocapsules on microneedles for enhanced transcutaneous vaccine delivery, ACS Nano 6 (2012) 8041–8051.
[26] B. Slutter, S.M. Bal, Z. Ding, W. Jiskoot, J.A. Bouwstra, Adjuvant effect of cationic liposomes and CpG depends on administration route, J. Control. Release 154 (2011) 123–130.
[27] S.M. Bal, S. Hortensius, Z. Ding, W. Jiskoot, J.A. Bouwstra, Co-encapsulation of antigen and toll-like receptor ligand in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination, Vaccine 29 (2011) 1045–1052.
[28] Z. Ding, S.M. Bal, S. Romeijn, G.F. Kersten, W. Jiskoot, J.A. Bouwstra, Transcutaneous immunization studies in mice using diphtheria toxoid-loaded vesicle formulations and a microneedle array, Pharm. Res. 28 (2011) 145–158.
[29] G. Du, R.M. Hathout, M. Nasr, M.R. Nejadnik, J. Tu, R.I. Koning, A.J. Koster, B. Slutter, A. Kros, W. Jiskoot, J.A. Bouwstra, J. Monkare, Intradermal vaccination with hollow microneedles: a comparative study of various protein antigen and adjuvant encapsulated nanoparticles, J. Control. Release 266 (2017) 109–118.