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Research paper

GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial

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ABSTRACT

For the treatment of melanoma DNA vaccines are a promising therapeutic approach. In our institute a plasmid encoding a melanoma-associated epitope (MART-1) and an immunostimulatory sequence (tetanus toxin fragment-c) termed pDERMATT was developed. In a phase I study the plasmid will be administered intradermally using a newly developed tattoo strategy to assess the toxicity and efficacy of inducing tumor-specific T-cell immunity. To facilitate this study a Good Manufacturing Practice (GMP)-compliant plasmid manufacturing process was set up and a pharmaceutical dosage form was developed. Each batch resulted in approximately 200 mg plasmid DNA of a high purity >90% supercoiled DNA, an A260/280 ratio 1.80–1.95, undetectable or extremely low residual endotoxins, *Escherichia coli* host cell protein, RNA, and DNA. In the manufacturing process no animal derived enzymes like RNase or potentially harmful organic solvents are used. After sterile filtration the concentration of the plasmid solution is approximately 1.1 mg/mL. For the scheduled phase I study a concentration of 5 mg/mL is desired, and further concentration of the solution is achieved by lyophilisation. The formulation solution is composed of 1 mg/mL pDERMATT and 20 mg/mL sucrose in Water for Injections. Upon reconstitution with a five times smaller volume an isotonic sucrose solution containing 5 mg/mL pDERMATT is obtained. Lyophilised pDERMATT is sterile with >90% supercoiled DNA, an A260–280 ratio 1.80–1.95, content 90–110% of labeled, and residual water content <2% (w/w). The product yields the predicted profile upon restriction-enzyme digestion, is highly immunogenic as confirmed in an in vivo mouse model, and stable for at least six months at 5 °C. We have not only developed a reproducible process to manufacture pharmaceutical grade plasmid DNA but also a stable dosage form for the use in clinical trials.

1. Introduction

Malignant melanoma is a highly aggressive type of cancer. In the past 10 years its incidence and mortality rate are rapidly increasing compared to other cancers [1,2]. Surgical resection and systemic chemotherapy are the main therapeutic strategies for the treatment of malignant melanoma. However, these approaches are insufficiently effective and may be associated with significant adverse effects.

In the past decades, evidence has accumulated for a role of T lymphocytes in the host immune response against cancer in general and against melanoma in particular [3]. MART-1 is a melanoma-associated antigen (melanocyte lineage-specific) that is expressed in a large fraction of melanomas and that is frequently recognized by tumor-reactive cytotoxic T lymphocytes (CTLs) in melanoma patients [4]. Previous studies with MART-1 peptide vaccines have shown that it is feasible to boost immune responses against the MART-1 epitope [5,6]. However, while peptide vaccination can enhance melanoma-reactive CTL responses, it has become clear that the vaccine-induced responses are insufficiently potent to induce clinical responses in advanced-stage patients [7,8].

In parallel work it has been shown that naked DNA injected into muscle tissue is expressed in vivo [9], and that the introduction of immunogenic sequences can result in the induction of vaccine-specific T cell and B cell responses [10,11]. DNA vaccines are currently in development for the prevention and treatment of a series of human diseases, including cancer, acquired immune deficiency syndrome (AIDS), malaria and hepatitis B [10,12]. Because of the potential of DNA vaccines in the induction of melanoma reactive T cell responses [13,14] and because of the marked potency of a recently developed DNA tattoo technology [15], we sought to devel-

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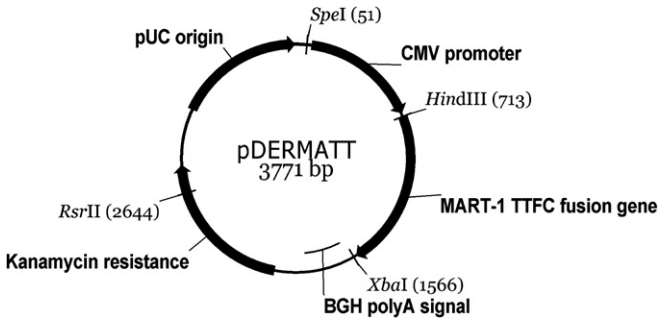

Fig. 1. Plasmid DNA map of pDERMATT (plasmid DNA encoding recombinant MART-1 and Tetanus Toxin Fragment-c) including selected restriction sites.

op a GMP production process for DNA vaccines encoding melanoma-associated antigens.

To this purpose we developed a novel DNA vaccine termed pDERMATT (plasmid DNA Encoding Recombinant MART-1 and Tetanus toxin fragment-c) (Fig. 1). In pDERMATT the MART-1 (26–35) E27L (ELAGIGILTV) major histocompatibility complex (MHC) class I epitope is fused to the carboxyl terminus of tetanus toxin fragment-c. Prior work has demonstrated that such a genetic fusion of single epitopes to the COOH-terminus of carrier proteins yields superior CTL responses [16]. Furthermore, the tetanus toxin fragment-c contains the well described "universal" helper epitope p30 [17,18]. This epitope binds to a range of mouse and human MHC class II alleles [19], and the resulting CD4+ T cell stimulation [20] is necessary for the induction of robust CD8+ T cell responses by DNA vaccines. The plasmid that is described here will be administered intradermally using a newly developed tattoo strategy, which has been shown to lead to a rapid and sustained development of both T-and B-cell responses [15]. In a phase I study toxicity and efficacy of inducing tumor-specific T-cell immunity of pDERMATT will be assessed.

To facilitate this study an in-house plasmid manufacturing process to obtain pharmaceutical grade plasmid DNA was set up and a pharmaceutical dosage form was developed. The challenge was to design a scalable, robust and reproducible manufacturing process, resulting in a pharmaceutical product meeting current quality standards. This means that the pharmaceutical production of pDERMATT needs to meet the requirements for Good Manufacturing Practice (GMP) [21]. The plasmid product must be of high purity, essentially in its supercoiled form and free of host-cell proteins, chromosomal DNA, RNA (preferably without the use of ribonuclease A [22–24]) and endotoxins [10,23–28]. As pharmaceutical formulation a sterile, injectable pharmaceutical dosage form containing 5 mg/mL pDERMATT was required.

In this article we describe the GMP production of pDERMATT for vaccination in which we use an RNase free, one-step purification and organic solvent free protocol. Tangential Flow Filtration (TFF) in combination with lyophilisation was used in order to achieve a highly concentrated and stable pDERMATT dosage form.

2. Materials and methods

2.1. General

The plasmid DNA production facility for manufacturing of the Master Cell Bank (MCB) and bulk drug consists of two class 100 (B) cleanrooms (Interflow, Wieringerwerf, The Netherlands). One room is dedicated to handling bacteria and the second room is dedicated to purification. Both cleanrooms contain a class 100 (A) biosafety cabinet and the fermentor is placed in a class 100 (A) down-flow booth. Manufacturing of pDERMATT final product is performed in a third class 100 (B) cleanroom, containing a class 100 (A) down-flow cabinet, as described earlier [29]. All cleanrooms are subjected to a monitoring program for viable and non-viable particles at operating and at resting state [30].

During manufacture only product dedicated glassware and sterile disposables are used. Buffer components, pharmaceutical excipients and primary packaging materials used in the manufacture of pDERMATT were of European Pharmacopeia (Ph. Eur.) grade (if possible) and provided with a Certificate of Analysis (CoA) by the supplier. All materials and excipients were approved on the basis of in-house quality controls carried out according to monographs in the mentioned pharmacopoeia (if applicable).

2.2. Plasmid design and Master Cell Bank (MCB) generation

The insert of the pVAX-based plasmid pDERMATT (Fig. 1), a plasmid of 3.8 kb with a kanamycin resistant marker, was designed at the Netherlands Cancer Institute (NKI-AvL, Amsterdam, The Netherlands) and subsequently manufactured by GeneArt (Regensburg, Germany) to obtain a small quantity of plasmid produced under GMP/Good Laboratory Practice (GLP) conditions. The insert was synthesised and cloned into a pVAX1 backbone (Invitrogen, California, USA) using HindIII and NotI (Roche, Mannheim, Germany).

Escherichia coli DH5 cells (ATCC, Teddington, Middlesex, UK) were made competent with CaCl2 and subsequently transformed with the plasmid pDERMATT using a standard heat shock method [31]. These cells were plated on Luria Bertani (LB) plates containing 100 μg/mL kanamycin (Biotrading Benelux, Mijdrecht, The Netherlands) and grown in a Refrigerated Incubator Shaker Innova 4230 (New Brunswick Scientific BV, Nijmegen, The Netherlands). One single colony was isolated and grown in 175 mL LB-Miller broth (Sigma–Aldrich Chemie B.V., Zwijndrecht, The Netherlands) containing 100 μg/mL kanamycin (Roche Diagnostics Nederland B.V., Almere, The Netherlands) at 37 °C in a 1L sterile baffled shake flask (Nalgene) at 200 rpm. When an OD600 of more than 0.7 was reached, glycerol (BUFA, Uitgeest, The Netherlands) was added to the culture (30% v/v) and aliquots of 1 mL were frozen to −80 °C, forming the MCB.

The MCB was verified to contain kanamycin resistant *E. coli*, no adventitious viruses and no mycoplasma by MicroSafe B.V. (Leiden, The Netherlands) according to Ph. Eur. [32] and FDA guidelines (CBER, 1993) [33]. Plasmid identity was confirmed by sequencing (in house).

2.3. Fermentation

The inoculum of the fermentor consisted of a shake flask culture. One vial of the MCB was transferred into a baffled shake flask containing 500 mL LB-Miller with 100 μg/mL kanamycin, which was subsequently grown for approximately 8 h at 37 °C and 200 rpm. This culture was inoculated into a BioFlo 3000 benchtop fermentor with a working volume of 10 L (New Brunswick Scientific BV, Nijmegen, The Netherlands) containing 500 mL 500 g/L glucose (B. Braun, Melsungen, Germany), 100 μg/mL kanamycin (as selection agent), 5 mL gamma irradiated silbione antifoaming agent (BUFA, Uitgeest, The Netherlands), and 9 L LB-Miller. During fermentation, pH was controlled at 7.0 with 10% NaOH (BUFA, Uitgeest, The Netherlands). Air inflow was set at 5 L/min and agitation speed (300–800 rpm) was automatically feed-back controlled based on dissolved oxygen (DO) at a set point of 35%.

2.4. Downstream processing

The lysis, pre clarification and purification procedures were performed according to Qiagen protocols [34]. All buffers for bacterial lysis and column chromatography were prepared by the

production facility of our pharmacy department. The composition of the buffers was kindly supplied by Qiagen. Equilibration buffer contained 750 mM KCl and 50 mM KAc. pH was adjusted to 5.0–5.2 with acetic acid and conductivity was adjusted to 90 mS/cm with 3 M KCl. Wash buffer contained 1.7 M KCl and 50 mM Tris–Cl, pH was adjusted to 7.2 with HCl. Elution buffer contained 2.0 M NaCl and 50 mM Tris–Cl, pH was adjusted to 7.5 with HCl.

2.4.1. Recovery

The cells were harvested from the fermentor by mechanically pumping the culture into a Flexstand Tangential Flow Filtration (TFF) system equipped with a Hollow Fibre Cartridge model UFP-300-E-6A with a membrane area of 0.28 m^2 (GE Healthcare, Diegem, Belgium). The cells were concentrated from 10 L to approximately 1.5 L. Subsequently LB-Miller was exchanged for sterile buffer P1 (50 mM Tris–HCl pH 8.0, 10 mM EDTA) without addition of RNase A. After exchanging eight times, the suspension was harvested and divided equally over three 5 litre bottles (Schott). To each bottle additional P1 buffer was added to a total volume of 1.5 L.

2.4.2. Lysis and pre clarification

Lysis was performed at room temperature by adding 1.5 L of sterile buffer P2 (200 mM NaOH, 1% SDS) and inverted mixing by hand. Eight minutes after lysis, cellular debris, gDNA and proteins were precipitated by gently adding and mixing 1.5 L of pre-cooled (4–10 °C) buffer P3 (3 M KAc pH 5.5). The contents of the three bottles were transferred into a sterile 20 L vacuum bottle (Schott) and a small vacuum of 500 mbar was applied to this bottle. After 15 min, the precipitated material floated on top and formed a distinct upper face [35]. The pre-cleared lysate was harvested through a tube that reaches to the bottom of the 20 L bottle and subsequently filtered through an Opticap 4 capsule filter, 1.2/0.5/0.22 μm 9/16 in. (Millipore, Amsterdam, The Netherlands) to obtain a clear solution. To this solution 1/10th volume of endotoxin removal buffer was added and subsequently the conductivity of the solution was adjusted to approximately 90 mS/cm with a 3 M KCl solution.

2.4.3. Purification

Plasmid purification was performed using anion exchange chromatography. A BPG-100/500 column with 10 cm I.D. × 50 cm height and a maximum bed volume of 2 L (GE Healthcare, Diegem, Belgium) was packed with 520 mL Ultrapure resin, containing positively charged DEAE groups on the surface (QIAGEN, Venlo, The Netherlands) according to the instructions of the manufacturer. The column was connected to an Äkta Pilot (GE Healthcare, Diegem, Belgium) and equilibrated for 20 min at a flowrate of 120 mL/min. The cleared lysate was loaded onto the column with a flowrate of 14 mL/min. The column was washed in two steps, first 20 min (120 mL/min) equilibration buffer followed by 55 min (120 mL/min) wash buffer. pDERMATT was eluted at 50 mL/min, a total of 1 L elution fraction was collected. During the run the following parameters were monitored: UV at 260 and 280 nm, pH, conductivity and the pressure (ΔP) over the column.

2.4.4. Concentration and sterile filtration

After elution the plasmid solution was transferred into a Quixstand TFF system equipped with a Hollow Fibre Cartridge model UFP-300-E-3MA with a membrane area of 0.011 m^2 (GE Healthcare, Diegem, Belgium) to concentrate the solution. The concentration of the elution fraction was determined with an Eppendorf BioPhotometer 6131 (Hamburg, Germany). The solution was concentrated to a concentration of approximately 1.1 mg pDERMATT/mL. Subsequently, the elution buffer was exchanged for Water for Injections (Wfl, B. Braun, Melsungen, Germany) in eight steps. After exchanging eight times the solution was sterile filtered through a 0.2 μm Mini Kleenpack filter (Pall, Hitma B.V., Uithoorn, The Netherlands). The final plasmid solution was stored at −80 °C in sterile United States Pharmacopeia (USP) grade poly propylene (PP)-bottles (Nalgene, VWR, Amsterdam, The Netherlands) until further processing.

2.5. Formulation

2.5.1. Formulation development

Small-scale test batches of lyophilised formulations of four different compositions containing sucrose (Merck, Darmstadt, Germany), trehalose (Ferro Pfanstiehl, Waukegan, IL, USA), mannitol (Bufa, Uitgeest, The Netherlands) or Polyvinyl Povidone (PVP) (Plasdone C15 [36], ISP, Waalwijk, The Netherlands) were manufactured and subjected to quality control and stability studies. Cake appearance, residual water content, pDERMATT content and purity were determined after manufacture and after 2 weeks, 1, 2, 3, and 6 months of storage at 25 ± 2 °C/60 ± 5% relative humidity (RH), and 40 ± 2 °C/75 ± 5% RH in climate chambers (HEKK 0057, Weiss Technik Ltd., Buckinghamshire, UK).

The formulation solutions for the test batches contained 1 mg pDERMATT/mL. Aliquots of 2.0 mL were filled into 8 mL colourless glass injection vials (hydrolytic class 1 Type Fiolax-clear, Aluglas, Uithoorn, The Netherlands) and grey butyl rubber lyophilisation stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) were inserted into each injection vial, subsequently the formulations were lyophilised. Vials were frozen to −35 °C, primary drying was started at a shelf temperature of −35 °C and a chamber pressure of 0.4 mbar. During the primary drying phase temperature was elevated to −12 °C. Secondary drying was carried out at a shelf temperature of 25 °C and a chamber pressure of 0.2 mbar. Vials were sealed with aluminium caps.

2.5.2. Manufacturing process

The formulation solution contained 1 mg/mL pDERMATT and 20 mg/mL sucrose. After complete dissolution of the sucrose, the solution was adjusted to final volume with sterile water for injection and subsequently filtered through a sterilising 0.2 μm Mini Kleenpack filter (Polyethersulfone (PES) membrane). Aliquots of 2 mL were filled into the same vial-stopper system used for the manufacture of the test batches. Platinum cured silicone tubing (Watson Marlow, Cheltenham, UK) was used during filtration and filling. Three vials were equipped with thermocouples and all vials were loaded into a Model Lyovac GT 4 freeze-dryer (GEA lyophil GmBH, Hürth, Germany) at ambient temperature and lyophilised. The product temperature, shelf temperature, chamber pressure and condenser temperature were monitored. The product was sealed with flip-off caps (West Pharmaceutical, Germany) and labelled.

2.6. Quality control

All chemicals used for quality control were of analytical grade and used without further purification.

2.6.1. Agarose gel electrophoresis including restriction endonuclease digestion

Agarose gels were run in a Horizon 20 25 horizontal gel electrophoresis unit coupled to a Whatmann Biometra power supply (Westburg B.V., Leusden, The Netherlands).

IPCs and final product were analysed by electrophoresis using 25 cm, 1% agarose (ABGene, Epsom, Surrey, UK) self cast gels. Running buffer was a 40 mM Tris–Acetate, 1 mM EDTA, pH 8.3 solution and electrophoresis was carried out at 30 V for 21 h. After electrophoresis, gels were stained for 1.5 h with a 1× Sybr green I solution (Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands) and then

visualized and photographed under UV light (GeneGenius, Westburg B.V., Leusden, The Netherlands).

Open circular and linear standards of the plasmid DNA were prepared using N.BstNBI (New England Biolabs, Hertfordshire, England, UK) and HindIII (Roche Diagnostics Nederland B.V., Almere, The Netherlands), respectively, according to the instruction of the manufacturer.

For AGE the samples were diluted, if necessary, to approximately 20 ng pDERMATT/μL; for determination of residual *E. coli* host RNA/DNA in the bulk and lyophilised product the sample was diluted to 50 ng pDERMATT/μL. Of the resulting solutions 10 μL was mixed with 2 μL of 6× loading dye and this was subsequently loaded onto the gel.

2.6.2. Anion exchange chromatography

Analytical chromatography was performed with an HPLC system that consisted of an 1100 series binary HPLC pump, Model G1312A (Agilent technologies, Amstelveen, The Netherlands), a model spectraSERIES AS3000 automatic sample injection device, equipped with a 20 μL sample loop (Thermo Separation Products, Breda, The Netherlands), a photodiode array detector (PDA) Model Waters™ 996 (Waters Chromatography B.V., Etten-Leur, The Netherlands). Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).

Plasmid purity was determined by a validated anion-exchange chromatography (AEX-HPLC) on a TSK DNA-NPR column (75 mm × 4.6 mm I.D. particle size 2.5 μm) protected by a TSK DNA-NPR guard column (5 × 10 mm, Anachem House, Luton, England). Injection of 10 μL of sample was followed by a linear gradient of 0.45–0.53 M NaCl in 20 mM Tris, 10% isopropyl alcohol (IPA), pH 9.0 for 32 min. The flow rate was 0.5 mL/min. Absorbance was monitored at 260 and 280 nm. Furthermore, a UV spectrum was recorded from 340 to 190 nm using a diode-array detector. Samples were diluted, if necessary, to approximately 100 μg/mL.

Standards of plasmid pDERMATT were derived from the first plasmid batch. Under the conditions used the TSK-DNANPR column was able to resolve open circular, supercoiled and linear plasmid (manuscript in progress).

2.6.3. UV analysis

UV analysis was used to determine the concentration and purity of the plasmid DNA sample. The HPLC samples were diluted to approximately 30 μg/mL and measured with a Biofotometer (Eppendorf, Hamburg, Germany). Absorbance was measured at 230, 260, 280 and 320 nm.

2.6.4. Protein analysis

A bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA) was used to measure residual protein content.

2.6.5. Endotoxin analysis

A Pyrochrome® limulus amoebocyte lysate assay (Cape Cod Associates, Cape Cod, MA, USA) was used to measure endotoxin content in the bulk material.

2.6.6. Sequencing

Primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [37]. A total of 26 primers were designed to cover the whole sequence. First, PCR products of the forward and reverse couples were generated. Results of the PCR reaction were analysed on a 2% agarose gel. For sequencing the PCR products were purified with ExoSAP-IT (GE-Healthcare, Diegem, Belgium). After purification, DNA cycle sequencing was carried out as described by the manufacturer (Applied Biosystems, Foster City, CA, USA) in 20 μL reactions on a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA). The forward and reverse primers were identical to those used in the PCR amplifications. Both DNA strands were sequenced. Sequences were analysed on an Applied Biosystems 3100-Avant DNA sequencer. For sequence alignment Seqscape v2.1 (Applied Biosystems, Foster City, CA, USA) was used.

2.6.7. Bioburden

The bioburden of the solution was determined using the total viable aerobic count Ph. Eur. <2.6.12> using 10 mL of the bulk product.

2.6.8. Sterility and bacterial endotoxins

Sterility of the final lyophilised product was checked by the filtration method and the presence of bacterial endotoxins with the limulus amoebocyte lysate (LAL) test (Cape Cod Associates, Cape Cod, MA, USA), both carried out according to the Ph. Eur. (2.6.1 and 2.6.14) [32].

2.6.9. Mice

Human Human Db (HHD) mice (2–4 months) transgenic for the Human Leukocyte Antigen (HLA)-A2Kb fusion gene [38] were obtained from the experimental animal department of The Netherlands Cancer Institute. Animals were kept under normal conditions. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Laboratory Animal Research Committee of the Institute.

2.6.10. DNA immunisation

Mice were vaccinated with pDERMATT final product (2 mg/mL in Wfl), or with empty pVAX vector as a control. For intradermal DNA vaccination, the hair of the left hind leg of the mice was removed with depilatory cream (Veet sensitive). Next, 10 μl of the pDERMATT solution was applied to the skin and a sterile disposable 9-needle cartridge (MediUm-Tech, Berlin, Germany) mounted on an Aella® tattoo machine for medical use (MediUm-Tech, Berlin, Germany) was used to apply the vaccine. Needle depth was adjusted to 1.0 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were punched into the skin by a 30 s tattoo. Mice were vaccinated with a standard vaccination scheme on day 0, 3 and 6 [15]. All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment.

2.6.11. Cytotoxic T-cell assay

To measure specific CTL responses against the MART-1 epitope, peripheral blood lymphocytes were stained at the indicated time points with Phyco Erythrin (PE)-conjugated A2Kb-ELAGIGLTV-tetramers and Allo Phyco Cyanin (APC)-conjugated CD8 (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1 × Phosphate Buffered Saline (PBS), 0.5% Bovine Serum Albumin (BSA) and 0.02% sodium azide) as described before [39]. CD8+ cells expressing the MART-1 epitope-specific T-cell receptor will bind to the tetramer and can be detected by flow cytometry. After incubation cells were washed three times in FACS buffer and analyzed. Living cells were selected based on propidium iodide (PI) exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) using CellQuest software (Becton Dickinson, Franklin Lakes, USA).

3. Results

3.1. Production process

An overview of our plasmid production process is shown in Figs. 2A and B. One fermentation run consisted of a total of 10 L media

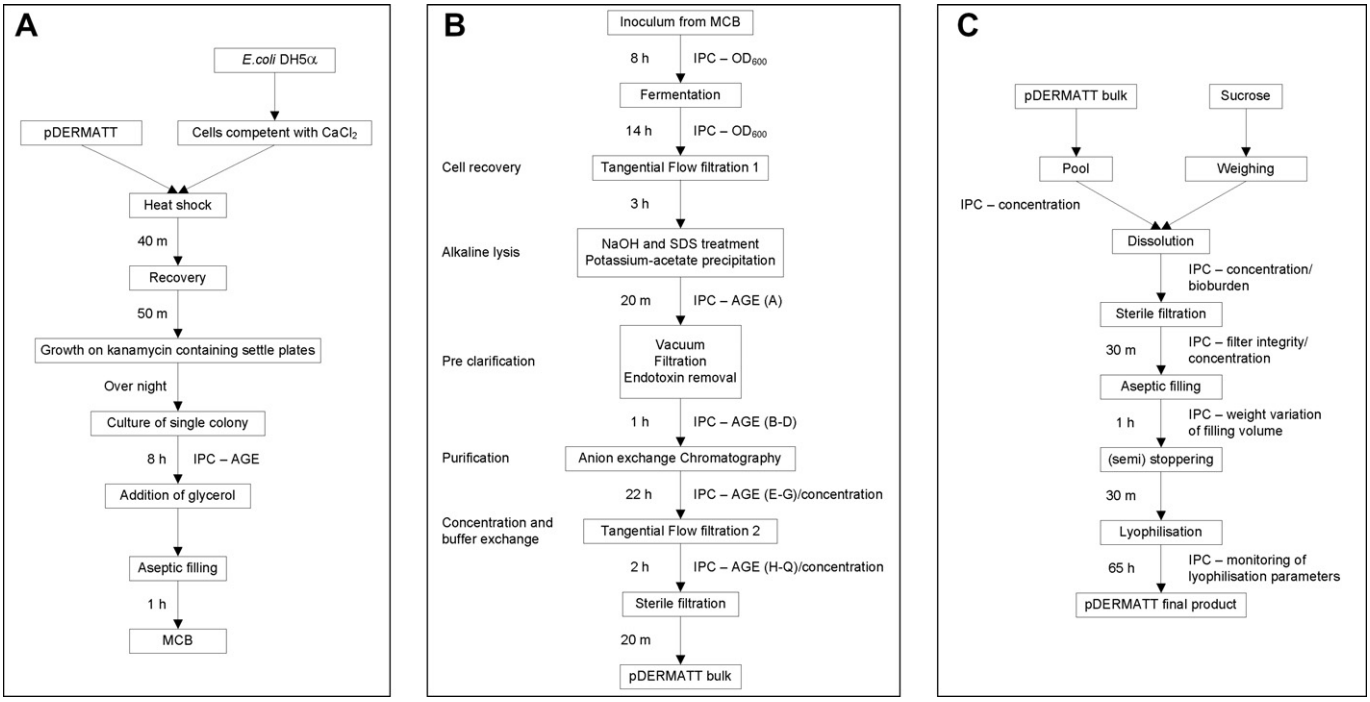

Fig. 2. Process flow sheets for the production of pDERMATT at our facility, production of Master Cell Bank (MCB) (A), pDERMATT bulk (B) and pDERMATT final product (C). Approximate time in hours (h) or minutes (m) for each step are displayed on the left of the arrow, while in process controls (IPCs) are indicated on the right side. The letters behind the IPC controls in figure B correspond to the letters in Fig. 3. Abbreviations: OD600, optical density at 600 nm; AGE, agarose gel electrophoresis; SDS, sodium dodecyl sulphate.

incubated for 13–15 h. Five fermentation runs allowed for the production of 780 mg pDERMATT, a sufficient amount of plasmid DNA for the clinical trial. A total of six fermentation runs were performed (Table 1). For all fermentations the final OD600 was approximately 7, irrespective of OD600 of inoculum or fermentation time. With the aim to obtain higher yields in future studies,

Table 1
Quality control performed on produced bulk plasmid including set specifications

Test item	Specification	Bulk product (batch number)					
		1	2	3	4	5	6
IPC							
OD600 inoculum	⩾ 0.7	0.721	2.94	1.67	2.46	3.275	2.51
Fermentation time (hh:mm)	For information	15:07	13:27	15:40	15:45	15:20	14:51
OD600 fermentation	For information	6.84	7.52	7.48	6.54	7.46	7.20
A260/280 eluate	1.80–1.95	1.90	1.94	1.90	1.89	1.89	1.95
Amount DNA in eluate (mg)	For information	154	201	195	202	212.8	171.1
A260/280 after 2nd TFF	For information	1.78	1.68	1.74	1.70	1.72	1.70
Recovery after 2nd TFF (%)	⩾ 85%	95.5	88.5	106.4	112	102.5	96.1
Amount DNA final product (mg)	For information	134.1	164.9	191.2	214.1	207.4	149.3
QC							
Appearance	Clear, colourless solution	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Sequencing	Conform reference	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
HPLC analysis							
Identification	Rt identical to standard	Conforms	Conforms	Conforms	Double peak	Conforms	Conforms
Purity	⩾90% supercoiled	98	90	91	88	94	96
UV analysis							
Concentration	For information (mg/ml)	1.048	1.024	1.180	1.252	1.152	1.044
Purity	A260/280 = 1.80–1.95	1.86	1.88	1.83	1.84	1.84	1.87
AGE analysis							
Identification	3395–4149 bp	3700	3728	3756	3785	3756	3609
Restriction map	Compares to theoretical	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Residual *E. coli* host DNA	<5% (<0.05 mg/mg plasmid)	<5%	<5%	<5%	<5%	<5%	<5%
Residual *E. coli* host RNA	⩽4% (⩽0.04 mg/mg plasmid)	<4%	<4%	<4%	<4%	<4%	<4%
BCA assay	<5 μg/ml protein	nd	nd	nd	nd	nd	nd
Residual ER buffer	None detected	nd	nd	nd	nd	nd	nd
Total viable count	0 cfu	0	0	0	0	0	0
Endotoxin	<10EU/mg	<2	<2	<2	<2	<2	2
Approved?	Complies to all test items	Yes	Yes	Yes	No	Yes	Yes

Abbreviations: OD600, optical density at 600 nm; AGE, agarose gel electrophoresis; AEX-HPLC, anion exchange high performance liquid chromatography; UV, ultra violet; BCA, bicinchoninic acid; nd, not detected; RP-HPLC, reversed phase high performance liquid chromatography; ER, endotoxin removal; cfu, colony forming unit; Ph. Eur., European Pharmacopoeia; EU, Endotoxin Unit.

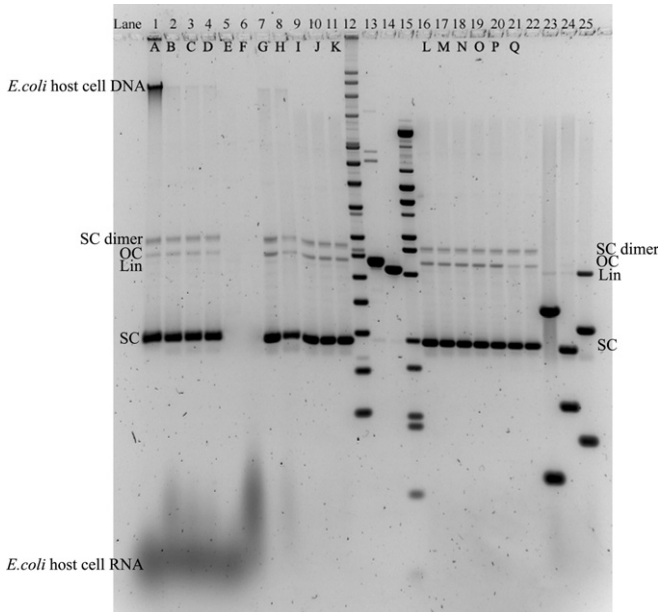

Fig. 3. Analysis of IPCs, bulk product and restriction fragments by 1% agarose gel electrophoresis: lane 1–11 and 16–21 contain IPCs, letters corresponding to letters in Fig. 2B; lane 12 contains a supercoiled DNA ladder; lane 13 open circular standard; lane 14 linear standard; lane 15 lambda DNA BstEII digest; lane 22 pDERMATT bulk product; lane 23 HindIII and XbaI digest; lane 24 SpeI and XbaI digest; lane 25 RsrII and SpeI digest.

new fermentation strategies are currently being developed. Preliminary data using fed-batch culture with glycerol as carbon source and a luciferase encoding plasmid showed that an OD600 of 105 can be reached with a plasmid content of 135 mg/L.

Pre-manufacturing experiments showed that a column bed volume of 520 mL was sufficient for purification. Table 1 and Fig. 3 show that *E. coli* host RNA can efficiently be removed during purification, even when cellular RNA is not removed by RNase A. As is shown in Figs. 3E and F *E. coli* host RNA is released from the column during two wash steps by increasing pH and salt concentration. Subsequently, the plasmid DNA is released by a further increase in salt concentration and pH (Fig. 3G). HPLC and AGE data show no alterations in topology during the downstream processing (Table 1, Fig. 3G vs 3H and 4 A). During downstream processing, in particular with the tangential flow filtration step, the A260/280 (DNA/protein) ratio decreases. However when the bulk product is diluted in Tris-EDTA (TE) buffer instead of Wfl the A260/280 ratio returns to a value between 1.80 and 1.95.

Table 1 represents the Quality control (QC) results of the pDERMATT batches including in-proces controls (IPCs). The specifications for pDERMATT bulk are based on specifications found in literature [26,40–45]. As is shown in Fig. 4A the topology as determined with HPLC analysis does not change during production, however one of the batches showed a double peak in the HPLC analysis (Fig. 4B). AGE analysis showed a slight difference in migration between both peaks (data not shown). Additionally, the purity of this batch is below the set specification. Therefore the batch was rejected, and further research is being performed as to see what caused the formation of the second peak and to characterise this peak. Possibly the peak consists of supercoiled plasmid DNA with a lower amount of supercoils, as has been described earlier [46]. Linear plasmid was not seen in any of the samples measured. The last peak in the chromatogram still has to be fully characterised, but this is probably a dimer. All samples show a band just above the open circular band in AGE. When compared to the supercoiled ladder the size of this band is approximately twice the size of the supercoiled plasmid, confirming that this is a dimer [35] (Fig. 3). The endotoxin removal detergent is completely removed during chromatography, resulting in <0.0025% (limit of detection) in the final eluate. Endotoxin level of the six batches is ⩽2EU/mg pDERMATT (Table 1), well below the specification of <10 EU/mg.

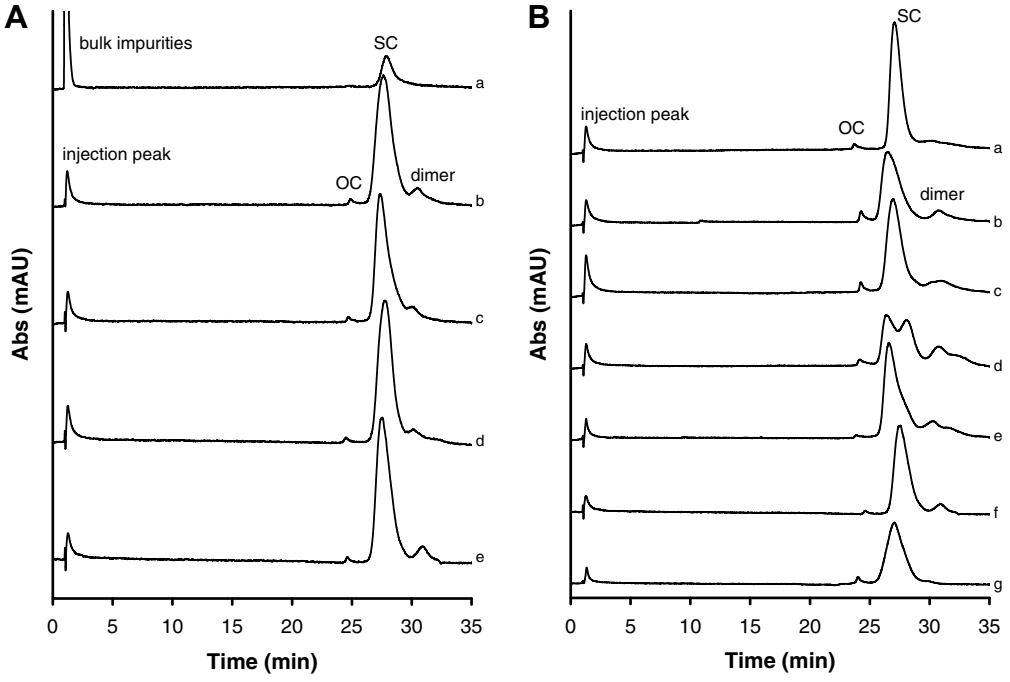

Fig. 4. Typical HPLC chromatograms; IPCs and bulk product a = after lysis, b = after concentration with 2nd TFF system, c = after exchanging elution buffer for Wfl in 8 steps, d = before filtration, e = after sterile filtration (A), pDERMATT bulk and final product a = 1st bulk batch, b = 2nd bulk batch, c = 3rd bulk batch, d = 4th bulk batch, e = 5th bulk batch, f = 6th bulk batch, g = final product (B).

3.2. Formulation

3.2.1. Formulation development

The vials were filled with 2 mg pDERMATT and upon reconstitution with 400 μL an isotonic solution containing 5 mg/mL pDERMATT was achieved. Four different bulking agents were selected for the test batches; sucrose, trehalose and PVP (2%) as non-crystallising and mannitol (1%) as crystallising excipients. The initial pDERMATT SC content of the formulation solutions was within 95.0–105.0% with exception of the PVP formulation (90.7%). pDERMATT content and purity after one month of storage at 25 ± 2 °C/60 ± 5% RH or 40 ± 2 °C/75 ± 5% RH are given in Table 2. The supercoiled purities are normalised to the purity of the formulation solution. The residual water content of the mannitol formulation is relatively high. It is not clear what caused the rapid decrease in content and purity of pDERMATT in this formulation, either mannitol is not a suitable bulking agent, or the high water content or both. For the mannitol containing formulation the lyophilisation process needs to be optimised.

Even though pDERMATT content and purity decrease over time in all formulations, the formulation containing only sucrose is the most stable. Therefore development of this formulation was further pursued.

3.2.2. Manufacturing process

An illustrative flowchart of the lyophilised plasmid DNA production process is shown in Fig. 2C. First, the different pDERMATT bulk products were pooled and gently mixed, to ensure that the material for the phase I clinical trial originated all from the same batch. The pDERMATT concentration of the formulation solution before and after filtration and filling was shown to be equal, indicating no retention of pDERMATT in the filter or tubing.

The QC results for pDERMATT 2 mg/vial final product with their specifications are presented in Table 3.

3.2.3. Immunogenicity

To test the immunogenicity of the produced pDERMATT DNA vaccine, HHD-transgenic mice were vaccinated with pDERMATT final product by intradermal DNA tattooing. Fig. 5 shows the CTL response upon dermal vaccination. All vaccinated mice developed a strong MART-1 specific CTL response, with a peak level at day 12, similar to previous data [15]. The control group did not develop a specific immune response.

3.2.4. Stability upon storage

Stability of pDERMATT 2 mg/vial lyophilised product was evaluated. Samples were taken at different points in time. Stability studies were initiated at −20 °C, +5 ± 3 °C and the accelerated storage condition of +25 ± 2 °C/60 ± 5% RH (climate chamber HEKK 0057, Weiss Technik Ltd.) in the dark. Table 4 shows the shelf life results obtained thus far for the stability studies. Although the residual water content increases during storage, content and purity of all samples still comply to the specifications (content 90–110% of labelled content and purity ⩾90% supercoiled). To date, pDERMATT 2 mg/vial final product proved stable for 6 months at all storage conditions.

Table 3
Overview of in-process controls (IPC) and quality control (QC) results of pDERMATT 2 mg/vial final product with the derived specifications

Test item	Specification	Result
IPC		
Concentration of pool (mg/ml)	1.0–1.1	1.039 (0.75)
Concentration formulation solution (mg/ml)	0.9–1.1	0.92 (1.44)
Concentration after filtration (mg/ml)	0.95–1.05	1.001 (0.12)
Bioburden before filtration (cfu)	<2.7 × 106 cfu/ml (retention capacity filter)	0
Filter integrity test, bubble point (psi)	⩾1200	1576
Weight variation of filling volume (%)	⩽3.0	0.31
QC		
Visual inspection	White, freeze-dried cake	Conforms
Reconstitution	Complete, leaving no visible residue as undissolved matter and resulting in a clear colourless solution	Conforms
HPLC analysis		
1. Identification	Rt reference standard = Rt final product	Conforms
2. Purity	⩾90% supercoiled	95.7 (0.5)
UV analysis		
1. Content	90.0–110.0% of labelled content	99.6 (0.3)
2. Purity	A260/280 = 1.80–1.95	1.8
AGE analysis		
1. Identification	3395–4149 bp	3773 bp
2. Residual *E. coli* host DNA	<5% (<0.05 mg/mg plasmid)	<5%
3. Residual *E. coli* host RNA	⩽4% (⩽0.04 mg/mg plasmid)	<4%
Uniformity of dosage units	Conforms Ph. Eur. <2.9.40> Acceptance value <15%	Conforms 8.3%
Residual water content (%w/w)	⩽2%	0.7 (0.07)
Endotoxin level (EU/vial)	<10	Conforms
Sterility	Sterile	Conforms

Mean values are given with standard deviations within parentheses.

4. Discussion

We chose not to make a Working Cell Bank (WCB) because a MCB batch consisting of 250 vials was deemed sufficient for the planned experiments. If more pDERMATT would be required for subsequent clinical studies, a WCB can readily be made from the existing MCB.

Table 2
Composition of pDERMATT test batches per ml of formulation solution

No.	Excipient	Amount (mg)	Water content (%) (t = 0)	% of initial content		Purity (%)	
				25 ± 2 °C/60 ± 5% RH	40 ± 2 °C/75 ± 5% RH	25 ± 2 °C/60 ± 5% RH	40 ± 2 °C/75 ± 5% RH
1	Sucrose*	20	1.03 (0.3)	100.3 (2.3)	96.0 (1.4)	98.4 (0.6)	91.6 (0.5)
2	Trehalose	20	0.77 (0.07)	86.7 (1.0)	68.1 (1.6)	92.8 (0.5)	76.1 (1.2)
3	Mannitol	10	4.87 (0.3)	83.4 (1.9)	39.8 (1.8)	90.9 (0.3)	50.8 (1.6)
4	PVP**	20	0.89 (0.3)	63.5 (3.4)	17.6 (2.7)	67.5 (3.3)	26.7 (1.2)

All formulations contain 2 mg/ml pDERMATT. Residual water content is given at t = 0, just after manufacturing. The pDERMATT content (% of initial) and purity after 1 month of storage is given, with exception of formulation 1 and 4 as is indicated by asterixes. Mean values are given with the standard deviation within parenthesis.

* Results after 6 months in the stability program.

** Results after 2 weeks in the stability program.

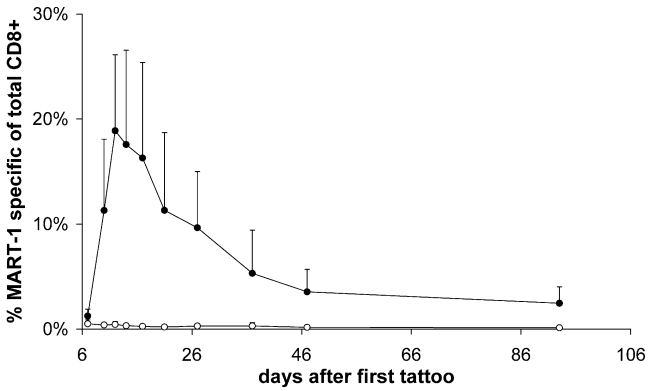

Fig. 5. MART-1 specific CD8+ T-cell responses in peripheral blood lymphocytes in HHD mice upon vaccination with pDERMATT (n = 7, filled circles) by DNA-tattooing. Control mice were vaccinated with empty pVAX vector (n = 5, open circles). Values represent the Mean + Standard Deviation (error bars).

For fermentation a minimal medium (Luria Miller Broth), supplying only minimum nutritional requirements, was used. Glucose was added to the medium to provide an extra carbon source and extra energy for achieving a higher cell density [47]. Since fermentation was performed in the batch mode, growth of bacteria was limited by the nutrition supply in the growth medium. Following fermentation bacterial cells can be harvested from the fermentor by using either centrifugation or filtration [48]. In our process we used TFF for harvesting, since it gave the possibility of concentrating the cell paste and subsequently exchanging the culture medium for buffer P1, thus avoiding an additional resuspension step before lysis. Usually RNase A is added to this first buffer to ensure complete RNA removal, however the use of animal derived enzymes is not preferred for the production of clinical grade plasmid DNA [22–24]. The suspension was harvested from the membrane and equally divided into three lysis bottles. To minimise loss of bacterial cells in the membrane, the membrane was subsequently rinsed with one litre of fresh buffer P1. Each bottle contained approximately 100 g bacterial wet cell paste to ensure efficient lysis of the bacterial cells. The high salt neutralisation solution promotes the formation of large, flock-like particles of gDNA, host cell protein, and cell debris [49], some of which float while others precipitate [48]. By applying a small vacuum (approx. 500 mbar) to the bottle even the precipitated flocks start to float [35,50]. With this separation technique air escapes from the solution under vacuum to encourage the formation of a compact bed of flocculent material. The vacuum clarification only takes 15 min, and preclears the lysate sufficiently to allow filtration using a standard, commercially available capsule filter [51].

Plasmid DNA can be purified with many different techniques [41,52], and a number of purification procedures are on the market (e.g. QIAGEN, PlasmidSelect Xtra from GE-Healthcare and Mustang Q from Pall). We selected the QIAGEN purification resin as it is a single step method [44] and approved for production of clinical grade DNA since 1996 [34]. For every purification run a fresh column was prepared, since the resin cannot be sanitised with standard agents (e.g. sodium hydroxide) [51]. The BPG column is easy to clean and has sanitary end fittings, all according to GMP. Instead of using the standard Qiagen endotoxin removal (ER) buffer we used a different triton-X based buffer. The advantage of this ER buffer is that endotoxin removal does not require incubation of the solution on ice, and therefore it is time saving. Furthermore cooling on ice of the cleared lysate during loading onto the column is not necessary.

The conductivity and pH of the equilibration buffer and the lysate are equal and of such a value that plasmid DNA will bind to the column material [53]. Because we are using an RNase free process not only plasmid DNA but also *E. coli* host RNA will bind to the column, since both are polyanionic molecules [22]. Fortunately RNA is efficiently removed as can be seen in Table 1 and Fig. 3. The QIAGEN resin has an exceptionally high charge density and plasmid DNA remains tightly bound to the DEAE groups over a wide range of salt concentrations. The broad separation range of the resin makes efficient separation of plasmid DNA from RNA possible [54]. pH of the buffer is also important for efficient separation of RNA from DNA on DEAE resins [22]. For the wash steps pH and salt concentration are chosen such that only RNA elutes from the column, while DNA stays bound to the resin. Following purification plasmid DNA is usually precipitated using ethanol or isopropanol and then resuspended in the desired volume [54]. However the use of potential toxic organic solvents during manufacture of plasmid DNA intended for human applications is not preferred [22–24]. TFF in combination with lyophilisation is used as alternative methods to concentrate the purified DNA. With the second TFF step the solution is concentrated to approximately 1 mg/mL pDERMATT, and elution buffer is exchanged for Wfl. During this buffer exchange step the A260/280 ratio of the solution decreases. The pH of Wfl is slightly acidic compared to the elution buffer, which has a pH of 7.2. It is known that pH and ionic strength of the spectrophotometric solution significantly alters the A260/280 ratio of nucleic acids, because the degree of ionisation of the bases is strongly pH-dependent [55]. Specifications for A260/280 are set using a slightly basic solution, therefore the A260/280 ratio returns to a value within specifications when using TE-buffer (pH 8.3).

The final amount of DNA (134.1–214.1 mg) cannot be correlated to the OD600 (6.54–7.52) see Table 1. It is known that during cell division plasmid DNA, of high copy number plasmids like pVAX, is distributed randomly between the new born cells. Therefore plasmid copy number may vary among individual dividing cells in the population [56]. Furthermore, fermentations can be characterised into two distinct phases. First, a phase of rapid growth occurring from inoculation, followed by a phase characterised by a reduced growth rate to the end of the cultivation cycle. It is during the second phase that most of the plasmid production is achieved [57]. Possibly some of the batches were in the second phase of fermentation for a prolonged period, thus generating more plasmid DNA.

Table 4
Stability of pDERMATT 2 mg/vial final product Lot 112106SQ1

Test item	Initial	3 months		6 months		
		5 ± 3 °C, dark	25 ± 2 °C/60 ± 5% RH, dark	−20 °C, dark	5 ± 3 °C, dark	25 ± 2 °C/60 ± 5% RH, dark
Appearance	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Reconstitution	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
HPLC analysis, purity (% supercoiled)	95.7 (0.5)	98.5 (0.08)	98.3 (0.09)	97.2 (0.3)	96.2 (0.4)	95.1 (1.1)
UV analysis, content (%)	99.6 (0.3)	98.0 (0.2)	98.3 (0.2)	97.5 (0.4)	100.8 (4.7)	97.5 (0.0)
Residual water content (%w/w)	0.7 (0.07)	nd	nd	1.27 (0.05)	1.96 (0.2)	2.41 (0.2)

Mean values are given with standard deviations within parentheses. All test items were analysed in triplicate. *Abbreviations:* RH, relative humidity; nd, not determined.

For the scheduled phase I clinical trial a plasmid DNA solution of 5 mg/mL was required. Since the pDERMATT bulk material could only be concentrated to approximately 1 mg/mL, lyophilisation was chosen for further concentration [58]. A second advantage of lyophilisation is that the product has a longer shelf life than when kept in solution [59]. During storage of plasmid DNA in solution at 2–8 °C a conversion of SC DNA towards OC DNA was observed within a week. Furthermore, after prolonged storage the OC DNA was converted into linear species, as was described previously [59]. This was prevented by storage at −20 °C, however repeated freeze thaw cycles did also affect the topology.

The four selected bulking agents in the formulation development phase are well known for protein and non-viral DNA vector stabilisation during lyophilisation [60–62]. Since naked DNA is more stable than proteins or non-viral DNA vectors we assumed that the selected bulking agents would also be able to stabilise pDERMATT during lyophilisation. The lyophilisation program for the mannitol formulation contained an annealing step in order to completely crystallise the mannitol. Amorphous mannitol may crystallise during transport or storage, releasing the sorbed water. This water may then be available for interaction with the other formulation ingredients including the active pharmaceutical ingredient (API) [63].

For the final lyophilised product, containing sucrose as bulking agent, a less tight specification limit (90–110% in stead of 95–105%) concerning content is applied to prevent unnecessary batch rejection, since the product is an investigational agent [29]. As additional batches will be manufactured, tightening of the specification will be considered on the basis of the resulting risk of batch failure that is estimated from the quality control results obtained (process mean, process standard deviation) [29,64].

Immunogenecity was tested using HHD-transgenic mice, which express a chimeric HLA-A2.1 heavy chain (α1 and α2 human, α3 mouse) linked to a human β2-microglobulin with a double knockout of mice H-2KbDb. These mice are a suitable model to test the immunogenicity of pDERMATT as the encoding MART-1 epitope is HLA-A2.1 restricted [65]. Fig. 5 shows that pDERMATT is a very potent DNA vaccine in this animal model. Vaccination with the lyophilised product resulted in a rapid and strong MART-1 specific CTL response against the MART-1 epitope in all vaccinated animals.

In conclusion the production process used at our facility is capable of producing pharmaceutical grade pDERMATT, under GMP conditions, at a relatively large scale. The process is generic and will be optimised in the future to obtain even higher yields. Quality control of the bulk product revealed a high purity of the plasmid even though an RNase free process is used. Furthermore the product is chemically more pure since the use of organic solvents is avoided.

A sterile and stable, injectable final product for pDERMATT has been developed. Lyophilisation of 1 mg/mL pDERMATT with sucrose as bulking agent in a concentration of 2% (w/v) resulted in a stable product. Furthermore upon reconstitution with a five times smaller volume an isotonic product with a DNA concentration of 5 mg/mL was obtained. This product showed immunogenicity and a strong MART-1 specific CTL response in a murine model. The lyophilised pDERMATT 2 mg/vial final product will soon be used in Phase I clinical trials.

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