Investigation of Virus-Like-Particles and Antigen-Loaded Poly-Lactic-Acid Particles for Transcutaneous Vaccine Delivery

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Table of Contents

Table of Contents

Selbstständigkeitserklärung

Hiermit erkläre ich, Zahra Afraz, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin,

Zahra Afraz

Abbreviations

A

APCs	Antigen-Presenting Cells

B

BPV-1	Bovine PapillomaVirus Type 1

C

c	Concentration
CD	Cluster of Differentiation
CFDA	Carboxyfluorescein Diacetate
CLSM	Confocal Laser Scanning Microscopy
CMV	Cytomegalovirus
CSSS	Cyanoacrylate Skin Surface Stripping
CTL	Cytotoxic T Lymphocyte

D

DAPI	4´, 6-Diamidino-2-Phenylindole
DCs	Dendritic Cells
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide

E

E. coli	Escherichia coli
EP	Electroporation
eGFP	Enhanced Green Fluorescent Protein

F

FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate

G

g	Gram or Gravitational Acceleration of Earth
GIMP	GNU Image Manipulation Program
GMP	Good Manufacturing Practice
gp	Glycoprotein
GTU®	Gene Transfer Unit

H

h	Hour
HaCaT	Human Adult Low Calcium High Temperature Cultured Keratinocytes
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HF	Hair Follicle
HLA	Human Leukocyte Antigen

I

ID	Intradermal
IgG	Immunoglobulin G
IM	Intramuscular

K

KGM	Keratinocyte Growth Medium

L

LCs	Langerhans Cells
LN	Lymph Node

M

MACS	Magnetic-Activated Cell Sorting
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	Minute
MLV	Murine Leukemia Retrovirus

P

PFA	Paraformaldehyde
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pGTU®	Plasmid GTU®
PLA	Polylactic Acid

R

rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature

S

SC	Stratum Corneum
SCC	Side-Scattered Light
SD	Standard Deviation
SDS	Sodium-Dodecyl Sulfate

T

TC	Transcutaneous
TEM	Transmission Electron Microscopy

V

VLP	Virus-Like Particle

Introduction

1. Introduction

1.1. The state of the art of vaccination

The field of disease prevention has seen substantial improvement over the past years. Vaccination has prevented the morbidity and mortality induced by many infections. However, many diseases still do not have associated vaccines, including chronic infectious diseases caused by human immunodeficiency virus (HIV) or hepatitis C virus (HCV), and malaria (1).

Different pathogens require different defense mechanisms. E.g., prevention against intracellular organisms, like viruses, needs both, cellular and humoral immunity (2). While antibodies maybe helpful in preventing virus entry, cell-mediated, especially type I cytokine driven immunity is effective against the cells already infected by the intracellular pathogens. Key actors in this regard are antigen presenting cells (APCs), which are capable of antigen presentation (3).

One challenge in vaccine development is to find a mixture of antigenic compounds, which reflect the pathogen well enough to provide effective protection. In order to cover more diseases by means of vaccination, antigenic compounds, which are representative of the pathogen to the body, are needed. For example, eradication of viral diseases like smallpox was achieved using live attenuated vaccines. Since the vaccine was extremely similar to the original pathogen, a long term immune response was achieved against the preliminary infection. However, pathogen reactivation, especially in immune-compromised patients is a major concern when using such vaccine compounds (4).

Subunit antigens, toxoids, and killed organisms are promising alternatives for live attenuated vaccines. However, they are less antigenic, frequently unstable in the *in vivo* environment, and tend to favor humoral immunity (5). Therefore novel vaccine candidates that are safe, induce a broad immune response, and are cost-effective are needed (6).

1.2. Conventional or routine methods of vaccination

Injection is the routine method used for vaccine administration. Injections in the subcutaneous or intradermal (ID) compartments of the skin tissue, and intramuscular (IM) injections are the most commonly used methods of vaccination. Although these methods have been used in different vaccination plans, major associated concerns are the need for trained personnel, and the increased risk of needle-stick or -reuse. The pain of injection also decreases patient compliance, which impacts vaccination coverage (7, 8). A needle free, low or non-invasive method would be helpful in increasing the acceptability and the access to vaccination. As such, transcutaneous (TC) vaccination by means of low-invasive methods such as patch applications on the skin surface could be an advantageous alternative vaccination route (9, 10).

Increasing evidence from experimental and clinical studies suggest that the site of injection affects the immune response outcome based on the presence, and type of APCs in the tissue (11). Many cell populations in the body are capable of presenting the antigens to the immune system, but only professional APCs can effectively prime the naïve T cells by presenting the antigens along with costimulatory molecules (12). The low presence of APCs at the inoculation site, e.g. muscular or subcutaneous fat tissue, could be one limiting factor for immune response induction (13). Therefore, adjuvants are being co-administered with the immunogen to induce inflammation. As a result, naïve dendritic cells (DCs) infiltrate the injection site from blood circulation in response to inflammation, process the antigens, and after activation, initiate the immune response (14-16).

1.3. The transcutaneous route

The direct contact of the skin with external pathogens makes it a suitable organ for vaccination. Edward Jenner discovered the first use of this vaccination route, as an empirical finding, using the skin for vaccination of smallpox. The mechanism underlying his experiment was only uncovered in the last few years. Several immunologically active cell populations can be found in different skin layers, from keratinocytes, Langerhans cells (LCs) in the epidermis to the DCs, fibroblasts, endothelial cells and others in the dermis. In a non-inflammatory condition these cells create the cellular defense mechanism of the skin. The keratinocytes are mainly involved in innate immunity, whereas LCs and DCs are involved in stimulation of

antigen specific immune response. The stratum corneum (SC) on the other hand acts as the physical barrier of the skin (8, 17).

The presence of APCs in epidermis and the dermis makes the skin an immunologically active organ suitable for immunization purposes (18-21). The LCs are specialized DCs characterized by human leukocyte antigen (HLA)-DR, the adhesion marker epithelial-cadherin, unique cytoplasmic organelles named Birbeck granules or Langerhans granules, and they strongly express the cluster of differentiation CD1a (8, 17). Immature LCs are typically located in the suprabasal layers of the epidermis (8). They capture the antigens in the skin and process them. As a result, they become activated and enter the maturation phase. The processed antigens will be presented as a complex with major histocompatibility complex (MHC) proteins on their surfaces (8, 17). The activated LCs migrate to the skin lymph nodes (LN), and present antigens to the resident lymphocytes. They also stimulate the helper or cytotoxic activity in naïve resting T cells.

Epidermal LCs and the immature dermal DCs are capable of antigen delivery to the LN to initiate an immune response *in vitro* and *in vivo* (22-25). Evidence is increasing that delivery of an antigen to LCs may favor cellular immune responses, suggesting that the route of vaccine administration, e.g. patch application versus ID or subcutaneous injection, could have a major impact on the type of immune response (24, 26, 27).

The TC vaccination is a simple method for delivery of antigens to the abundant APCs present in the tissue. The large surface area of the skin is easily accessible, which makes the targeting of the skin immune cells more feasible. Vaccination through the skin could even improve the response compared to conventional IM strategies. Previous studies have shown that cutaneous vaccination by ID injection could induce the same immune response using smaller doses of antigen compared to the muscular route (28, 29). This could be because of a higher number of resident APCs and an easier availability of APCs in the skin tissue in comparison to muscular tissue.

It has been shown that TC vaccination is a safe and tolerable method, and effectively induces antibody responses in animal and human studies (21, 30-36). The TC vaccination's benefits are derived from the simplicity of the method and its minimally invasive nature. Therefore, TC vaccination could facilitate mass vaccination in

developing countries and during epidemic outbreaks. Yet, few topical application systems have reached clinical trials so far (37, 38). Studies in the vaccination field mainly focus on the humoral response as a sign of adequate immune response. However, the cellular response can contribute to an effective immunization especially against intracellular pathogens.

Translational work related to TC strategies has just begun, because the vaccine penetration in sufficient amounts is a challenge. Since the skin is exposed to the external environment, the barrier of this tissue is responsible for impeding the penetration and invasion of foreign pathogens (39, 40). The SC of the skin is the main physical barrier of the skin, which prevents pathogens from penetrating the tissue. The lipid components of the skin barrier have limited permeability to hydrophilic macromolecules including hydrophilic proteins, DNA plasmids and other vaccine compounds. This is especially interesting with regard to the fact that many, newly developed vaccines contain large molecules or particle structure. Hair follicles (HF) as physiological breaks in this barrier are widely accepted as important reservoir structures and penetration pathways for large molecules and particles (41, 42). Since the SC has discontinuations in the lower HF infundibulum, accumulation of particles in the HF canal, brings them in close contact with abundant LCs around the HF infundibulum, hereby creates a suitable environment for uptake of the topically applied vaccine (43-45).

In order to overcome the barrier and enhance the penetration via the TC route, different approaches could be used to facilitate the antigenic-delivery across the SC. A concentration gradient between the applied antigen and the skin could facilitate passive diffusion into the skin. Hydration of the skin leads to transient separation of lipid bilayers in the SC intercellular area, and improve penetration of water-soluble substances (46). In fact, increased water content in the SC decreased electrical resistance of the barrier and were reported to form aqueous pores in the SC (47, 48).

The single and short-term pulses induced by electroporation (EP) create microchannels in the cell membrane or within the lipid layer of the SC. These pores then act as local transport region for delivery of the molecules into the cells and also increase the permeability of the skin (49-53). The antigen-specific immune response induced by TC immunization using EP could even be equivalent to those induced by

ID injection (51). Although EP needs specific devices, it is a promising method to improve vaccination with plasmid deoxyribonucleic acid (DNA) (54). Iontophoresis promotes penetration through the SC (55-57). Combination of EP and iontophoresis makes the substance penetration and cellular uptake even more effective (58). The problem with these methods is that they increase risk of infection through changes in the barrier function of SC and are not practical in developing countries due to device requirements. Sonophoresis disrupts the SC structure by using low-frequency ultrasound. Application of this method for TC immunization have shown to induce an antigen-specific immunoglobulin G (IgG) antibody response by activating immunocompetent cells. However, sonophoresis requires power-supplied equipment and there are still concerns about its safety regarding the extent of barrier disruption especially in poor hygienic environments (59-62).

1.3.1. Patch systems

For TC antigen delivery, several mechanical and chemical enhancing technologies are being used (8). Patch systems are TC application methods that use adsorption, passive distribution and diffusion mechanism to cross the SC. They were shown to enable penetration of antigenic proteins through the SC (63), and induce antigen-specific immune responses (21, 64-67). E.g., patch-based TC methods with whole inactivated viruses such as influenza (68) or herpes simplex virus (69) can induce humoral and cellular immune responses. Moreover, intact *Escherichia coli* (*E. coli*) particles were used to activate APCs and T cells, thereby acting as natural adjuvants for TC usage (70). Since penetration of vaccine compounds across an intact skin barrier is frequently limited, different mechanical and physical pretreatment methods are being explored to improve the delivery of large molecular compounds through the skin SC into the viable epidermis and the dermis.

1.3.2. Cyanoacrylate Skin Surface Stripping

Mechanical techniques include mechanical abrasion, such as shaving or friction, sandpapering, and stripping, including conventional adhesive tape stripping but also cyanoacrylate skin surface stripping (CSSS) (8). In contrast to conventional tape stripping using multiple adhesive tapes, CSSS combines the tape stripping method

with application of a small amount of superglue. The advantage is an increase of removed SC material, and probably more importantly, an effective opening of HFs. The CSSS method is a safe and tolerable method, which decreases the thickness of the interfollicular SC and enhances the transepidermal penetration by opening the HF orifices through removal of debris and sebum from HF orifices (43). In order to improve penetration, CSSS procedures could disrupt the SC before patch application (21, 65, 67, 71). TC vaccination using CSSS enhances particle penetration into the skin and induces migration of APCs after minor SC removal (72). By limiting damage to the skin barrier this method is much less invasive than microneedles or ID injections and could help reduce risks associated with use of needles. The CSSS method enhances the transfollicular penetration, and improves the cellular uptake by cutaneous APCs (73-77). Transfollicular penetration via HF into the skin has also been reported for nanoparticles (77-79). Penetration through the HF and also the interfollicular epidermis are shown to be effective for antigen delivery by different methods. Combining all of these methods could promote penetration of protein or vaccine compounds into and across the SC (63). As one example, the gene expression and plasmid DNA uptake increased when HF is in the induced anagen stage (80).

Despite increasing knowledge in the field of immunology we still need more information to find the exact host-organism interaction. The selected route of vaccination, the candidate antigen and the carrier will shape the immune response. In studies performed on animal models, TC penetration of the compounds was shown in the sections of skin tissue (41). The presence of the antigen in the LN after TC administration could suggest the capturing of the antigen by LCs and the migration of activated LCs to the LN for activation of the immune response (41, 81). Experiments on the human skin are necessary since the structure of the human skin is different from that of the animal model.

1.3.3. Microneedles

Complete disruption of the SC barrier by microneedles is another method to promote the penetration of particles by creating local micro-injuries through the SC. Methods using microneedles (including solid, coated, hollow, and dissolvable microneedle arrays) are under development with the goal of creating transient pores in the SC (82).

Basically arrays of micrometer-sized needles designed as microneedles painlessly create a transport pathway through the skin (83). Consequently, the vaccine compounds can penetrate through the SC barrier and deliver the component to skin cells. Different forms of the microneedles offer various applications for cutaneous delivery. Solid microneedles increase the skin permeability and could be used to directly bring the vaccines across the skin barrier. Coating of microneedles with drugs, which dissolve in the skin, or encapsulating the drug on the polymeric particles, could be used for drug delivery into the skin layers. After entering the skin the hollow microneedles deliver the compounds directly into the skin tissue through the holes designed within each needle.

Despite the massive efforts in developing microneedle arrays, the clinical use of them is still limited. The fragments of the conventional microneedle arrays could fracture in the skin. Therefore, microneedles created from biocompatible or biodegradable dissolving material are safer for clinical trials. The biodegradable materials could breakdown into molecules within the cellular environment. These molecules could later be metabolized or eliminated from human body. The biocompatible materials on the other hand are safe and harmless, i.e don't induce an adverse effect in contact with the cells (84). Hence, using microneedles still risks contamination with environmental pathogens, which can insert the skin through the breaks in the cutaneous barrier. In the manufacturing of dissolving microneedles, the technical hurdle is to incorporate the antigen into the matrix of microneedle material without a decrease in antigenicity or compromise in material strength. In the current study pricking of the skin was used to simulate the mechanism of microneedles by creating several minor injuries in the skin tissue. The pricking method applied here is similar to the routine allergy test using 1 mm Lancet.

1.3.4. Clinical studies on transcutaneous vaccination

Large-scale vaccination, especially in developing countries, is facilitated when easier methods are available. Patch preparations and microneedles are among the most promising approaches. Recent research in the field focuses on clinical studies of the utility of patch or microneedles in providing low-invasive vaccination through the TC route. In a clinical phase I trial by Glenn et al. a powerful systemic antibody response

after the application of heat-liable toxin (LT) patch in humans was detected (21). In a phase II trial, they showed that the LT-patch vaccination was capable of reducing the severity of the traveler's diarrhea in 59 recipients (85). In a phase I/II clinical study, Etchart et al. showed a patch containing live-attenuated measles vaccine was able to induce measles-specific salivary IgA. The T cells also produced IFN-γ against the measles virus, which indicates the specific cellular response against the virus. In contrast to the subcutaneous injection, the TC method failed to induce serum antibodies, probably targeting of the skin LCs might activate the Th1-immune response more effectively (65). A hydrogel patch containing diphtheria toxoid (DT) and tetanus toxoid (TT) was used by Hirobe et al. resulted in an IgG increase against DT and TT in humans. This indicates that inducing a humoral response after TC immunization is possible. The application of a second patch increased the antibody response in volunteers who showed no immune response after the first patch, which confirms that repeated application will increase the efficacy of the TC (86).

In phase I trials, the groups of Vogt and Combadiere showed that the inactivated influenza vaccine induce significant CD8 responses after the opening of the HF canal for the TC route when compared to the needle-injection for the IM route (36, 74). The key element of this approach is the opening of the HFs, which was performed using a CSSS to enhance the vaccine delivery to the skin and enhance the uptake by skin APCs.

Various types of microneedles are being used in clinical trials for TC vaccination. E.g. a hollow microneedle, which can inject vaccines into the dermis, has been used by Van Damme et al. for influenza vaccination (87). Because of the more invasive nature of the injecting device, local reactions were more frequent compared to the IM application, but the injury due to microneedles was mostly mild, and also resolved more rapidly. Interestingly the humoral response after immunization detected in volunteers after the microneedles was similar to that of the IM injection, while CSSS seemed to favor cellular CD8 reactions (36). In order to prove the efficacy and safety of the microneedles for TC vaccination several methods and devices have been produced by different researchers (88). These methods and devices will result in more clinical studies to find the more effective method.

1.4. Vaccine design and formulation

To provide an effective vaccination via the TC route, the vaccine compounds should be presented to the skin professional APCs. The size and the chemical characteristics of the compound affect the penetration into the skin. For example, due to the presence of the lipid layers in the SC, penetration of hydrophilic compounds to the skin is limited. As a result, penetration properties and the formulation of the vaccine compounds could have a tremendous impact on their efficacy. Manipulation of the skin barrier could change the integrity of the SC and improve the permeation. Furthermore, degradation of compounds in contact with the skin surface, intercellular or intracellular environment (i.e. in case of DNA plasmid) reduces the efficacy of topical vaccination (89). Especially, in case of DNA plasmid the delivery of the whole compound to the cells is necessary. Encapsulation of vaccine preparation in particulate formulations could improve the antigen stability, enhance the penetration into the SC, and consequently improve the TC immunization.

In order to achieve an effective immunization through the cutaneous route, uptake of the candidate antigen by DCs and/or LCs at the site of inoculation is desired. The structural and chemical property of the particulate vaccine plays an important role in this regard (90). E.g., the size of the particle affects the cellular uptake (higher uptake by cells in smaller particles) (91). The release of the compound over time and the specific design, which results in higher uptake by specific cell populations (for example, APCs), have led these delivery systems to gain great attention in the past few years. Particle-bound antigens trigger the DCs' activation and increase their antigen presentation capacity compared to free antigens. Additionally, particle-based delivery systems could aggregate and remain in HF openings to facilitate the transfollicular targeting and penetration (92). Nanoparticles preferably accumulate in the HF canal for several days. There is a high density of APCs, which upon contact with the antigens can prime immune responses.

It has been shown that most particulate forms could not penetrate the intact skin (93). The physicochemical characteristics of the particles including particle's size and formulation are determining factors. E.g., lipid-based particles like liposomes (94) and especially the ones with enhanced elasticity (e.g. transfersomes) (95) were shown to penetrate the intact skin (96). An interaction between the lipid layers of these particles and flexibility of the particles' structure enhance their penetration through

intercellular junctions of the SC (97, 98). When mixed with SC lipids, flexible liposomes can change the lipid structure of the SC and therefore could promote cutaneous drug delivery after altering the permeability of the skin barrier (99). Liposomes have also been reported to stimulate a TC immune response by acting as an adjuvant (100). Solid nanoparticle on the other hand need enhancing methods for penetration into the skin. Several invasive and non-invasive techniques are therefore being used to enhance the delivery of materials to the skin cells (93).

There are numerous nanoparticle systems available, including biodegradable polymeric poly (lactic-co-glycolic acid) and polylactic acid (PLA) nanoparticles, and biological nanoparticles such as virus-like particles (VLPs) (101-103). Biodegradable particles are known to degrade in the environment into small nontoxic compounds (104). The biodegradable polymers have already been tested in animal models and approved to be used in forms of implants and resolvable sutures in humans, which makes them more reliable options for clinical use (105). The VLPs are mainly produced by transfection of cultured cells with a plasmid encoding capsid protein of a virus. The encoded proteins afterward attach to the lipid layer of the host cells, gain a lipid envelope from the host cell and release in form of VLPs. The plasmid encoding VLPs could produce several antigens or stimulatory factors. The encoded proteins will be incorporated in VLPs structure. Since the formed VLPs don't contain the DNA, they are not able to replicate.

The particles could carry the antigen as a whole through the viable skin layers and follicular pathway, increase the penetration and form a residue of intact particles for uptake by APCs (73). Furthermore, the particulate delivery of antigens improves the adsorption of vaccine by cells, stimulates the activation of APCs and subsequently increases the immune response and enhances cellular stimulation (36). Although the method of particle uptake is different considering particle type, the cell-particle interaction affects the delivery to different cell structures and also presentation to the immune cells.

The TC route has shown not only to induce humoral responses but also the cellular immune response, while the IM administration induced only the humoral response (36). It seemed that the penetration of nanoparticles and the uptake by LCs in the epidermis, most likely by LCs surrounding the HF using the follicular route,

preferentially induced the CD8 response in human and animals (36, 43, 74, 77). Several methods have been used for TC vaccination. However, there are differences with regard to the carrier, the experimental model, and the vaccine compound. Currently studies on the TC route are mainly performed on animal models. Interspecies differences between the skin structure of humans and animals necessitate using a human skin model to validate the results obtained from animal models (106).

In summary, the design of novel nanoparticle-based carrier systems protects the antigen from the external environment and maintains long-term activity through the depot formation in the HF. These properties are favorable to the application of TC vaccine. However, the main adversity in development of novel nanoscale systems for TC is to improve their efficacy to elicit robust immune responses.

1.5. Challenges regarding vaccination against HIV

As outlined earlier, some pathogens are especially hard to target by means of immunization. Preventing and treating infection with HIV still remains a major challenge. The main problem with vaccination against HIV-1 is the genetic diversity of the virus strains, which impede effective vaccination. The HIV-1 genome changes rapidly due to recombination and mutation, as well as, the combination of different strains during co-infection in one individual (107). The whole inactivated organism is too dangerous to be used for vaccination purposes and effective component vaccines are still to be identified. Using an antigenic combination of different parts of the virus not only increases the chance of covering the virologic-diversity but also increases the chance of immune response coverage (101, 108). In order to gain effective immune response, both arms of the immune system should be activated. Cellular immunity is critical to impede the virus replication and the humoral immunity should be activated to produce neutralizing antibodies against circulating viruses.

1.5.1. Novel vaccine candidates for HIV

1.5.1.1. GTU®-DNA plasmid

Genetic immunization is an immune response to an antigenic protein, which is delivered in the form of DNA encoded in a plasmid. The gene transfer unit (GTU®)-DNA, a technology developed by FIT Biotech, Tampere, Finland, is a DNA plasmid

coupled to a nuclear targeting unit to express a specifically encoded gene in a desired cell population (e.g. keratinocytes). By introducing the plasmid-DNA to the skin, the goal is to produce the antigen continuously in the cutaneous tissue to increase the stimulation of the T-cells and to enhance the immune response. The GTU® especially benefits from the bovine papillomavirus function based on the viral E2 protein and multimeric E2 binding sites. These parts were shown to facilitate the expression of the gene in the epithelium (109-111). The GTU®-DNA used in this study is a DNA-GTU®MultiHIV encoding multiHIV fusion protein. This protein contains different parts of HIV-1 to increase the efficacy of the immune response.

In previous studies, the DNA-GTU®MultiHIV vaccine induced an increase in the CD4 cell count and decreased the viral load in HIV-infected patients (112). However, the mechanism underlying the efficacy of the vaccine in the therapeutic clinical trial is still unclear. At the beginning of this project GTU®-DNA-vaccines were among the two candidate vaccines available in good manufacturing practice (GMP)-grade quality for up-scale production.

1.5.1.2. Virus-like particle based HIV vaccine candidates

The VLPs resemble the structure of viruses, but miss the viral genome part. These non-replicating particulate elements are quite stable and can be manufactured easily, and are therefore a desirable alternative to live-attenuated viruses (113). Antigenic proteins could be inserted in the structure of the VLPs, especially for unstable protein structures, to increase the immunogenicity of the vaccine (114, 115). Even multiple HIV-1 antigens can be incorporated in the structure of the VLP. The VLPs are prepared by transfection of a cell line with a plasmid encoding for HIV capsid Env protein. The cells transfected with the encoding DNA-plasmid are able to assemble and release HIV-VLPs (116, 117). HIV Gag VLPs, which present HIV-specific CD4 and CD8 T-cell epitopes, are able to induce T-cell response as well as to stimulate the innate immunity (115, 118-121). The presence of Nef or Env epitopes of HIV in the structure of the particle enhances the efficient induction of cytotoxic T-cells and the immune response (122, 123). Furthermore, the visualization of the VLPs was made possible by chemical linkage of the fluorescent markers to evaluate the cellular uptake (124). This marker is produced by enhanced green fluorescent protein (eGFP) encoding sequence in the designed plasmid-DNA.

1.6. Objectives of the current study

1- To study the possible penetration of the candidate particles through the skin barrier on the human skin explants, the possible uptake of the particles by skin cells and the depth of penetration of the particles.

2- To compare the non-invasive and invasive TC vaccination methods on the penetration into the skin, their effect on target cell population. And to investigate the penetration and uptake of vaccine compounds after administration by TC after CSSS pretreatment, by pricking the skin, and ID injection.

3- To study the stability of VLPs after contact with skin and the possible release of the antigens before uptake by DCs (transmission electron microscopy (TEM) analysis).

4- To evaluate the activation of the APCs by the migration of DCs in a human skin culture model after VLP administration. The migration of DCs evaluated as the first stage of the cascade for stimulation of the immune response.

5- To investigate the efficacy of the plasmid vaccine model for TC use on human skin explants. The penetration of the DNA-GTU®-MultiHIV plasmid was analyzed by detecting the expression of encoded eGFP gene.

6- To analyze the effect of particle formulation on plasmid GTU®-DNA expression in HaCaT cell line.

7- To evaluate PLA particles for delivery of antigen compound on the skin tissue and HaCaT cell line.

Material and Methods

2. Material and Methods

The following tables show materials and devices used during the PhD project.

2.1. Devices

Device	Manufacturer
Analytical Balance, KERN® 770	Kern & Sohn GmbH, Balingen, Germany
Flow Cytometer, FACSCalibur™	BD Bioscience, Heidelberg, Germany
Axioplan Fluorescence Microscope	Carl Zeiss AG, Oberkochen, Germany
Heater Block, QBT	Grant Instruments Ltd, Cambridge, England
Incubator, HERAcell, Heraeus	Thermo Fisher Scientific Inc., Waltham, MA, USA
Inverted Microscope, IX 50	Olympus Corporation, Tokyo, Japan
Cryostat, HM 560 CryoStar	Microm International GmbH, Walldorf, Germany
Magnetic Stirrer, IKAMAG® RCT	IKA®-Werke GmbH, Staufen, Germany
Hemocytometer	Paul Marienfeld GmbH, Germany
Battery Powered Pipette Filler, Pipetus®	Hirschmann Inc., Louisville, KY, USA
Microplate Shaker, MTS4	IKA®-Werke GmbH, Staufen, Germany
Suction Pump, MEDAP P7010	MAQUET GmbH, Rastatt, Germany
Shaker, MR-1 Mini-Rocker	Biosan, Riga, Latvia
Sterile Bench, HERAsafe™	Thermo Fisher Scientific Inc., Waltham, MA, USA
Vortex, MS1 Minishaker	IKA®-Werke GmbH, Staufen, Germany
Centrifuge, Biofuge™ Fresco™ Heraeus™	Thermo Fisher Scientific Inc., Waltham, MA, USA
Centrifuge, Multifuge™ 3S-R, Heraeus™	Thermo Fisher Scientific Inc., Waltham, MA, USA
Zeiss Microscope, EM906	Zeiss, Oberkochen, Germany
Zeiss LSM 700	Zeiss, Oberkochen, Germany

2.2. Consumption materials

Material	Manufacturer
Adhesive Tapes	Tesa SE, Hamburg; Germany
Aluminium Foil, Rotilabo®	Carl Roth GmbH, Karlsruhe, Germany
Cell Culture Inserts, 8 µm	BD, New Jersey, USA
Cell Scraper, 3010	Corning Incorporated, NY, USA
Cell Strainer, 70 µm	BD Bioscience, Heidelberg, Germany
DAKO Pen	Dako Deutschland GmbH, Hamburg, Germany
Disposable Microtome Blades C35	Feather Safety Razor Co., Ltd., Osaka, Japan
Disposable Scalpels	Feather Safety Razor Co., Ltd., Osaka, Japan
Falcon™ 6-Well Cell Culture Plate	BD Bioscience, Heidelberg, Germany
Falcon™ Cell Culture Dish (different sizes)	BD Bioscience, Heidelberg, Germany
Falcon™ Cell culture flask, Easy Flask 25 cm²	BD Labware, Franklin Lakes, NJ, USA
Falcon™ Cell Strainer, Nylon, 70 µm	BD Bioscience, Durham, NC, USA
Falcon™ Conical-Bottom Tubes, 15 &. 50 ml	BD Bioscience, Heidelberg, Germany
Falcon™ Conical Tubes 5 ml	BD Bioscience, Heidelberg, Germany
Falcon™ Disposable Pipets 5,10 & 25 ml	BD Bioscience, Heidelberg, Germany
Lancets	ALK Abelló, Hørsholm, Denmark
Microscope Cover Slips	Gerhard Menzel GmbH, Braunschweig, Germany
Microscope Slides, SuperFrost®	Gerhard Menzel GmbH; Braunschweig, Germany
Needle, Microlance™ 3	BD Bioscience, Heidelberg, Germany
Nunc™ Cell Culture, EasYFlasks™ 75 cm²	Thermo Fisher Scientific Inc., Roskilde, Denmark
Parafilm®	Bemis Company Inc., Oshkosh, WI, USA
Permanent Marker, 404	Edding, Wunstorf, Germany
Pipettes	Gilson, Inc., Middleton, WI, USA
Pipette Tips	Carl Roth GmbH, Karlsruhe, Germany

SafeSeal Tubes, 0,5, 1,5 u. 2 ml	Sarstedt, Nümbrecht, Germany
Super Glue	UHU GmbH, Bühl/Baden, Germany
Trucount™, Absolute Counting Tubes	BD, Heidelberg, Germany

2.3. Chemical substances

Chemical substance	**Manufacturer**
Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L)	Invitrogen, Ltd., Paisley, UK
Anti-BDCA-1 (AntiCD1c) Antibody	Miltenyi Biotec, Bergisch Gladbach, Germany
APC Mouse Anti-Human CD1a	BD Bioscience, Heidelberg, Germany
APC Mouse Anti-Human HLA-DR	BD Bioscience, Heidelberg, Germany
Bovine Serum Albumin (BSA)	SERVA GmbH, Heidelberg, Germany
Calcium chloride Dihydrate	Sigma-Aldrich Chemie, Munich, Germany
Collagenase Type II	Biochrom GmbH, Berlin, Germany
Culture Medium RPMI 1640	Biochrom GmbH, Berlin, Germany
Culture Medium RPMI 1640 w/o Phenol Red	PAA Laboratories, Pasching, Austria
DAPI Nuclear Counterstain	Thermo Fisher Scientific Inc., Waltham, MA, USA
Dispase® II	Roche Diagnostics, Mannheim, Germany
DNase	Roche Diagnostics, Mannheim, Germany
Dulbecco´s Phosphate Buffered Saline (PBS)	Biochrom GmbH, Berlin, Germany
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich Chemie, Munich, Germany
Ethanol	Sigma-Aldrich Chemie, Munich, Germany
FcR-Blocking Reagent, human	Miltenyi Biotec, Bergisch Gladbach, Germany
Fetal Bovine Serum, (FBS)-Gold	PAA Laboratories, Pasching, Austria
Fluorescein Anti-Mouse IgG (H+L)	Vector Laboratories, Inc., California, USA
Fluorescein Anti-Rabbit IgG (H+L)	Vector Laboratories, Inc., California, USA
Hyaluronidase	Sigma-Aldrich Chemie, Munich, Germany

Keratinocyte Growth Medium, KGM-Gold™	Lonza, Walkersville, MD, USA
L-Alanyl-L-Glutamin	Biochrom GmbH, Berlin, Germany
Monoclonal Mouse Anti-Human CD1a	Dako Deutschland GmbH, Hamburg, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie, Munich, Germany
PBS Dulbecco, Without Ca^{2+}, Mg^{2+}	Biochrom GmbH, Berlin, Germany
Penicillin/Streptomycin	Biochrom GmbH, Berlin, Germany
Protein Block, Serum-Free	Dako Deutschland GmbH, Hamburg, Germany
Rabbit Anti-HIV-1 gp120	US Biological, Massachusetts, USA
Sheath Fluid, FACSFlow™	BD Bioscience, Heidelberg, Germany
Texas Red® Anti-Rabbit IgG (H+L)	Vector Laboratories, Inc., California, USA
Tissue Freezing Medium	Leica GmbH, Wetzlar, Germany
Triton X-100	Sigma-Aldrich Chemie, Munich, Germany
Trypsin Powder Substance	Biochrom GmbH, Berlin, Germany
Lipofectamine® LTX & Plus™ Reagent (transfection)	Invitrogen™, Carlsbad, CA, USA

2.4. Particles

Particle	Properties	Manufacturer*
VLP-Pr55Gag-CFDA	Size: 100–160 nm Concentration: 0,75 and 0.31 µg/µl Fluorescent labeling: CFDA Antigen: HIV-Pr55Gag	Wagner et al. University of Regensburg, Germany
VLP-Gag-eGFP	Size: 90-100 nm Concentration: 1 µg/µl Fluorescent labeling: eGFP Antigen: MLV-Gag	Bellier et al. University Pierre and Marie Curie, Paris, France
VLP-Gag-gp140-eGFP	Size: 90-100 nm Particle concentration: 1 µg/µl Fluorescent labeling: eGFP Antigen: MLV-Gag and HIV-gp140 fusion protein	Bellier et al. University Pierre and Marie Curie, Paris, France

PLA-pGTU®-gp140	Size: 242 nm Particle concentration: 15 µg/µl Plasmid: Auxo-GTU®8-eGFP-2A-Luc: 6 µg/ml Antigen: HIV-gp140: 0.045 µg/µl	Verrier et al. Institut de Biologie et Chimie des Protéines, Lyon, France
GTU®-DNA Plasmid vectors	Plasmid: Auxo-GTU®8-eGFP-2A-Luc Size: 8954 base pairs Concentration: 2.91 µg/µl	FIT Biotech, Tampere, Finland

* All candidate vaccines were made available through the CUT'HIVAC project consortium. www.cuthivac.eu

2.4.1. Investigated vaccine candidates

2.4.1.1. Virus-like particles

The VLPs are very promising vehicles to present the antigens to APCs. Multiple antigens can be incorporated in one carrier, and they are capable of inducing strong humoral and cellular responses without the help of adjuvants (119, 125). In the current study, three different kinds of fluorescently labeled VLPs were used as carriers for HIV-1 proteins.

VLP-based immunogens presenting HIV GagPol and Env antigens in the capsid were provided by partners of the EUFP7 large-scale research project CUT'HIVAC. Epitope-enriched HIV GagPol were produced by the Wagner group from University of Regensburg, while murine leukemia retrovirus (MLV) Gag-based VLPs were provided by the Bellier group from University Pierre and Marie Curie, Paris. In addition, corresponding VLPs were loaded with the HIV-1-Env fusion glycoprotein (gp)140.

Fluorescent markers, eGFP or carboxyfluorescein diacetate (CFDA), enabled the visualization of VLPs by flow cytometry and fluorescence microscopy. This design made it possible to localize the particles on skin sections and to investigate cellular uptake of VLP.

a) VLP-Pr55Gag-CFDA

The VLP-Pr55Gag-CFDA was received from Wagner group (Institute of Medical Microbiology and Hygiene, University of Regensburg, Germany).

Particle characteristics

The HIV type I (HIV-1) *gag* gene encodes a polyprotein called Pr55Gag. This Gag protein contains important precursor proteins, which are essential for the formation of the virus. It includes p17 matrix-, p24 capsid-, p7 nucleocapsid-, and p6 linker protein and also small p2 and p1 peptides (Fig. 1). The *gag* gene produces an unprocessed Gag precursor, the Pr55Gag, which can form non-infectious VLPs when expressed in host cells (117).

These Pr55Gag-based VLP are candidate carriers for the delivery of foreign immunogens. In order to produce HIV-1 vaccine candidates, the transfected cells with the designed *gag* gene synthesize the Pr55Gag polyprotein. This protein is capable to attach the inner layer of the plasma membrane, acquire an envelope, and bud in form of VLPs (118). The 100–160 nm sized VLP can be produced with about 80% purity (117, 126).

To evaluate the efficient distribution and penetration of VLP across the skin barrier, fluorescent VLPs were used for tracking in tissue explants. Purified VLPs were labeled with CFDA dye, which was loaded non-covalently on the particulate candidate VLP. The CFDA signals could be recognized by flow cytometry and fluorescence microscopy and indicated the location of VLPs within the tissue or inside the cells.

The VLPs were administered at a concentration of 0.31 µg/µl for penetration experiments and cellular isolation by means of magnetic-activated cell sorting (MACS) (16 cm^2 skin area, in the humidified chamber), 0.75 µg/µl for migration experiments (1-2 cm^2 skin area, in the skin tissue culture plate as described in the chapter 2.16.). The total amount of 6.2 µg/cm^2 (volume: 20 µl/cm^2) for experiments on the humid chamber and 19.5 µg/cm^2 (volume: 26 µl/cm^2) for migration studies were used respectively.

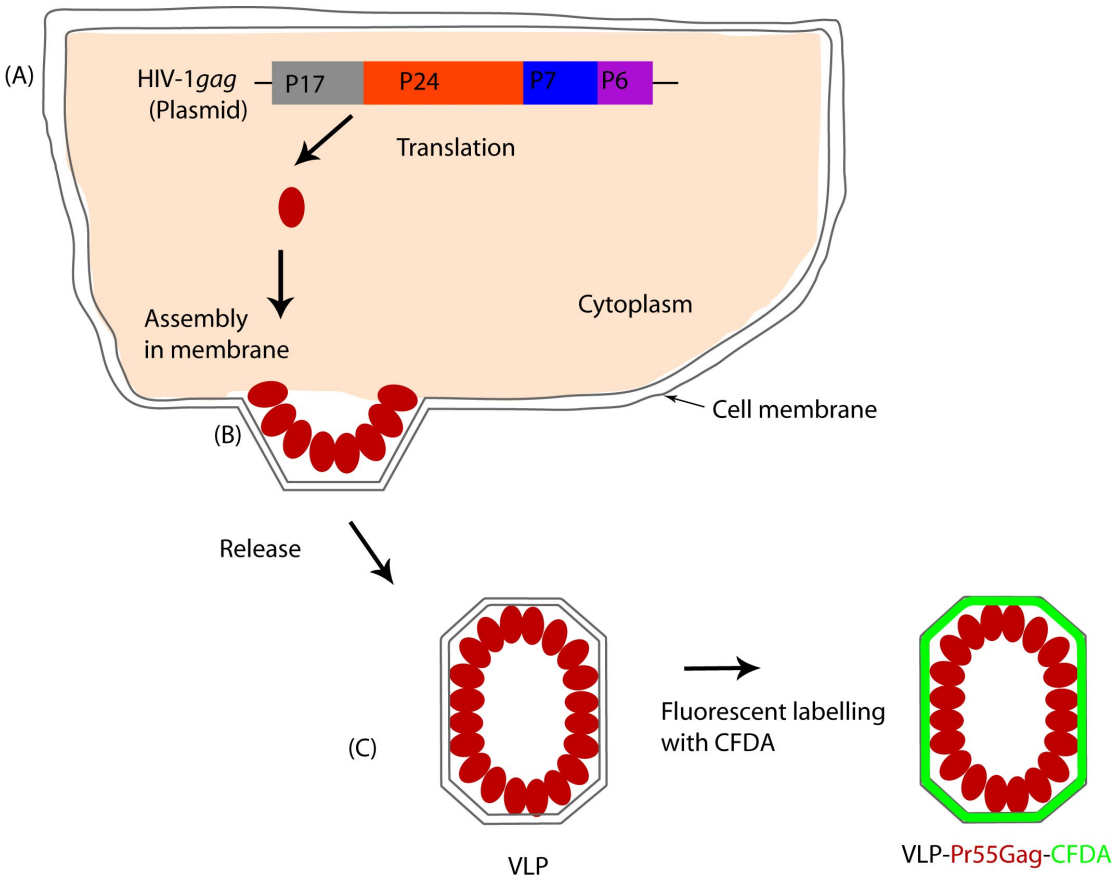

Figure 1. Schematic figure of the HIV Gag polyprotein and VLP-Pr55Gag-CFDA formation: The HIV Pr55Gag polyprotein consisting of the p17 matrix-, the p24 capsid-, the p7 nucleo- and the p6 linker protein (A) includes all information to target to the inner leaflet of the membrane of the producer cell (B) and bud in the form of enveloped VLP (C). (Picture adapted from (113)).

The production process

Media, cells and viruses

A plasmid DNA constructed of chimeric genes encoding Pr55Gag was transferred to baculoviruses *Autographa californica* according to standard procedures described by Wagner et al. 1996 (127). The recombinant viruses then were disseminated on *Spodoptera frugiperda* (SF9) insect cells in TC100 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ml) as described by Wagner et al. 1996 (128). The recombinant viruses then were used to infect HighFive™ insect cells derived from *Trichoplusia ni* egg cell homogenates (Invitrogen Inc., San Diego, CA, USA) (127, 129). The insect cells then proliferated in Insect-Xpress medium (Cambrex, Walkersville, MD, USA), a

serum free medium, supplemented with 100 µg/ml kanamycin sulfate (PAN, Oberasbach, Germany) in 24-27 °C (129).

Purification of VLP-Pr55Gag-CFDA

Contaminated insect cells synthetized the chimeric proteins, and secreted them after creating VLPs into the culture medium. After 3 days, the cell culture supernatant was cleared by centrifuge process (2000 ×*g,* for 10 min at 4 °C). The VLPs were isolated further via centrifuge of the suspension through a 30% sucrose cushion for 2.5 h at 100,000 ×*g* at 16 °C as described by Wagner et al. (127). The concentrated VLPs were resuspended overnight in phosphate buffered saline (PBS) (Biochrom, Berlin, Germany), and were purified by separation on 10–50% sucrose gradients as described by Wagner et al. 1994 (118). Pooled antigenic peak fractions of VLPs were analyzed using separation on a 12.5% denaturing sodium-dodecyl sulfate (SDS) polyacrylamide gel followed by polyacrylamide gel electrophoresis and Coomassie brilliant blue staining to detect the antigens. To detect the proteins produced with VLPs the proteins were gathered after SDS-gel electrophoresis and moved onto nitrocellulose membranes (Millipore, Bedford, MA, USA) by electroblotting in a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). The concentration of produced VLPs was then calculated using a Biorad protein assay (Bio-Rad Laboratories, Munich, Germany) as described by Spech te al. 2008 (129).

b) VLP-Gag-eGFP

The VLP-Gag-eGFP were provided by B. Bellier group (Institute Immunology, Immunopathology, Immunotherapeutic (UMRS-959), University Pierre and Marie Curie, Paris, France).

For this study, the plasmid DNA encoding recombinant MLV-Gag-protein-based VLPs was added to cultured 293T cells (Fig. 2). The cells were transfected using a calcium phosphate transfection protocol and produced the VLPs. After 16 h post-transfection the medium was replaced to ensure cellular vitality. The supernatant medium were gathered after 24 h, filtered through 0.22-µm pore-sized membranes and concentrated with Centricon Plus-70 devices (Millipore, Molsheim, France). VLPs formation was confirmed *in vitro* by electron microscopy. The gene for eGFP was fused to the recombinant plasmid, leads to eGFP production incorporated to the VLPs, which

enables the recognition of particles by flow cytometry and fluorescence microscopy (130, 131).

VLPs were administered at concentration of 1 µg/µl for skin sections studies and migration experiments. Particle volumes of 20 µl were used on one cm^2 (20 $\mu g/cm^2$).

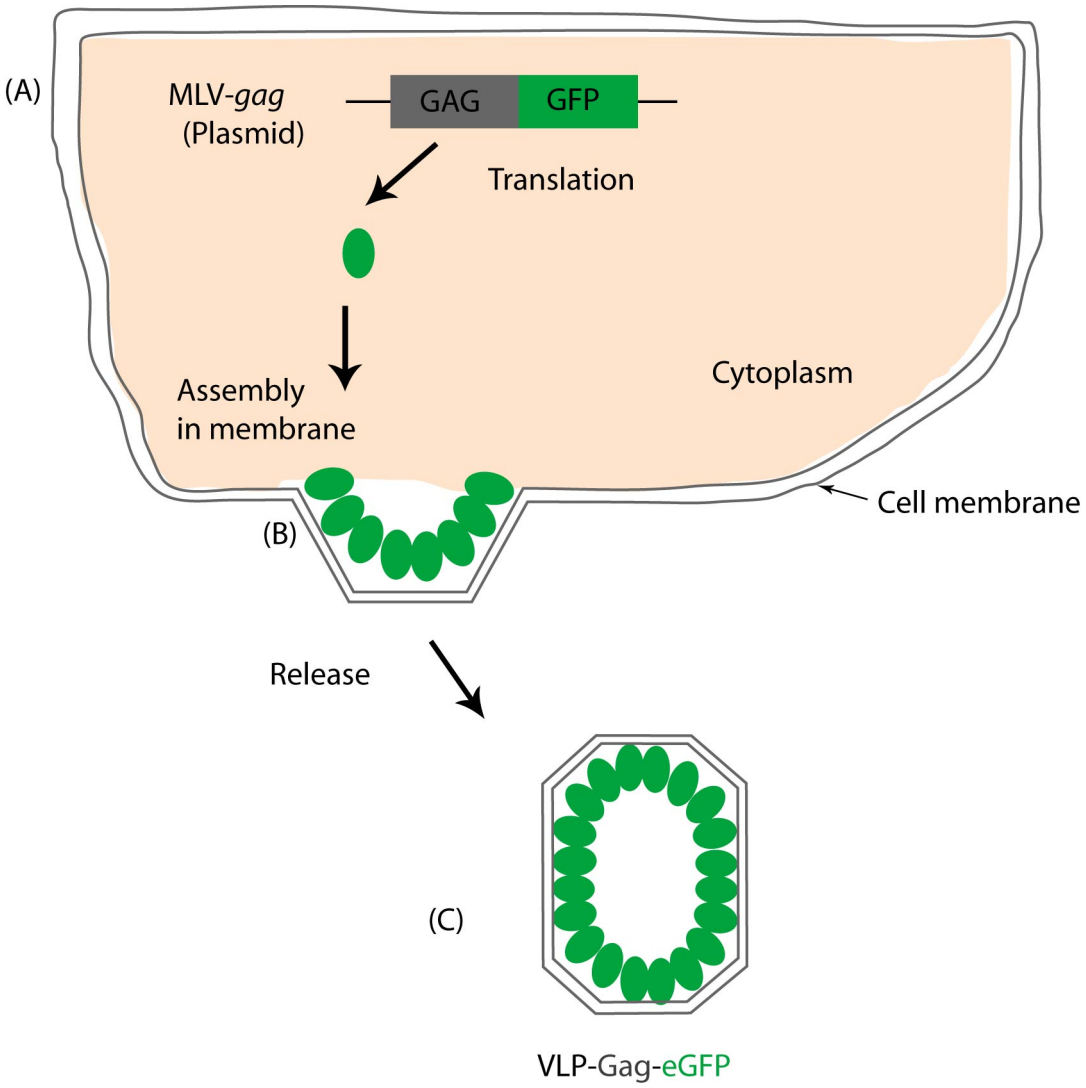

Figure 2. Schematic figure of VLP-Gag-eGFP: A highly immunogenic carrier for the presentation of foreign polypeptides. VLPs are generated by co-expressing the particle forming MLV-Gag protein together with the eGFP protein (A, B). The eGFP protein is located inside the lipid membrane along with the Gag protein (C) (Picture adapted from http://epostersonline.com/aidsvax2013/?q=node/4728&page=4).

c) VLP-Gag-gp140-eGFP

The VLP-Gag-gp140-eGFP was provided by B. Bellier group (Institute Immunology, immunopathology, immunotherapeutic (UMRS-959), Paris, France).

The MLV-based VLPs were generated by transfection of 293T cells with 50 µg antigen-encoding plasmid DNA (2/3 of MLV-Gag protein and 1/3 of HIV $gp140^{TM}$) using a calcium phosphate transfection protocol (Fig. 3). The medium was replaced 16 h after transfection. Supernatants were gathered 48 h later, filtered through 0.45-µm pore-sized membranes and concentrated with Centricon Plus-70 devices (Millipore, Molsheim, France) as described by Bellier et al. the supernatants afterward ultracentrifugated through a 20% sucrose cushion (Sigma-Aldrich, Saint-Quentin Fallavier, France) by 25000 rpm for 2 h. The $gp140^{TM}$ envelope protein is a chimeric form derived from gp160 of HIV clade B (JR-FL). It corresponds to the gp140 part of JR-FL fused to the transmembrane G protein of the vesicular stomatitis virus (VSV-g) in order to facilitate its pseudo-typing. The native conformation of the $gp140^{TM}$ onto VLPs was confirmed by an inhibition test of binding human peripheral blood mononuclear cells (PBMC) with the neutralizing antibody. Since the eGFP gene was incorporated to the plasmid DNA, the VLP could be visualized by fluorescence microscopy (130, 131).

VLPs were administered with a concentration of 1 µg/µl for skin sections studies and migration experiments on whole skin tissue culture model. Particle volumes of 20 $\mu l/cm^2$ were used on administration area.

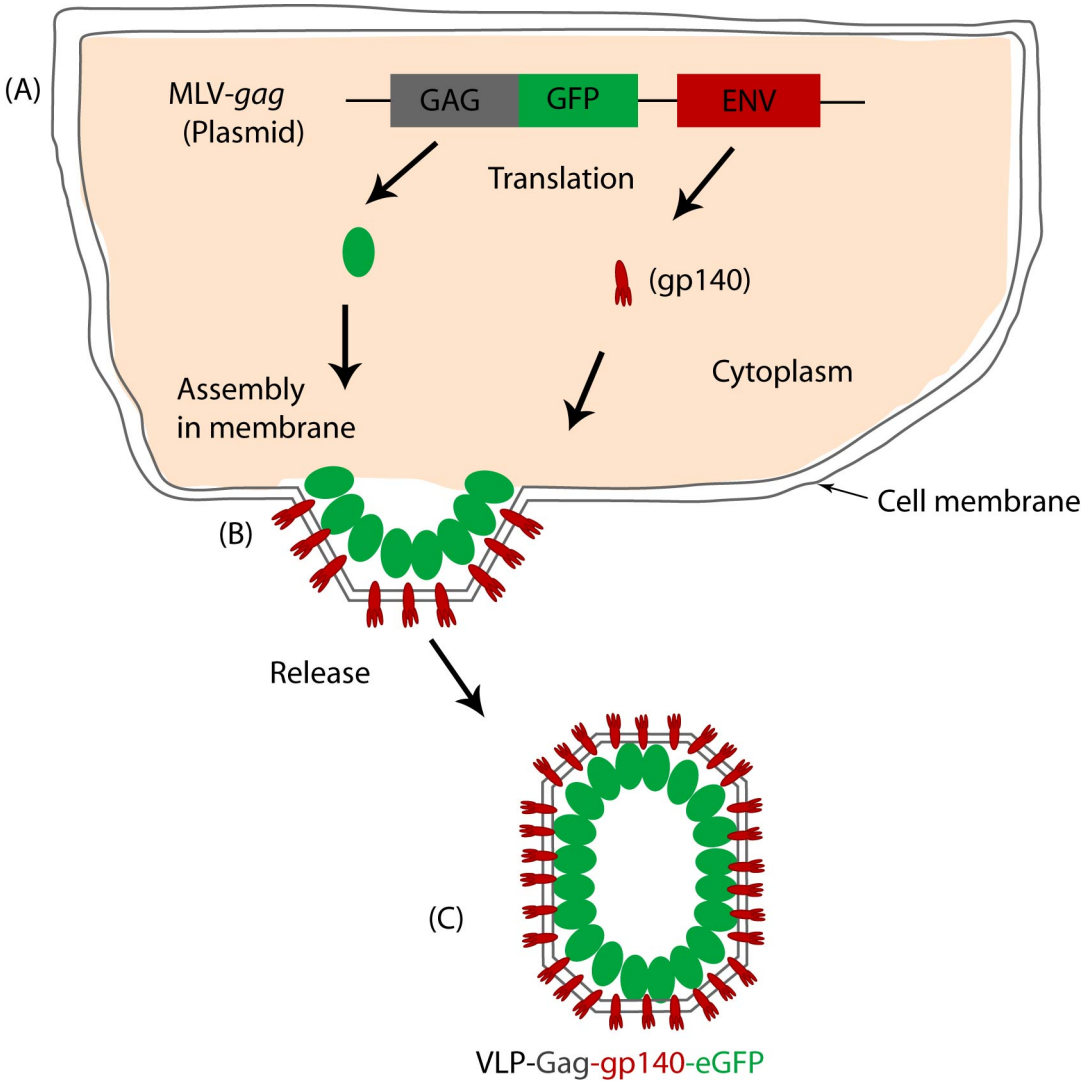

Figure 3. Schematic figure VLP-Gag-gp140-eGFP: Schematic overview to introduce complete envelope proteins into Gag particles. VLPs are generated by co-expression of particle-forming MLV-Gag and eGFP proteins together with the Env protein (A, B). The Env proteins were incorporated at the outer surface of the Gag VLPs in the form of the HIV Env precursor gp140 (C) (Picture adapted from http://epostersonline.com/aidsvax2013/?q=node/4728&page=4).

2.4.1.2. GTU®-DNA vaccine

DNA vaccine constructs plasmid DNA vaccines using the GTU® technology and were developed and provided by FIT Biotech Plc (Biokatu, Tampere, Finland) (109, 111, 132). GTU® is a technology platform that produces a non-replicating DNA plasmid vector, which can be expressed in targeted cell populations. The plasmid DNA was delivered by the partner company FIT Biotech and was designed to be expressed in epidermal cells e.g. keratinocytes. The idea behind this technology stems from the partitioning function of the bovine papillomavirus type 1 (BPV-1) genome. The viral E2

protein of BPV-1, a transcriptional regulator that causes the prolonged gene expression in differentiating cells, is used in GTU® plasmids. This vaccine candidate has genes encoding for HIV-1 capsid and envelope proteins as well as for the eGFP. The detection of eGFP fluorescence after the administration of plasmid DNA to the skin explants indicates successful uptake and expression by keratinocytes. Using this method, the transferred gene can be expressed in the proliferating cells for a long time. Prototype GTU® vaccine containing limited HIV genes was shown to be safe and well tolerated in Phase I and II trials in HIV infected individuals (112, 133). In previous studies, the GTU®-MultiHIV plasmid has been shown to generate strong HIV-specific cytotoxic T lymphocyte (CTL) immune responses in BALB/c mice (111).

The GTU®-MultiHIV plasmid in this study, a plasmid construct encoding multi-proteins from HIV-1, is supposed to be expressed in the differentiating epithelium. The plasmid expresses a recombinant fusion protein including regulatory proteins (Rev, Nef and Tat), structural protein Gag (p17 and p2), and also selected T-helper and CTL rich clusters from Pol and Env polypeptides of the HIV-1. These synthetic gene segments (encoding p17, p24, and CTL) are gene codons, which are optimized to improve their expressions in human cells (Fig. 4).

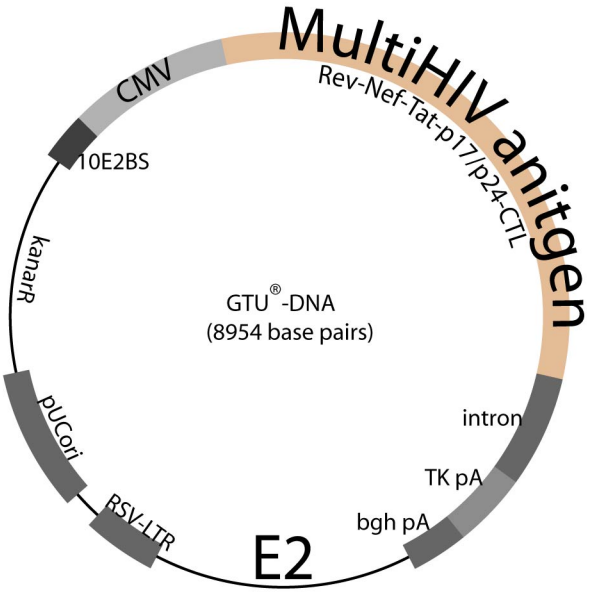

Figure 4. Schematic figure of the GTU®-DNA plasmid vector: (figure adapted from (109)).

Plasmid construction and purification

GTU®-MultiHIV is the synthetic bacterial plasmid, which contains different parts of the HIV-B clade. The multiHIV gene is driven by immediate early promoter of cytomegalovirus (CMV) modified replicon. The vector of the GTU® contains a gene encoding for the BPV-1 E2 site, responsible for nuclear attachment. In addition, vector especially contains high-affinity E2 binding sites from the BPV-1 genome (named as 10E2BS) prior to the CMV promoter.

For effective proliferation in the *E. coli* cells the vector also contains resistance markers of bacterial cells to kanamycin (kanarR), and pUCori (109).

To evaluate the efficacy of the model plasmid that the GTU®-DNA encodes, the gene of eGFP was used to track plasmid expression in skin explants.

The GTU®-MultiHIV plasmid was produced in the *E. coli* as described by Blazevic et al. 2006. Afterward the cells were centrifuged and lysed. The plasmid was purified from the endotoxin by anion-exchange chromatography using Qiagen Ultrapure Columns (Qiagen, Hilden, Germany). Finally the plasmids were dissolved in PBS (2.91 μg/μl) columns according to the manufacturer's instructions (109).

2.4.1.3. PLA-pGTU®-gp140

The PLA-pGTU®-gp140 were provided by B. Vellier group (The Laboratory of Tissue Biology and Therapeutic Engineering (LBTI), CNRS, UMR 5305 University of Lyon, Paris, France). The GTU®-MultiHIV was provided by FIT Biotech (Tampere, Finland) as described previously.

The PLA particles containing the GTU®-DNA vaccine were produced as one option to improve DNA delivery by putting it in a particulate form as an alternative for delivery of complex vaccine compositions e.g. DNA combined with protein antigens. In addition to the plasmid DNA, the HIV Envelop protein gp140 was inserted on the particle surface of the PLA (Fig. 5). These biodegradable nanosystems were used as a delivery system for the GTU®-DNA vaccine model to facilitate the uptake by APCs via TC route. PLA particles possess immune stimulatory properties and assist in TC penetration and cell targeting (134). These particles are taken-up well by APCs and have the appropriate size for penetration through the follicular duct (242 nm). In summary,

proteins and plasmids were added to the PLA solution and gently mixed at room temperature (RT). Since the PLA particles have a negative surface charge, the positively charged proteins attached to the surface of the PLA. The negatively charged plasmid DNA attached afterwards to the surface of the particles.

Synthesis of the PLA-gp140-GTU®

Preparation of anionic PLA nanoparticles

Negatively charged PLA particles were produced as described by Ataman-Onal et al. (135). Briefly surfactant-free PLA was produced using dialysis method described by Jeon et al. For this reason PLA (PLA50, Phusis, Grenoble, France) was dissolved dimethyl sulfoxide (DMSO, Calbiochem, Fontenay-sous-Bois, France). The solution then was dialyzed for 6 h at RT, and continued overnight. When nanoparticles gathered after final washing process no residual DMSO was detected at the final solution. The nanoparticle size was evaluated by quasi-elastic light scattering using a Zeta Sizer 3000HS (Malvern instruments, Malvern, UK).

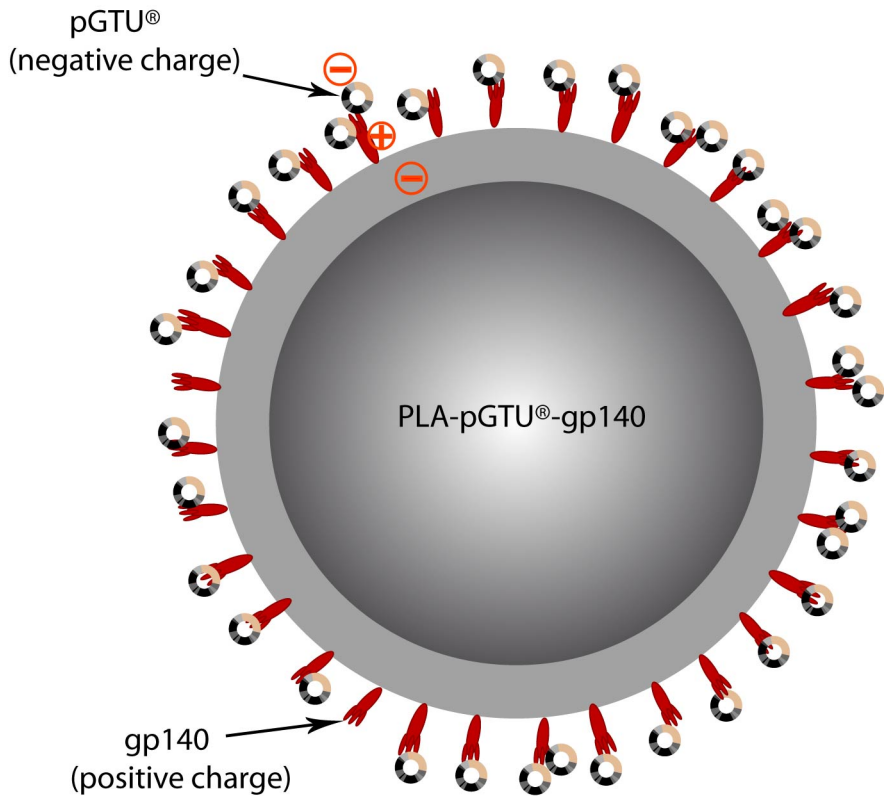

Figure 5. Schematic figure of the PLA-pGTU®-gp140: The positively charged gp140 protein is adsorbed to the negatively charged surface of the PLA particle, while the plasmid (pGTU®) is adsorbed on the positively charged gp140 protein. The PLA particle has no dye incorporated in its structure. For detection of the PLA immunohistochemistry methods were used after the experiments.

Adsorption of gp140 onto PLA particles

The anionic PLA was coated afterward by HIV-1 gp140 proteins. The gp140 was diluted in PBS. The negatively charged PLA was added to the protein solution by and briefly vortexed. The adsorption process to the surface of the nanoparticles occurred over night with mild stirring at RT. The free proteins, which were not adsorbed onto the particles, were isolated from the nanoparticles by 2 times centrifuge (16000 ×*g*, 3 min). The positive charge of the adsorbed proteins (gp140) makes them a suitable bounding site for adsorption of the negatively charged DNA plasmid (135).

Adsorption of plasmid DNA onto PLA-gp140 particles

Different concentration of plasmid DNA was added to the PLA solution in NaCl (final concentration of the PLA 1 mg/ml), the adsorption process performed for 10 min in RT, and the solution was centrifuged (3000 ×*g*, 10 min) afterward. The amount of adsorbed plasmids to the PLA was analyzed after hydrolysis of the PLA particles pellet in NaOH (0.2 M, at 120 °C for 10 min) by means of UV-spectrophotometry at 260 nm as described by Munier et al. (136). The size of PLA-gp140-GTU® particles were determined by Zeta Sizer (136).

Particle characteristics

The investigated PLA particles were approximately 242 nm in size. The concentration of DNA, PLA, and gp140 antigen were 0.006 µg/µl, 15 µg/µl, and 0.045 µg/µl respectively. For penetration studies, in humidified chamber incubation model, particle volume of 20 µl was administered on 1 cm^2 human skin (i.e. 300 µg of PLA particle, which contains 0.9 µg of gp140 and 0.12 µg of DNA plasmid). For *in vitro* studies, 200,000 HaCaT cells were incubated with 1 ml of PLA particles (1.5 µg/µl), previously diluted in RPMI medium.

2.5. Tissue samples

Human skin explants (breast, abdomen, upper arm, and face-lifting) were obtained from healthy volunteers undergoing plastic surgery within 6 h post operation after written informed consent. The study was conducted in compliance with the ethical rules stated in the Declaration of Helsinki principles, and was authorized by the ethics committee of Charité-Universitätsmedizin Berlin [ethic approve No. EA1/135/06]. Skin tissues with any macroscopic or microscopic injury were excluded from experiments, and the

application area was delineated using a permanent marker (Edding International GmbH, Ahrensburg, Germany).

The application area was selected based on the experiment type. A 1 cm² application area was used for both microscopic studies on tissue sections (see chapter 2.16.4.a) and also for migration studies on the full-thickness human skin tissue culture model (see chapter 2.16.). A 16 cm² application area was used for particle penetration studies in the humidified chamber, in order to be able to obtain sufficient cells for subsequent MACS separation and cellular uptake analyses by flow cytometry. For all experiments, 0.5 cm on each border was left as safety margin to avoid non-specific sideway leak of particles into the tissue. Different particle administration strategies were performed on each application areas as described in chapter 2.7.

2.6. Human skin tissue

Three different forms of vaccine administration were investigated in human skin explants. CSSS, skin pricking and ID injection. As described before, CSSS represents the least invasive route by opening HF and decreasing SC thickness. Skin pricking (see chapter 2.7.2) sets multiple epidermal/dermal vaccine depots while ID injection delivered the entire amount of vaccine to one single site in the dermis. CSSS was performed using cyanoacrylate (super glue, UHU GmbH, Germany). Candidate VLPs were applied on the samples afterward. For pricking, particles were applied on the skin surface and distributed on the application area. Pricking was performed after distribution using disposable 1 mm tips lancets (ALK Abelló, Hørsholm, Denmark), with approximately 1 mm interspaces between each disruption point. For ID administration, 20 µl/cm^2 of the solution was injected (one injection of 20 µl volume in each 1 cm^2 skin area) using 1 ml insulin syringe (Becton Dickinson GmbH, Heidelberg, Germany) as described in chapter 2.7.3. Samples were incubated (37 °C, 5% CO_2, 20.95% O_2, 100% humidity) 16 h in a humidified chamber for penetration experiments or 40 h in whole skin culture model for migration studies. In order to remove the rest of particles from the skin surface and reduce the possibility of particle uptake during tissue digestion procedure, tape stripping was performed 5 times on CSSS and pricked samples. For cryosections, 1 cm^2 sample tissue was divided into 4 pieces and frozen in liquid nitrogen. Cryosections (5-6 µm thickness) were studied using fluorescence microscopy and selected for immunohistochemical staining.

2.7. Particle administration routes

Three different administration strategies were evaluated for TC application. The methods help the vaccine candidate to bypass the SC and penetrate the skin. Barrier bypass methods explored were TC administration after CSSS, barrier disruption after pricking of the skin, and ID injection.

2.7.1. Cyanoacrylate skin surface stripping

The CSSS was used as a non-invasive method, which opens the HF orifices and simultaneously reduces the SC thickness (90). One CSSS was performed as previously described (137). In summary, after cleaning the skin surface with PBS a droplet of cyanoacrylate super glue (UHU GmbH, Germany) was applied on 1 cm^2 of the skin surface followed by application of an adhesive tape and was massaged 10 times using a soft seam roller to improve the adherence. The tape was removed after 20 min and the candidate vaccines were applied on the selected area.

2.7.2. Pricking

In the case of pricking, after cleaning the skin surface with PBS, particles were applied in form of tiny droplets on the marked application area. Pricking was performed through each droplet using 1 mm metal lancet (ALK-Abelló, Hørsholm, Denmark) with approximately 1 mm interspaces between each point of injury (100 pricking/cm^2) in accordance with clinical standard procedures for allergy testing. Particles were subsequently applied and distributed over the skin tissue.

2.7.3. Intradermal injection

The ID injection was performed using the Mantoux technique. For this method the skin tissue was stretched and fixed on a block of expanded polystyrene foam covered by an aluminum foil and a parafilm layer. To prepare the injection an insulin specific syringe (1 ml), was filled with 20 µl of the solution. Since the volume is small, first 20 µl of the solution was put on the surface of a microscope slide using a pipette. Afterward, the needle was inserted in the droplet bevel downward. The plunger back on the syringe was pulled carefully to ensure only insertion of all 20 µl solution but not the air. The filled syringe was then used for ID injection. For ID injection, the needle was inserted

bevel upward and parallel to the skin surface into the tissue under the epidermis. The vaccine was injected (one injection of 20 µl compound per 1 cm^2) slowly to create a raised papule, which immediately appeared after injection of the vaccine. Successful injection, mainly depends on the experience of the person performing the injection, and will results in local insertion of the volume within the dermal tissue (138). The ID injection creates a point of pressure at the site of injection, which could help the particle suspension or the DNA plasmid to diffuse into the epidermal layer.

2.8. *In vitro* studies on excised human skin for particle penetration

After vaccine administration, samples were incubated in two different ways, in a humidified chamber for 16 h, or in a skin culture model for 40 h (see chapter 2.16.). For studying the penetration and the cellular uptake in epidermis and dermis, especially by CD1a-positive cells, 16 h incubation in the humidified chamber (37°C, 5% CO_2) was used primarily. Afterwards remnant particles were removed from the skin's surface, using tape stripping (5 times), to reduce the possibility of particle uptake during tissue digestion and cell isolation. The epidermis and dermis layers detached from each other, cells were then isolated from each layer (see chapter 2.9.) and the CD1a-positive cells separated utilizing MACS technique using anti-CD1c antibodies (see chapter 2.10.). Tissue sections were prepared and analyzed for microscopic evaluations.

In order to study the migrated cells, a method was established for gathering the cells that migrated out of the skin using a human skin culture method (see chapter 2.16.). After particle application, the skin tissue was incubated for 1 h in the humidified chamber (37 °C, 5% CO_2) to permit particle penetration through the barrier as well as HFs. The skin tissue was then placed onto an insert, which was located in 6-well plates filled with the keratinocyte growth medium-gold™ (KGM-Gold™) (keratinocyte basal medium plus 0.5 ml hydrocortisone, 0.5 ml transferrin, 0.25 ml epinephrine, 0.03 mg/ml gentamicin, 0.015 mg/ml amphotericin-B, 0.052 mg/ml bovine pituitary extract, 0.015 µg/ml human epidermal growth factor, insulin, Lonza, Walkersville, MD, USA). Due to the fat layer under the skin, the tissue stayed on the surface of the medium (see Fig. 12A). After 40 h of incubation (37 °C, 5% CO_2) remnant particles were removed from the skin's surface, dermal and epidermal cells were isolated (see chapter 2.9.) and stained with anti-HLA-DR antibodies. Cells migrated across the inserts through 8 µm pores were collected and after staining with HLA-DR marker evaluated by flow

cytometry analysis. Tissue sections were evaluated by means of fluorescence microscopy.

2.9. Isolation of epidermal and dermal cells from human skin tissue

The cell suspension from epidermis and dermis was obtained for analysis of particle uptake by flow cytometry or fluorescence microscopy as described by Peiser et al. 2003 (139). After incubation of the skin tissue explants and removing the remnant particle from the skin surface, the treated area were cut in small pieces (2×2 mm). The dermis and the epidermis layer of the skin tissue separated by placing the pieces in 2.4 U/ml dispase II (Roche, Mannheim, Germany) in RPMI solution (Biochrom GmbH, Berlin, Germany) for 2 h at 37 °C in the incubator. Afterward, the epidermis was gently detached from dermis using a fine-tip forceps, and epidermal and dermal cells were isolated from each layer as described below.

2.9.1. Epidermal cells

The epidermal sheets incubated with 10 ml of trypsin solution (0.2% trypsin, 1.5 mM $CaCl_2$ in PBS) for 10 min, 37 °C. The process stopped by adding 10 ml RPMI (plus 10% FCS). The epidermal sheets and the solution were mixed using 25 ml pipette to detach the cells from the epidermal tissue. The cell suspension was passed through 70 µm mesh (cell strainer, BD Bioscience, Heidelberg, Germany) into a new Falcon tube to remove the undigested pieces of epidermal tissue. The singular cell suspension then was centrifuged (184 ×*g*, 10 min) at 4 °C, supernatant removed and the cell pellet was resuspended in 500 µl MACS-buffer (PBS, 10 mM EDTA, and 5% BSA).

2.9.2. Dermal cells

The small pieces of the dermis was digested with 5 ml of enzyme cocktail (0.6 g/ml collagenase II, Roche, 0.3 g/ml hyaluronidase, and 1 µl/ml DNase in RPMI medium) for 2 h, at 40 °C. After digestion 5 ml PBS was added to the suspension and the cells isolated further by mixing the suspension with a 25 ml pipette. The cells separated from the undigested parts of the dermis tissue by passing through a 70 µm mesh as described by epidermal cell isolation (see chapter 2.9.1.). The solution then was centrifuged (362

×*g*, 15 min) at 4 °C, supernatant removed and the cell pellet was resuspended in 500 µl MACS-Buffer (PBS, 10 mM EDTA, and 5% BSA).

2.10. MACS separation and cell labeling

Cell separation was performed using anti-BDCA-1 (anti-CD1c) antibodies on the isolated epidermal and dermal cell suspension according to the manufacturer's recommendation (Milteny Biotec, Germany). Skin samples from 4, 6, and 3 donors were investigated for penetration studies on TC after CSSS, pricking of the skin, and ID administration respectively. Because CSSS targets epidermal APCs, pricking targets both epidermal and dermal APCs and ID sets dermal depots, focus of these first experiments was put on the following respective cell populations. In 4 treated samples with CSSS, CD1c cells from the epidermis were analyzed. On pricking studies, CD1c-positive cell-separation was performed on both epidermal and dermal cells. And with 3 ID treated donors, dermal DCs were investigated after separation by MACS technique.

Briefly, using MACS separation method the CD1c positive cells of the epidermis (LCs) were isolated from previously isolated cells according to the manufacturer's recommendation. For this reason, the epidermal cells were resuspended in 400 µl MACS-Buffer (PBS, 10 mM EDTA, and 5% BSA), marked with magnet marker (100 µl FcR-Blocking Reagent, CD19-Beads and anti-BDCA-1-Biotin antibody) and mixed for 12 min at 4 °C. Afterward 10 µl APC-anti-human CD1a antibody were added to the suspension and mixed (20 min, 4 °C). The labeled cells were kept in a vial while passing a magnetic field. Thereafter, the isolated LCs were washed with 5 ml PBS, centrifuged (362 ×*g*, 7 min, 4 °C), and resuspended in 400 µl MACS-Buffer. The MACS separation repeated for two times to ensure 90% enrichment of the LCs.

When no MACS separation was performed on the cells isolated from the epidermis or dermis, the APCs were labeled with anti-CD1a-APC or HLA-DR-APC antibody. The isolated cell suspension already resuspended in 500 µl MACS-Buffer (PBS, 10 mM EDTA, and 5% BSA) was incubated with 10 µl of the antibody (anti-CD1a-APC or HLA-DR-APC) for 20 min at 4 °C.

2.11. *In vitro* uptake experiments on isolated cutaneous cells

The epidermal layer of human foreskin explants was separated from the dermis after

dispase digestion (2.4 U/ml dispase II, Roche, Germany, 2 h, 37 °C). The epidermal cells were isolated after trypsin digestion (0.025% trypsin, 1.5 mM $CaCl_2$ in PBS, 10 min, 37 °C). Isolated cells were incubated with VLPs for 2 h *in vitro* (50 µg/ml, in RPMI 1640, 10% fetal calf serum, including 100 U/ml penicillin and 100 µg/ml streptomycin, 2 h). Cells were washed in PBS, stained with anti-CD1c-APC antibody, and analyzed by means of flow cytometry. Adult human skin and child's foreskin were used for LCs enrichment and processed as described in chapter 2.10. (77).

2.12. Cell culture

Human keratinocyte cell line (HaCaT) were used (Deutsches Krebsforschungszentrum, Heidelberg, Germany) for the studies on PLA particle and cellular transfection with plasmid DNA. The cell line is derived from primary human keratinocytes, and is capable of proliferating and differentiating *in vitro*. The cells were cultured in 75 cm^2 flasks in culture medium (RPMI plus phenol red, 10 µg/mL streptomycin, 100 IE/mL penicillin, 10% FCS, 4 mM glutamine) in incubator (37 °C, 100% humidity, 5% CO_2). When the cells were 80% confluent in the culture medium, they were passaged. The culture medium removed and the cells were washed with PBS. After removing the PBS, the cells incubated with trypsin/EDTA enzyme solution (0.2%/0.02%) for 5 min in 37 °C, in the incubator. The reaction stopped by adding the same amount of RPMI with 10% FCS, the cells detached from the culture flask, and centrifuged (184 ×*g*, 10 min, RT). The cells were resuspended in the culture medium, counted, and were cultured or were used for new experiments. For the *in vitro* experiments with PLA particles 200,000 cells were incubated in 6 well plates in culture medium for 16 h (37 °C, 100% humidity, 5% CO_2). After incubation, the medium was removed from the wells, and the wells were washed with PBS. Thereafter, 100 µl of PLA particles were diluted in 900 µl RPMI medium and incubated for different time periods. After the incubation the medium removed, the cells washed with 100 µl PBS, and the cells detached from the wells after 5 min incubation with 500 µl trypsin/EDTA suspension at 37 °C. The reaction stopped by adding the same volume RPMI/FCS to the wells, the cell scraper was used to ensure harvesting maximal cells from the wells. The cell suspension centrifuged (200 ×*g*, 7 min, 4 °C), and the cell suspension fixed by adding 400 µl of PFA (4%).

2.13. Intracellular immunostaining

The method was used to detect the gp140 loaded on the PLA particles. The fixed cell by the 4% PFA were diluted in PBS and centrifuged (200 ×*g*, 7 min, 4 °C), after removing the PBS, the cells pellet were permeabilized by 500 µl of Triton X-1000 (1%), in 0 °C (on the ice) for 5 min. The cells were washed once with PBS, centrifuged, and resuspended in blocking solution for 1 h to avoid nonspecific binding with the antibodies. Thereafter the cells were incubated with the first antibody (Rabbit anti HIV-1 gp120, diluted 1:100 in PBS/ FCS 5%, RT) for 1 h. This antibody detects the gp120 part of the protein gp140. After incubation with the first antibody, the cells were washed with PBS, centrifuged and incubated with the secondary antibody (Fluorescein Isothiocyanate (FITC) Anti-rabbit, 1:200 with PBS/ FCS 5%, RT). This secondary antibody attaches specifically to the first antibody and makes that visible to fluorescence microscopy. At the end the cells were washed and analyzed by means of flow cytometry and fluorescence microscopy.

2.14. Flow cytometry

Skin samples received from different donors were used to analyze the uptake of applied vaccine candidates using a FACSCalibur™ (Becton Dickinson, Heidelberg, Germany). Minimum of 20-30 × 10^3 events were measured per each cell population. The VLPs (eGFP and CFDA) uptake was detected by a left shift in FL1-H channel, and CD1a or HLA-DR positive cells were detected in FL4-H channel due to APC staining of the antibodies. The results obtained by flow cytometry, were analyzed for uptake of VLPs using the FCS-Express software.

2.15. Microscopy

2.15.1. Fluorescence microscopy

The fluorescence microscope is being designed to detect the light emitted by certain materials after being irradiated with lights from specific wavelengths. Excitation of fluorochromes at specific wavelengths leads to emission light to make up the image. Filters (Olympus®, Japan) allow for selection of wavelengths and selective detection. E.g. for the detection of FITC, a first filter used to get excitation light with 470-490 nm

wavelengths, while a second filter omits unwanted fluorescent signals and leaves only the specific green fluorescent emission in the range of 520-560 nm.

In the current project the presence of VLPs were detected based on fluorescent labels or presence of eGFP as a result of DNA penetration and expression by transfected cells.

Immunofluorescence is another common technique. It involves the use of antibodies to which a fluorescent marker has been attached (Fig. 6). Additionally, further characterization of cells was achieved by immunofluorescence labeling of cells separated from skin or directly on tissue sections.

E.g. an unlabeled primary antibody (e.g. monoclonal mouse anti-human antiserum CD1a) attached to the CD1a receptor of the cell, and was visualized by specific coupling of a secondary antibody conjugated with a fluorescent label (anti-mouse-Alexa Fluor®, or -FITC). For the staining of the gp140 protein on the VLP-Gag-gp140-eGFP or the PLA-pGTU®-gp140 the gp protein was first labeled with anti-HIV-1 gp120 and followed by a secondary antibody (anti-Rabbit/Texas-Red), carrying Texas-Red as fluorescent dye.

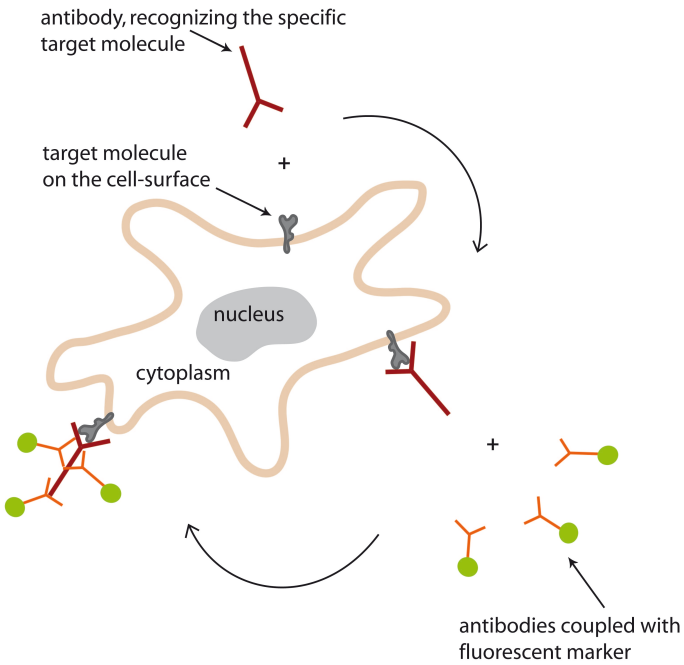

Figure 6. Immunohistochemical staining: The target protein first gets attached to a specific antibody, the second antibody which recognizes the first antibody is labeled with the fluorescent agent. The method is used to amplify the fluorescent signal.

The nucleus of the cells was stained with 4′,6-Diamidino-2-phenylindole (DAPI). In case of GTU®-DNA plasmid the expression of the plasmid was evaluated by evaluating the production of fluorescing molecule eGFP. Each section was photographed in 20, 40 - fold magnification using the program Axioversion (Soft Imaging System GmbH (SIS), Münster, Germany). The processing of the images was done in the by GNU image manipulation software (GIMP), and with Adobe Photoshop 7.0.

2.15.2. Confocal laser scanning microscopy (CLSM)

The uniform illumination of the whole samples by fluorescence microscopy often leads to reduced contrast. In order to obtain high-resolution images, and to confirm intracellular localization of particles, selected skin sections and cell samples were studied by CLSM. The CLSM allows the excitation of the fluorochrome by means of laser, which results in a better-focused fluorescence signal. By this method the illumination of the sample is focused on different parts of the sample and is not simultaneous. The fluorescence from this focal illuminated area returns to the detector, through a filter. The pinhole diagrams on this filter can effectively omit the un-focused light generated by the fluorescence emission of the sample, and simultaneously let the focus light to get to the detector. Image resolution is also improved due to the fact that the sample is scanned.

2.15.3. TEM on skin cryosections

The TEM design is basically like a basically like a light microscope, although it uses electrons instead of light to create higher resolution.

The electrons are produced by a “light source” located at the top of the microscope. The released electrons move in the column of the microscope with a help of a vacuum. The electromagnetic lenses located within the column focus the electrons exactly on the area. The electrons, which pass through the specimen, hit a fluorescent screen at the bottom of the device and produce a shadow image. The different density of the electrons that reach the screen will create an image with different spectrum of darkness (Fig. 7).

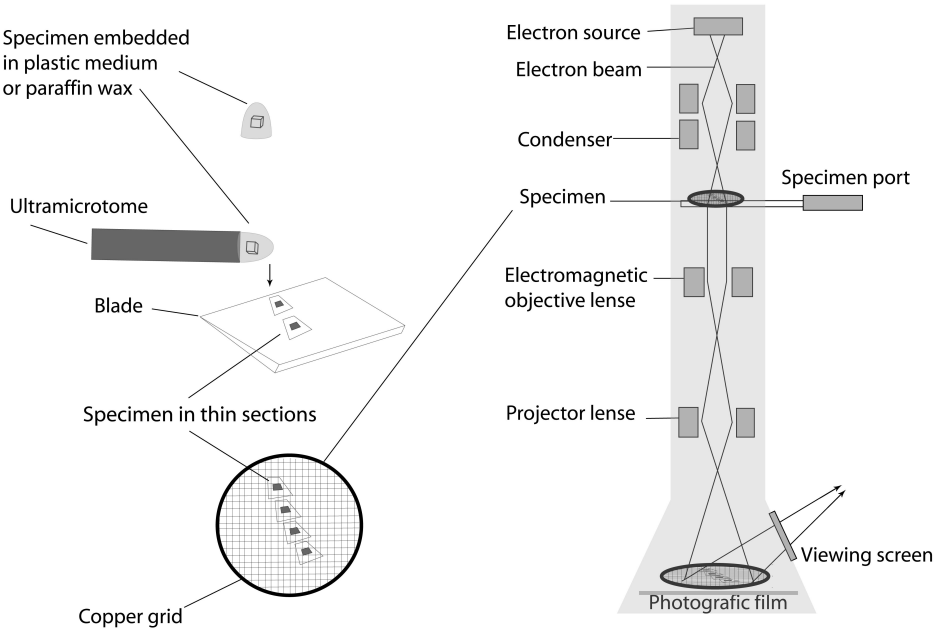

Figure 7. Schematic figure of the TEM and the preparation of the samples: the sample embeded in a plastic medium was placed on a ultramicrotome devive. Very thin section of the sample were put on a copper grid, and were analyzed under TEM (140).

TEM was used to evaluate the presence of the VLPs within the cells and their morphology. For these experiments isolated keratinocytes and the DCs (enriched by MACS separation method), and also the skin tissue after administration of VLPs by pricking were used. The *in vitro* incubation of the tissue was performed as previously described (16 h, humidified chamber). After removing the non-penetrated particles, skin samples were cut into small pieces. For *in vitro* experiments 2.5×10^5 cells were incubated for 2 h with 50 µg/ml VLP in RPMI medium supplemented with 10% FCS. The samples were afterward washed with PBS, and fixed in 2.5% glutaraldehyde and 0.1 Na-cacodylate buffer. The samples were then delivered to the TEM unit of the Charité-Universitätsmedizin (Prof. Bachmann), where they were further processed. First samples were refixed with 1% osmium tetroxide. For dehydration, the samples were washed afterward with ethanol at increasing concentrations (50%, 70%, 80%, 95%, and 100%). Thereafter, they were put in mixtures of propylene oxide and epon resin (SERVA Electrophoresis, Heidelberg, Germany) with ratios of 2:1, 1:1 and 1:2 for

1h, and then incubated in pure resin for 12 h. Samples were placed in capsules with fresh resin (48 h, 60 °C), and after polymerization of the resin, the blocks were sectioned and stained with Richardson stain. Ultrathin sections were made (70 nm) and placed in copper grids. The sections were then stained with Reynolds lead citrate (containing lead nitrate (Merck, Darmstadt, Germany) and sodium citrate (Merck)). The prepared sections were examined with a Zeiss EM906 (Zeiss, Oberkochen, Germany) microscope.

2.16. DCs migration studies using a full-thickness human skin tissue culture model

After evaluating the penetration and uptake of particles by APCs located in different layers of the skin, the next step was to evaluate if the particles induced cell migration as a measure of activation and capability to induce immune responses. We developed a novel method to evaluate the cells that migrated out of the skin tissue after particle application on the skin. The aim was to culture the whole skin tissue and gather cells, which migrated out of the skin tissue through 8 μm pores of inserts.

2.16.1. Incubation condition

To achieve this, the skin was cultivated in KGM-Gold™. Comparison of experiments using this model with previous set ups in a humidified chamber revealed no substantial differences suggesting that incubation in the medium did not lead to over-prediction of particle penetration. Preliminary experiments using inactivated fluorescently labeled *E. coli* particles were performed to identify the most suitable experimental conditions for cell migration (type and volume of the medium, incubation time, etc.). To ensure that the cultured skin receive proper amount of culture medium, cell culture inserts (8.0 μm, BD, New Jersey, USA) were used in culture well plates. The migrated cells should actively pass through 8.0 μm pores and gather in the medium beneath the inserts instead of being captured in the skin tissue, or simply shed from the tissue. In summary, 2 cm² undamaged skin area was selected and cut with a sterile scalpel (1 cm² in the center considered as application area for administration of particles and a 0.5 cm safety margin on each border). Subcutaneous fat was removed completely, and skin samples were stretched on expanded polystyrene sheets covered by plastic paraffin film (Parafilm®, Bemis Company, Oshkosh, WI, USA). Particles were then applied to the skin's surface, after one CSSS or before pricking. Pricking or CSSS were performed as previously

described, and for each method a control sample with PBS was prepared and compared to the sample with VLP. Samples were incubated in a humidified chamber (37 °C, 5% CO_2, 100% humidity) for 1 h to allow the VLP to penetrate in the HF and in the pricked areas. Thereafter the samples were placed on 8 µm pore Cell Culture Inserts (BD Falcon™ Cell Culture Inserts for 6-well plates, transparent PET membrane, BD, New Jersey, USA), the 6-well plate was filled with 5 ml KGM-Gold™ (as described by manufacturer's instructions: 2ml in wells and 3 ml in the inserts), and further incubated for 40 h in the incubator. To minimize medium's acidification, 500 µl KGM-Gold™ (37 °C) were added to each culture dish under sterile conditions after 24 h of incubation.

2.16.2. Collection of migratory cells

After 40 h of culture, the inserts were removed with the tissue from the culture wells with forceps. Epidermal and dermal cells were isolated from the skin as previously described. Medium in the culture dish was gathered and centrifuged. The cell pellet was mixed with RBC Lysis Buffer 1X (BioLegend, Fell, Germany) and incubated on ice for 5 min (shake for 2 seconds every 1 min). The reaction was stopped by diluting the Lysis Buffer with 10 ml of 1 X PBS, then centrifuged (184 ×*g*, 10 min, RT) and diluted into 500 ml of MACS Buffer. The number of migrated cells was calculated afterward by flow cytometry (see chapter 2.16.4.C).

2.16.3. Staining of cells isolated from the skin

For penetration studies, isolated cells were stained with anti-CD1a-APC (Pharmingen™ CD1a, APC, BD, Heidelberg, Germany).

For migration studies, staining was performed using anti-HLA-DR-APC (Pharmingen™ HLA-DR, APC, BD, Heidelberg, Germany) as recommended by the manufacturer.

2.16.4. Analysis of skin penetration and cellular uptake

For each skin sample a part of the treated skin was used to prepare cryosections (a), the rest of the skin was used for cell separation (b), and the cells collected from the culture plate were analyzed for migratory DCs (c).

a) Evaluation of tissue sections and immunohistochemistry

The investigated skin area of 1 cm² was spliced into four squares of 0.5 × 0.5 cm². Skin samples were immediately frozen by means of liquid nitrogen. Tissues were sectioned into 5 µm slices, utilizing a cryostat, followed by drying over night at RT before storage. Sections were examined under a fluorescence microscope (Axioplan, Zeiss, Thornwood, NY, USA). For immunohistochemistry, selected sections were fixed with 4% paraformaldehyde in PBS (Biochrom GmbH, Berlin, Germany) washed with PBS and unspecific binding sites blocked using blocking reagent (DAKO blocking reagent) for 1 h at RT. Subsequently, the sections were incubated with mouse anti-human CD1a monoclonal antibody (Dako Deutschland GmbH, Hamburg, Germany) for 1 h in RT. After washing with PBS (3 times), counterstaining was performed with anti-mouse allophycocyanin (APC) conjugated antibody (Vector Labs, California, USA) for 45 min and washed with PBS. Thereafter the sections were stained with the DAPI (1 mg/mL aqueous solution, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 2 min. After 3 times washing with PBS, the sections were dried overnight and observed by means of fluorescence microscopy.

b) Skin cells

The isolated cells from the epidermis and the dermis were evaluated by flow cytometry to detect particle-associated fluorescence as indicator of particle uptake. The different groups of cells were gated based on their HLA-DR presentation. The uptake in different groups of the cells was then analyzed using the FCS-Express (version 3, De Novo Software, Los Angeles, USA) by comparing the arithmetic mean of the samples to the control.

c) Migratory cells

HLA-DR-positive cells were gated, counted and analyzed for VLP uptake. The number of migrated cell was calculated by flow cytometry using Truecount™ Tubes (Absolute Counting Tubes, BD, Heidelberg, Germany) and utilizing the following calculation as recommended by manufacturer:

$$\frac{\text{\# of events in region containing cell}}{\text{\# of events in absolute count bead region}} \times \frac{\text{\# of beads per test}}{\text{test volume}} = \text{absolute count of cell}$$

The migratory cells after lysis of the RBC were evaluated for particle uptake. Particle-associated fluorescence was detected in FL1-H channel. The HLA-DR expression was detected in channel FL4-H. Cells positive for both FL1-H and FL4-H were considered as migratory DCs with internalized VLPs. The data analyzed by FCS-Express (version 3, De Novo Software, Los Angeles, USA).

2.17. Transfection of HaCaT cells with GTU®-DNA plasmid and Lipofectamine®

For *in vitro* transfection studies by means of Lipofectamine® HaCaT cells were prepared as previously described (see chapter 2.12.). The Lipofectamine® LTX-Plus™ reagent from Invitrogen™ (Carlsbad, CA, USA) was used as transfecting agent. The Lipofectamine® creates liposomal particles and is a known transfecting agent for plasmid and gene delivery purposes. One day before the experiment the cells were isolated from the culture plate, and 120,000 cells were incubated in 24 well plates with RPMI medium for 24 h (37 °C, 100% humidity, 5% CO_2). For transfection studies different volumes of Lipofectamine® reagent (2-5 µl) were mixed to 50 µl RPMI medium without phenol red supplemented with 10% FCS. The DNA plasmid (5 or 6.7 µg, 1.5 or 2.3 µl respectively) were mixed in a separate tube with 5 µl Plus™ reagent and 50 µl medium. Thereafter both solutions were mixed (1:1), and incubated at the RT for 5 min. Meanwhile, the medium from the cultured HaCaT cells was removed from each well, and after one time washing with 100 µl PBS, 500 µl RPMI medium without phenol red (supplemented with 10% FCS) were added to the cells. The mixture of the DNA plasmid and Lipofectamine® were added to the HaCaT cells and the samples were incubated for 24 to 48 h (37 °C, 100% humidity, 5% CO_2). For each experiment the control cells were incubated with medium or with the DNA plasmid without Lipofectamine®. The expression of the eGFP protein was measured afterward by fluorescence microplate reader or by means of flow cytometry. The isolated cells were investigated after under fluorescence microscopy.

Results

3. Results

3.1. VLP-Pr55Gag-CFDA

3.1.1. *In vitro* experiments on isolated cells

Uptake of HIV-1 VLP-Pr55Gag-CFDA by epidermal cells of the human foreskin after *in vitro* incubation with VLPs

In vitro experiments were performed using freshly isolated cells from human foreskin explants to evaluate the ability of epidermal cells (especially LCs) to internalize the VLP. The isolated epidermal cells from two different donors, incubated for 2 h with VLP-Pr55Gag-CFDA, were stained afterward with APC-conjugated anti-CD1a antibody, and examined for their capability to uptake VLPs by flow cytometry. Accumulated data, analyzed by FCS-Express, showed a clear shift of fluorescence indicating cellular uptake of VLPs in histograms (Fig. 8A). Both CD1a-positive cells (LCs), and CD1c-negative cells (keratinocytes), showed uptake after incubation with the VLP. The representative histograms of each cell populations are shown in (Fig. 8A). The ratio between the relative mean fluorescence intensity (MFI) of the sample group cells incubated with VLP-Pr55Gag-CFDA compared to the MFI of the corresponding control group incubated with PBS was calculated. A relative MFI higher than 1.1 was considered as indicative for particle uptake. The relative MFI for LCs were 3.80 and 4.21 whereas the relative MFI for keratinocytes were 3.90 and 4.21 in two donors. The histograms of one donor is shown in (Fig. 8A). Fluorescence microscopy of isolated cells further confirmed cellular association of VLP. Particularly the LCs population was loaded with green VLPs (Fig. 8B). The intracellular localization was proven using TEM (see Fig. 9B, C).

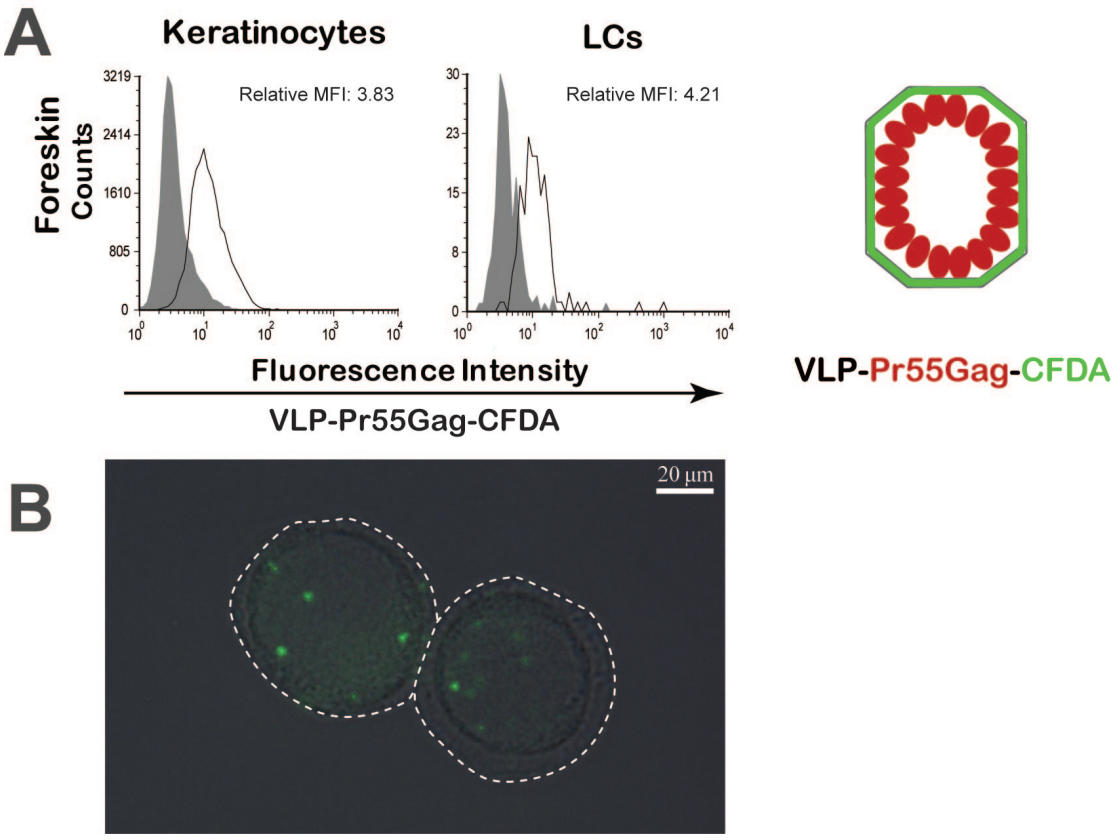

Figure 8. Uptake of VLP by human epidermal cells *in vitro*: Epidermal cells isolated from human foreskin explant were incubated for 2 h *in vitro* with VLP-Pr55Gag-CFDA (50 µg/µl). Representative flow cytometry analysis of epidermal cells shows an increase of cells associated fluorescence in keratinocytes as well as the isolated CD1a-positive LCs (gray = controls; black line = cells incubated with VLP) (A). Fluorescence microscopy shows VLPs (green) uptake of isolated epidermal cells of foreskin incubated with the VLP, which was further confirmed by TEM (see Fig. 9) (B).

Uptake of HIV-1 VLP-Pr55Gag-CFDA by cutaneous epidermal cells of breast skin after *in vitro* incubation with VLPs

To further investigate the capability of human adult skin cells to take up VLPs, epidermal cells isolated from human breast skin tissue were incubated with VLPs *in vitro* (n=2). LCs were enriched for CD1c-positive cells by means of MACS technique from the epidermal cell suspension. After 2 h incubation with VLPs, the cells were harvested, and stained with anti-CD1a-APC. After fixation of the cells by paraformaldehyde (PFA), they were investigated by flow cytometry. Uptake of the CFDA labeled VLPs was monitored in FL1-H channel and the anti-CD1c-APC antibody signal was detected in the FL4-H channel. A small shift was detected in the flow cytometry analysis for LCs as well as keratinocytes (Fig. 9A). The LCs showed higher cellular uptake of VLPs compared to the keratinocytes *in vitro* (LCs; relative

MFI = 4.80 / keratinocytes; relative MFI = 1.72). Under fluorescence microscopy VLPs were detectable both in epidermal cells and LCs, but the amount of VLPs in each cell was less in this adult keratinocytes when compared to the uptake observed in neonatal foreskin cells. This difference is most likely a result of higher functional and phagocytic capability of neonatal vs. adult epidermal cells. Also it has to be noted that detection of low uptake rates is limited due to the small size and low overall fluorescence intensity of the VLPs. LCs and keratinocytes were observed by means of TEM. VLPs were detected in both, keratinocytes and LCs (Fig. 9B, C). Birbeck granules were also detectable in some cells, confirming that the studied cells were LCs. These *in vitro* TEM studies also helped to identify VLPs in tissue sections (see chapter 3.1.2).

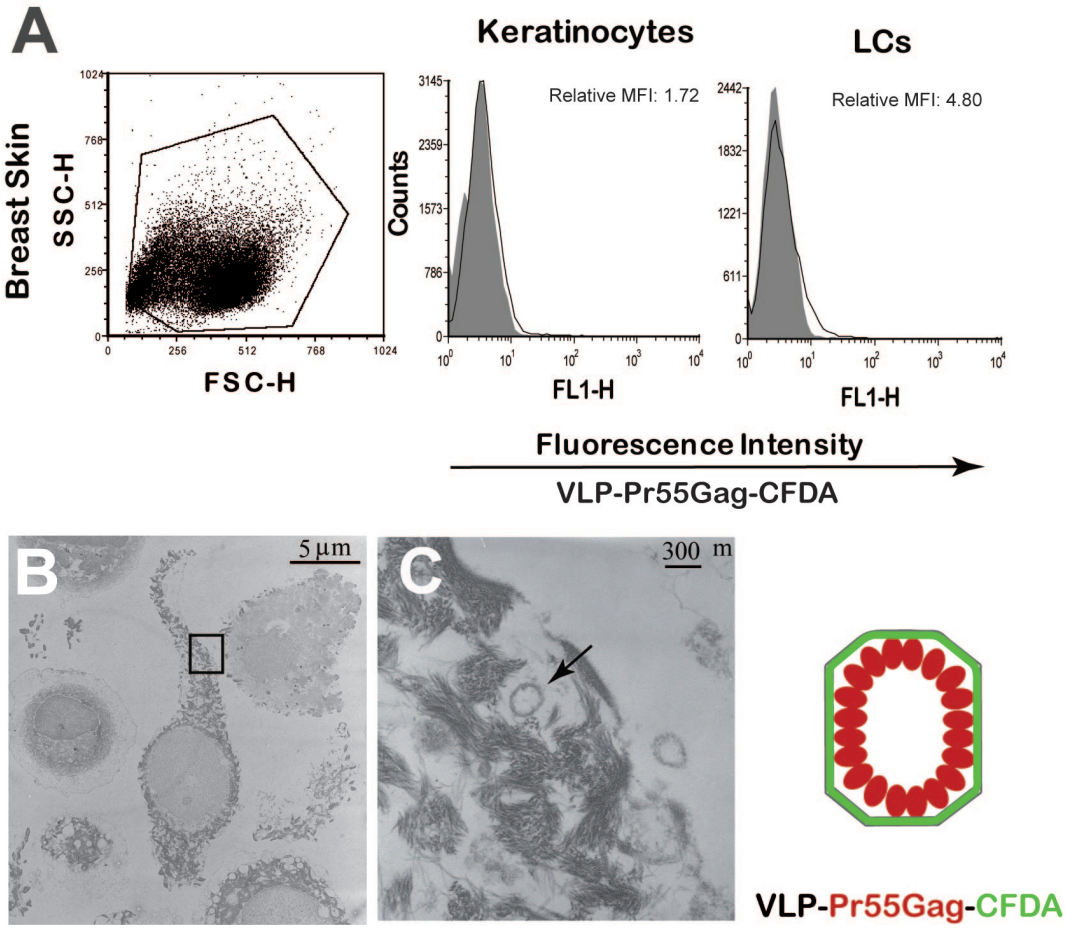

Figure 9. Uptake of VLP by epidermal cells *in vitro*: Representative flow cytometry analysis of epidermal cells shows slight shift in particle-associated fluorescence keratinocytes as well as the isolated CD1c-positive LCs (gray = controls; black line = cells incubated with VLP) (A). Epidermal cells isolated from human breast skin explants were incubated for 2 h *in vitro* with VLP-Pr55Gag-CFDA (50 µg/µl). TEM images of epidermal cells confirm internalization of the VLP particles (arrow) (B, C). The form of the cell in B is suggestive that the cell is a LC. Figure C shows the higher magnification of the selected area in B.

3.1.2. Penetration of HIV-1 VLP-Pr55Gag-CFDA into skin tissue explants

After TC administration of the VLPs, the skin sections were examined for presence of VLPs in the skin tissue by means of fluorescence microscopy. Three different methods were used for administration (TC after CSSS, TC after pricking, and ID injection). The location of the VLPs, on the skin surface or in the HFs, as well as their distribution across different skin layers was observed. Immunohistochemical staining of the cell nuclei (DAPI) and the immune cells (anti-CD1a-APC) also enabled detection of the LCs and DCs in the skin sections.

Following CSSS, the fluorescence microscopy studies revealed an inhomogeneous distribution of VLPs on the surface of the skin. The particle accumulated in the HF orifices, but no deeper penetration in HF infundibulum was detected (Figure 10B). The VLP were detected in the epidermal layer of the skin near LCs. In some sections, the dendrites of the DCs were even detected in close proximity to VLPs (Fig. 10A).

After particle application by pricking, particles showed a tendency to accumulate in the barrier-disrupted areas. VLPs could be detected on the skin surface and at the pricking sites, both in the epidermis and the dermis. The LCs in the epidermis and the DCs in the dermis were both in close contact with the particles (Fig. 10C, D). The study of skin sections under TEM confirmed that the particles were located in the cells as well as within the layers of the SC (Fig. 10F).

After ID injection, particles were mostly found in the ID depot at the injection sites in the dermis and not in the epidermis or near the LCs (Fig. 10E). Interestingly particles were still detectable at the injection site for up to 120 h of incubation, and did not disperse throughout the skin.

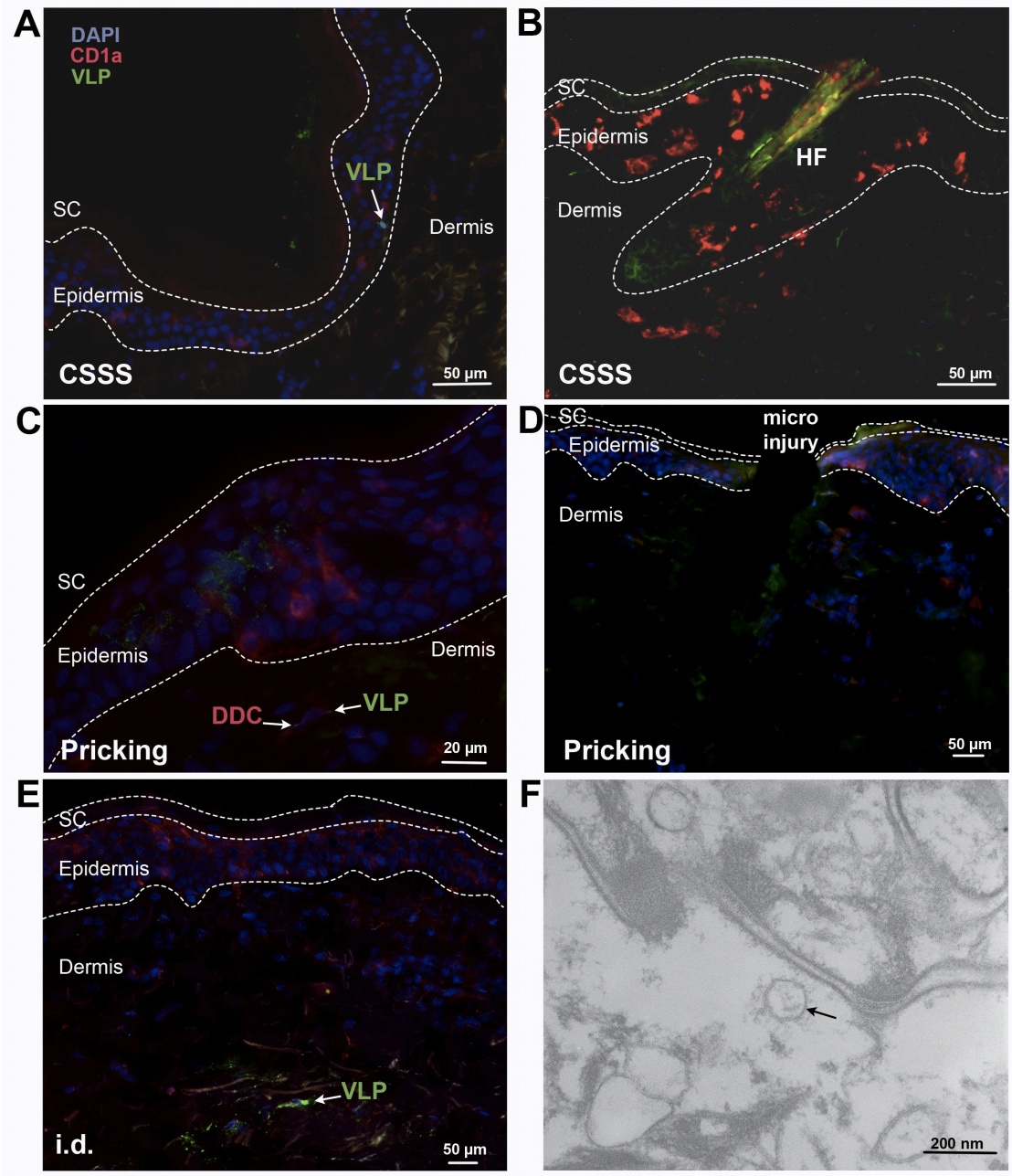

Figure 10. Skin penetration and distribution of VLP after topical application on human skin explants: Fluorescence microscopy of skin sections after topical administration of particles following CSSS (A,B). LCs and dermal DCs were visualized by immunohistochemical staining with anti-human CD1a antibody (Alexa Fluor®, red). Cell nuclei were stained with DAPI. After CSSS, VLPs (green) could be seen on the surface of the SC and near LCs in the epidermis (A), as well as along the HF canal (B). Section of the skin treated with pricking and VLP administration (C, D). CFDA-labeled particles could be found both in the epidermis and dermal layer. Arrows show VLPs near a CD1a-positive DC in the dermis. Image D shows accumulation of VLPs and CD1a+ cells along the pricking injury. After ID injection the VLPs are visible in the dermis (E). The TEM image shows that VLP (arrow) penetrate the SC layer and are still intact 16 h after topical administration (F).

3.1.3. Uptake of VLP-Pr55Gag-CFDA by cutaneous cells

Uptake of HIV-1 VLP-Pr55Gag-CFDA by epidermal and dermal cells following topical administration of VLPs on human skin explants was investigated after incubation in a humidified chamber for 16 h (Fig. 11), and in a skin culture model for 40 h (see chapter 3.1.4. and Fig. 12C).

To investigate if cellular uptake of VLPs was occurring after topical application and penetration across the skin barrier, epidermis and dermis cells isolated from VLP treated tissue samples were analyzed for VLP uptake by means of flow cytometry. The particle uptake by cells from different skin layers was examined following administration of particles by previously described methods, as representative for different ways of vaccine administration in the skin. The representative dot plots demonstrate the flow cytometry of dermal and epidermal cells after 16 h of incubation in humidified chamber (Fig. 11A). Three cellular populations detected in the cells suspensions after staining with anti-CD1a antibody are $CD1a^{high}$ (CD1a++), CD1a+, and CD1a-negative cells. The dot plots also shows the proportions in cell suspension without (w/o) and after MACS technique. The increase in cellular concentration of CD1a-positive cells after MACS could be seen in dermis and epidermis (Fig. 11A).

Following CSSS and 16 h of incubation in the humidified chamber, cellular uptake of VLP was detected both in epidermal LCs as well as the dermal CD1a-positive and $CD1a^{high}$ cells (n=4). Uptake was also detected in dermal and epidermal CD1a-negative cells (Fig. 11B). Donor 1 showed no uptake by CD1-negative keratinocytes (CD1a-), and a low uptake by CD1a-positive epidermal LCs, but VLP associated fluorescence was detected in both dermal CD1a-positive and -negative cells. Donor 2 showed a low particle uptake by epidermal and dermal CD1a- cells, but high particle uptake in CD1a+ dermal DCs, and especially the highest VLPs uptake among all donors treated with CSSS method was detected in CD1a+ epidermal cells of donor 2. The epidermal cells showed a low particle uptake in donor 3, but the VLPs was detected in CD1a-negative dermal cells, still no uptake in CD1a+ dermal cells. Donor 4 showed no particle uptake in any cell populations isolated from dermis and epidermis. Overall evidence of particle uptake by epidermal or dermal cells was detected in 3 out of 4 donors after TC administration of VLPs after 16 h incubation in humidified chamber. The mean MFI was highest in epidermal LCs and then in the

dermal DCs. Surprisingly the CD1-negative cells of the dermis showed higher VLPs uptake when compared to epidermal CD1a-negative keratinocytes.

Following VLP administration and pricking of the skin (see chapter 2.7.2.), VLP uptake was detected in cells isolated from all samples (Fig. 11C, n=6). Interestingly, donors with no detectable particles in epidermal LCs exhibited higher uptake in dermal DCs (CD1a+). Although, donor 1 showed no uptake of VLPs in the epidermal cells, VLPs were detected in both cell populations of the dermis. In donor 2 particle uptake was detected in all cell populations except epidermal CD1a-negative keratinocytes. The uptake was higher in dermal DCs and CD1a-negative cells in donors 2 when compared to the epidermal LCs. In donor 3 the particle was taken up by epidermal LCs and dermal CD1a- cells, but not by epidermal keratinocytes and the dermal DCs. Both epidermal and dermal cell populations of the donor 4 showed particle uptake, although the uptake was higher in LCs and DCs compared to dermal CD1a-negative cells, and only CD1-negative keratinocytes from epidermis showed no VLPs associated fluorescence. Donor 5 and 6 showed particle uptake in epidermal keratinocytes and dermal DCs, but not in the LCs or CD1-negative dermal cells. The results showed VLP uptake in all donors after pricking. In donors treated with pricking method, the highest mean MFI was detected in dermal DCs, dermal CD1a-negative cells, and epidermal LCs, respectively.

After ID, a highest uptake was detected in the dermal CD1a-postive and -negative cells, while no uptake was detected in the epidermal CD1a-positive cells (Fig. 11D, n=3). The donor 1 and 2 showed no particle uptake in epidermal cells, but the VLPs were detected in both dermal cell populations. Although the particle uptake was higher in dermal CD1a-positive DCs compared to dermal CD1a-negative cells in each donor. Hence, donor 3 showed no particle uptake in dermal cells, but only in epidermal keratinocytes. In summary after ID injection the particle uptake was detected in all donors. Dermal DCs, and dermal CD1a-negative cells showed the highest uptake of VLPs, respectively. Uptake was also confirmed by experiments on the skin organ culture model (see chapter 3.1.4.).

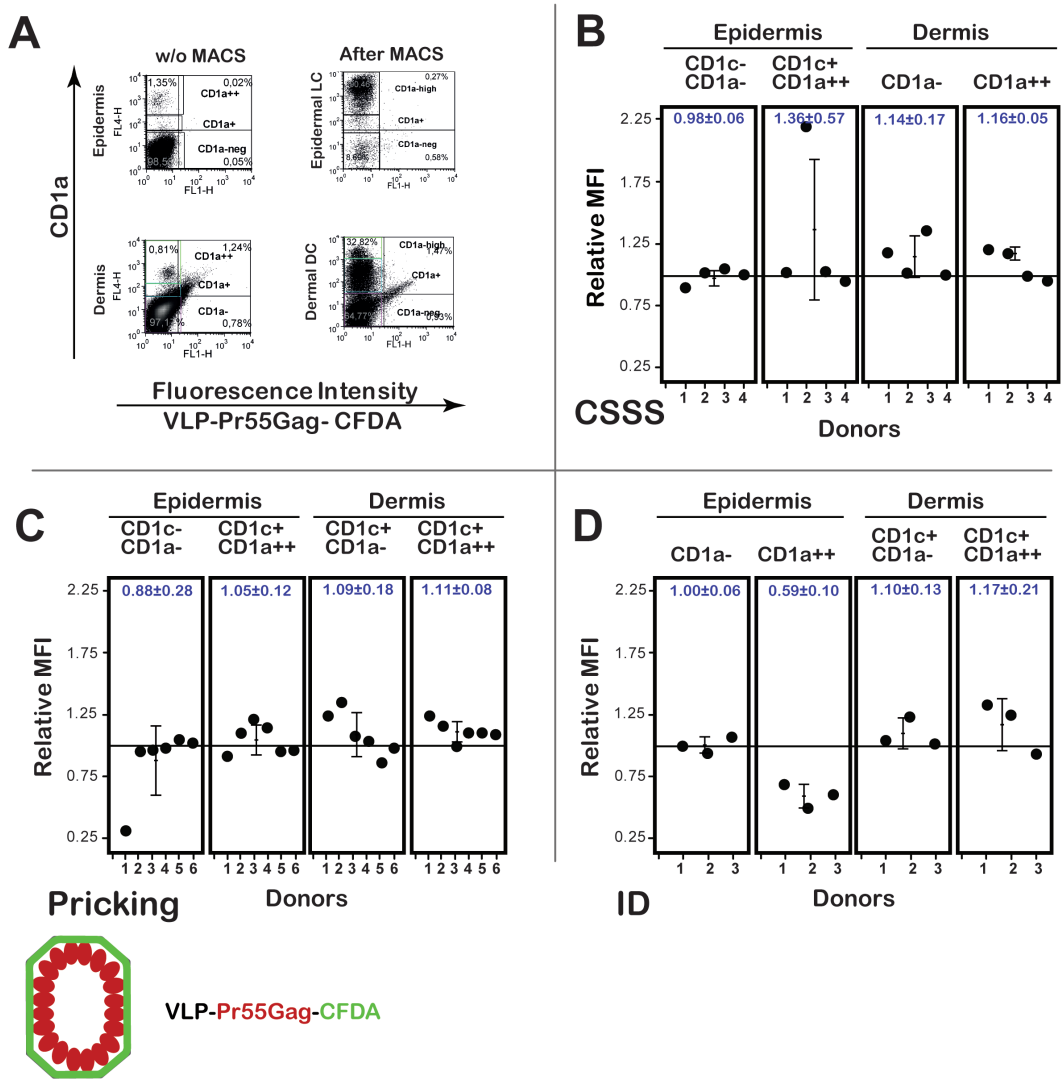

Figure 11. Particle uptake by skin cells after three different methods of skin administration: After topical administration, uptake of particles by non-DCs (CD1a and CD1c-negative epidermal and dermal cells) was compared to uptake by DCs (CD1a^{high} LCs and dermal DCs). After isolation of cells from epidermis and dermis, cells were either stained with an anti-CD1a antibody or separated by CD1c-MACS separation and then stained with an anti-CD1a antibody. Representative flow cytometry analysis of CD1c-positive cells, which were isolated by means of MACS separation from the epidermis and dermis after topical administration of VLPs (A). The dot plots shows the flow cytometry of dermal and epidermal cell suspensions without (w/o) MACS separation and the epidermal LCs and dermal DCs after MACS separation. The gated area of different cell populations within each group has been shown (A). The MFI of the gated cells (CD1a^{high}) for the control has been compared to the particle-treated skin by Relative MFI. Relative MFI values were calculated by normalizing sample MFI to that of the control and plotted in B-D. The relative MFI values are shown for different donors after administration of VLPs on excised human skin after one CSSS (B), by pricking (C) or by ID injection (D), respectively. Cellular uptake by DCs (LCs, and CD1a^{high} dermal DCs) was detected by all three administration methods and in most of the donors. For each cell population average relative MFI± standard deviation (SD) are shown (blue).

3.1.4. Migration of DCs after topical administration of VLP

The results of the penetration experiments, done in the humidified chamber, indicated a possible migration of LCs to the dermis after VLPs uptake. Thus, DCs migration experiments were performed using cultured full-thickness human skin. VLPs containing HIV1-Pr55Gag protein were used as an experimental model vaccine against HIV-1. Migration behavior of the skin APCs was investigated after culturing the whole human skin tissue on 8 μm tissue culture inserts in KGM-Gold^{TM} for 40 h (Fig. 12A). In order to track the migration process across the different skin layers, single cell suspensions were prepared from epidermis and dermis and in addition, migratory cells were harvested from tissue culture media. The results show that HLA-DR-positive cells migrated out of the skin and were found in the medium beneath the cultured skin after 40 h of incubation. HLA-DR protein is a MHC class II cellular marker on APCs, which expresses higher in stimulated and activated APCs. The viability of the cells was examined by the trypan blue exclusion-test, which indicated 80% viability in both the control and VLP treated samples. Similar to the penetration studies in the humidified chamber, VLPs were detected in the epidermal and dermal layers and the corresponding APCs after pricking or CSSS method. Epidermal and dermal cells were isolated after 40 h of incubation and stained with an anti-HLA-DR antibody to mark DCs.

In case of samples pretreated with CSSS method, particle uptake was detected in all donors in epidermis or the dermis (n=6). VLPs were detected in dermal cells in all donors, but only 5 out of 6 showed particle associated fluorescence in epidermal cells (Fig. 12C). In donor 1, minimal particle uptake in HLA-DR-negative epidermal cells, and no particle in HLA-DR-positive epidermal cells were detected. In the dermis the HLA-DR-positive cells showed higher uptake compared to HLA-DR-negative ones. Only donor 2 showed no uptake in epidermal cells. Hence, VLPs were detected in all dermal cell populations with particle uptake slightly higher in HLA-DR-negative cells compared to the HLA-DR-positive dermal cells. In donor 3, VLPs were detected in both epidermal cell populations, but not in the dermal cells. Although, in the epidermis HLA-DR-positive cells showed the highest uptake compared to all other 6 donors. In donor 4, minimal particle uptake in epidermal cells and HLA-DR-positive dermal cells, and a slightly higher particle uptake in HLA-DR-negative dermal cells were detected. In donor 5, VLPs were detected all epidermal and dermal cell

populations except HLA-DR-negative epidermal cells. In donor 6, VLPs were detected in epidermal HLA-DR-positive and -negative cells and HLA-DR-positive dermal cells but not in HLA-DR-negative dermal cells.

In the case of pricking, particles were detected in cells from different skin layers as well as in the migrated cells (Fig. 12C, D). All donors showed particle uptake by HLA-DR-positive and negative dermal and epidermal cell populations except donor 10 that only showed particle uptake in dermal cells. In donor 7 and 8 particle uptake was detected in epidermal and dermal cells, but the uptake was higher in dermal cells compared to the epidermal cells. In the dermis the HLA-DR-positives cells showed higher uptake compared to the HLA-DR-negative cells of each donor. In donor 9, particle uptake was detected in all dermal and epidermal cell populations, although the uptake was higher in epidermal compared to the dermal cells. The uptake was slightly higher in HLA-DR-positives cells compared to the HLA-DR-negative ones in each layer. Only donor 10 showed no uptake in epidermal cells. Hence, the highest VLP uptake was detected in the dermal cells among all other donors treated with pricking, and the particle uptake was higher in HLA-DR-positive dermal cells compared to HLA-DR-negative dermal cells. In donor 11, particle uptake was detected in HLA-DR-positive and -negative cells of epidermis. In the dermis, the uptake was detected only in HLA-DR-positive cell population and not in the HLA-DR-negative ones. In donor 12, particle uptake was detected in HLA-DR-positive and -negative cells of both epidermis and dermis, which was higher in HLA-DR cell populations in each layer compared to HLA-DR negative ones. In donors treated with pricking method, the mean MFI was higher in HLA-DR-positive cells compared to HLA-DR-negative ones in each layer, and the mean MFI of the HLA-DR-positive dermal cell population was higher compared to the HLA-DR-positive epidermal cells. The highest mean MFI were detected in HLA-DR-positive and -negative dermal cells and HLA-DR-positive epidermal cells, respectively.

Following administration after pricking, cells from the dermis and dermal DCs showed higher particle uptake, but surprisingly epidermal cells (EC, and LCs) showed lower uptake or no uptake in different donors (Fig. 12C). After 40 h activated cells containing the VLP had already migrated. In fact, in most donors VLP-positive cells that migrated out of the skin were found in the culture medium.

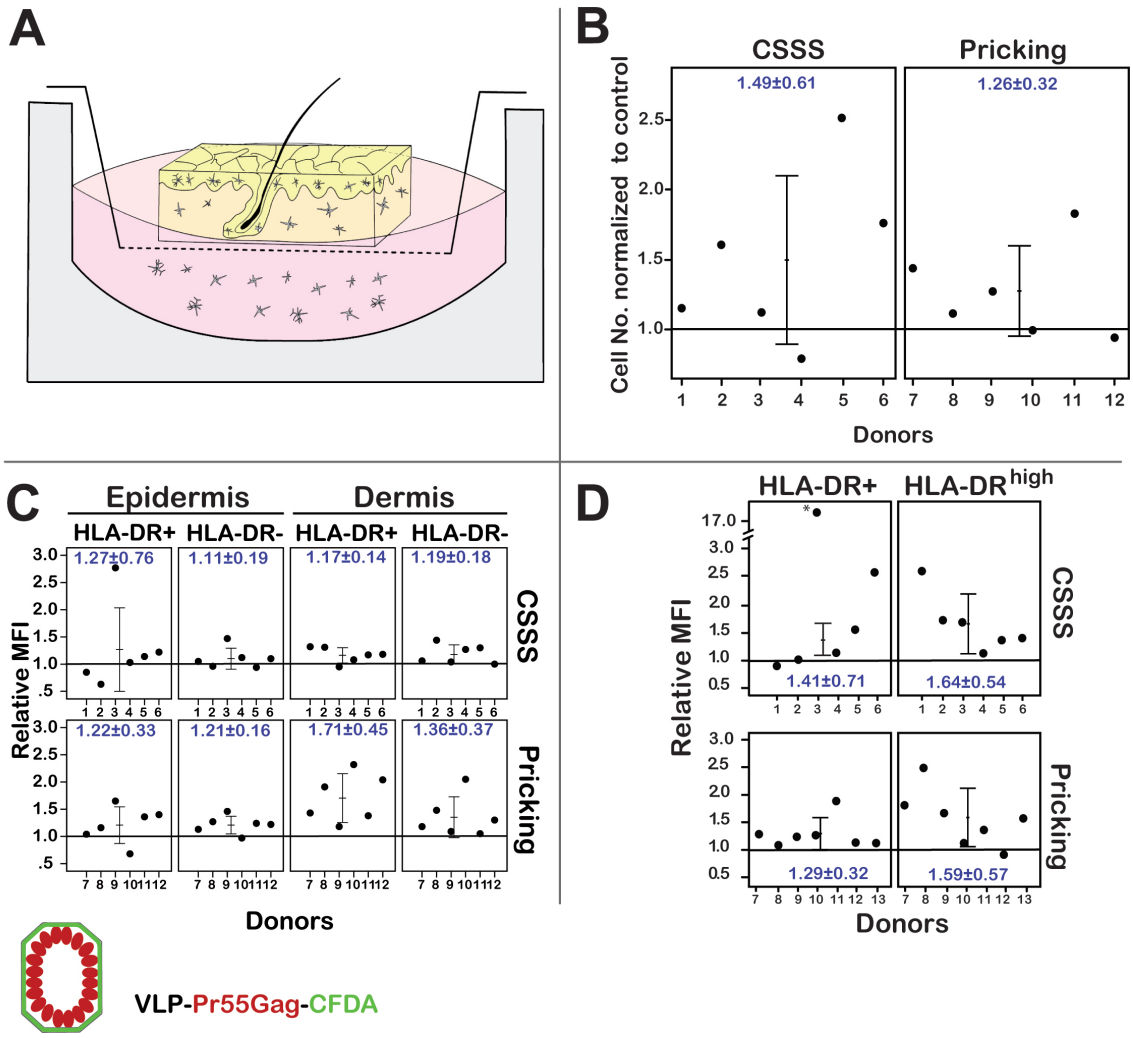

Figure 12. DCs migration after topical administration of VLP: Schematic figure of the established model for skin organ culture and DCs migration (A). Fluorescently labeled VLPs were applied transcutaneously after one CSSS treatment or pricking. After 40 h of whole skin organ culture, cells that migrated out of the skin into the culture medium were isolated, RBCs were lysed, and the cells were stained with anti-HLA-DR-APC antibody. The number of HLA-DR-positive cells was estimated by means of flow cytometry and counting beeds (see material and method). The graphic B reports the percentage of migrated cells with respect to controls. Comparison between the two administration methods is shown. Uptake of particles by HLA-DR-positive and negative cells was analyzed by means of flow cytometry after cell isolation from epidermis and dermis (C). Relative MFI was used to show uptake compared to control cells. Relative MFI higher that 1.1 indicates particle uptake by cells. VLPs' uptake by HLA-DR-positive cells of the epidermis and dermis is shown comparing CSSS and pricking treatments. Uptake of VLPs by HLA-DR-positive and HLA-DR^{high} cells that migrated out of the skin and collected in the acceptor medium (D). For each cell population average relative MFI±SD are shown (blue). The results show that VLPs were taken-up by cells of different skin layers and APCs migrated out of the skin.

A comparison of the two administration methods revealed that cells, which had migrated out of the skin also showed different particle uptake levels (Fig. 12D). Two different populations of APCs were detected among the cells migrated into the culture medium: regular HLA-DR-positive cells, referred to as HLA-DR+, as well as a small population of the cells with very high HLA-DR expression, defined in the following as "HLA-DR^{high}".

Overall both HLA-DR+ and HLA-DR^{high} cells exhibited particle-associated fluorescence. After pricking, higher particle uptake was observed in HLA-DR^{high} migratory cells compared to HLA-DR+ cells in 4 out of 6 donors. Following CSSS, HLA-DR^{high} cells showed higher uptake compared to HLA-DR-positive migrated cells. CSSS and pricking show similar uptake patterns for the different skin cell populations, i.e. slightly lower particle uptake in epidermal HLA-DR-positive cells accompanied with higher uptake in dermal cells especially in HLA-DR-positive cells. Higher particle uptake in the dermal and migrated cells following CSSS could be due to the migration of activated cells after particle uptake away from the epidermis towards the dermis, and finally out of the skin tissue. After CSSS, the particle uptake in the epidermal and dermal layer is generally lower than after pricking, but migrated cells show the same level of uptake of VLPs for CSSS samples as for pricking ones. This result indicates that the CSSS method not only enhances the penetration of VLP into HF and epidermis but also is able of activating APCs and of stimulating migration out of the skin with a similar efficacy as pricking. The particle uptake of different cell populations for 6 donors after pricking and CSSS are shown in figure 12. Interestingly, higher uptake by migrated cells was accompanied by lower uptake in the epidermal and dermal cells, which is in accordance with the assumption that APCs take up VLPs, get activated either by skin pretreatment or particle uptake, and then migrate towards deeper layers until they reach the culture media.

The number of migrated cells was counted using Truecount® beads and flow cytometry, and confirmed increased HLA-DR-positive cell-numbers after skin treatment and particle application. It has to be noted that even in the control samples, which were treated with CSSS or pricking and topical administration of PBS, a number of migrated cells were detected due to physiological activation and migration upon skin injury after plastic surgery. There was no significant difference in the normalized number of cells migrating out of the skin comparing the CSSS (mean

normalized cell number±SD=1.49±0.61) and pricking (1.26±0.32) methods (Fig. 12B).

3.2. VLP-Gag-eGFP

3.2.1. Skin penetration

To investigate the uptake of particles across the skin barrier, the vaccine model VLPs were administered topically on excised skin maintained in culture. The uptake of the VLP, by epidermis and dermis cells was analyzed after pricking (method described previously). In previous experiments in our group, the penetration of the VLP-Gag-eGFP was evaluated on the skin maintained in a humidified chamber comparing the CSSS and pricking administration methods for 16 h and particle uptake was detected after both applications in different skin cell population. The pricking caused local disruptions of SC and local injuries in both epidermis and the dermis. In the tissue sections, the particles could be found in the disrupted areas along the canal formed by needle insertion, in close contact with DCs in both layers. The particles were also found to be unevenly distributed on the SC with preferential accumulation near pricking sites. Following 40 h of incubation in the skin culture model, the uptake of VLP was detected by means of flow cytometry in both epidermal and dermal cells after isolation from the treated skin. The uptake of VLP was detected in higher amounts in HLA-DR-negative epidermal cells compared to the dermal HLA-DR-negative cells. In contrast, the HLA-DR-positive cells from both layers contained VLP, although the dermis HLA-DR-positive cells showed lower uptake compared to the same population in epidermis (Fig. 13).

VLP uptake was detected in samples of all donors. Interestingly, when particle uptake was not seen in the epidermis (e.g. donor 1), it was detected in the dermis. In donor 4, high uptake was detected in the epidermal cell population but the dermal cells showed similar uptake to the other donors. The results obtained from 6 donors are shown (Fig. 13).

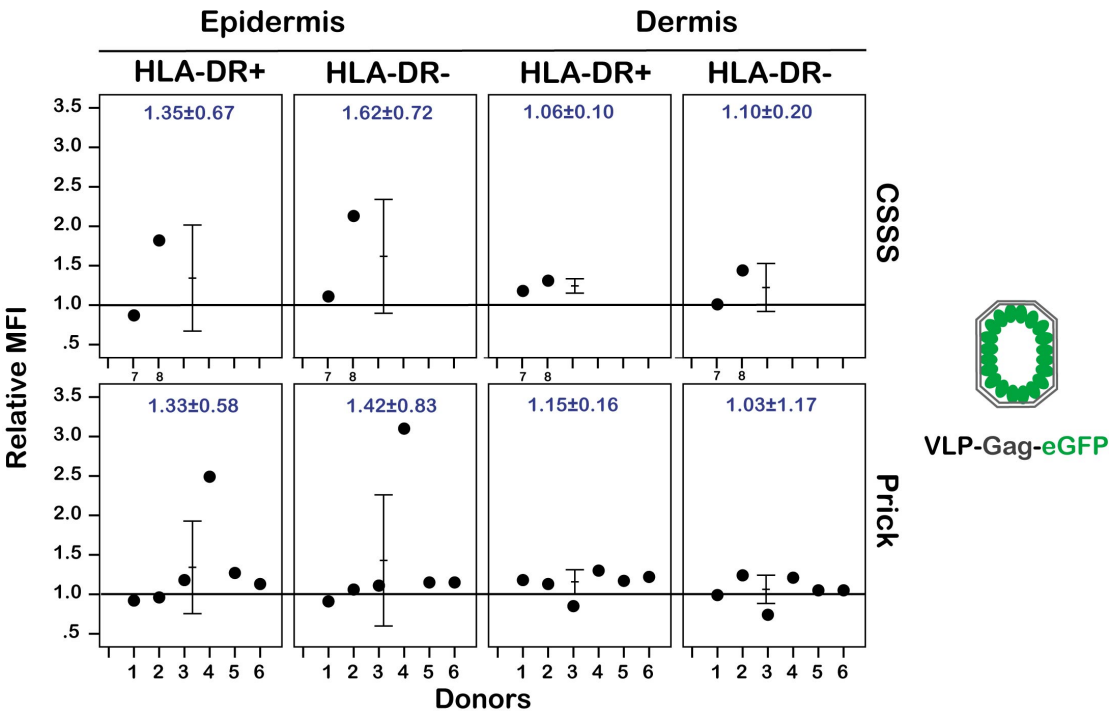

Figure 13. Uptake of VLP-Gag-eGFP by skin cells after TC administration by pricking (n=6) and CSSS (n=2) on human skin culture model: Fluorescently labeled VLPs were applied transcutaneously after pricking. After 40h of whole skin organ culture, cells that migrated out of the skin into the culture medium were isolated, RBCs were lysed, and the cells were stained with anti-HLA-DR-APC antibody. Uptake of particles by HLA-DR-positive and negative cells was analyzed by means of flow cytometry after cells were isolated form the epidermis and dermis. Relative MFI has been used to show the uptake compared to control cells. Relative MFI higher than 1.1 indicates particle uptake by cells. For each cell population average relative MFI±SD are shown (blue). The results show that VLPs were taken-up by cells of different skin layers, however with inter-individual differences.

3.2.2. Migration of DCs

The possible activation and migration of the APCs in reaction to the skin pretreatment and VLP application, was evaluated in the skin organ culture model. The particle uptake by the cells that migrated out of the skin tissue was evaluated after TC administration by pricking. The cells that migrated out of the skin barrier were gathered and stained with HLA-DR marker cells (see material and method). Amongst donors, cells that migrated out of the skin showed different particle uptake (Fig. 14B). In 5 donors out of 6 the particles were detected in the migrated cells. The VLPs uptake was particularly apparent in HLA-DR^{high} positive cells. Only e.g., 2 donors out of 6 donors also showed uptake by HLA-DR+ cells, whereas in 4 out of 6 donors

VLP were detected in activated HLA-DR^{high}. Interestingly, 50% of the donors who showed no VLP in the HLA-DR-positive cells (i.e. donors 1, 2, and 5) showed the highest amount of uptake in the HLA-DR^{high} positive group. Different patterns of uptake were detected amongst the different donors. E.g. donor 3 showed no particles in the migrated or dermal cells, but both cell populations in the epidermal layer were positive for the VLPs.

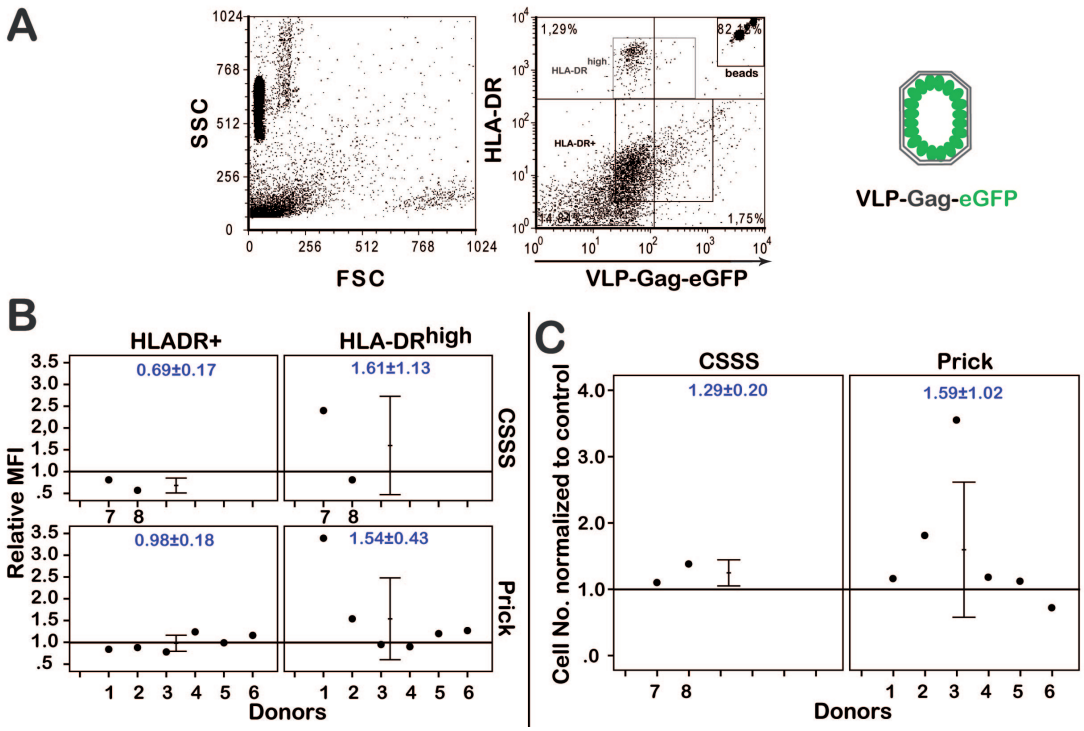

Figure 14. Uptake of VLP-Gag-eGFP by migrated cells after TC administration by pricking on excised human skin maintained in culture media model: Uptake of VLPs by HLA-DR+ and HLA-DR^{high} cells migrated out of skin and collected in the acceptor medium. The results show that VLPs were present in APCs that migrated out of the skin in 5 out of 6 donors, HLA-DR^{high} cells showed higher particle uptake compared to the HLA-DR positive migrated ones. For each cell population average relative MFI±SD are shown (blue).

Possible explanation is different cell activation and migratory capacity of the cells among various donors. Differences in the speed of cellular uptake and subsequent migration can lead to a situation where, at a given time point, cells are still in

superficial skin compartments such as epidermis, while in other samples, upregulation of activation markers and considerable migration towards deeper layers has already occurred. Perhaps VLP uptake after pricking treatment did not initiate the activation and migration process in the donor or the process takes longer in this donor compared to others. In contrast, donor 4 showed the highest particle uptake in the epidermal cells, a moderate uptake in dermal cells compared to the epidermal cells and only uptake in the HLA-DR-positive migrated cells, but not by HLA-DR^{high} ones. Donor 1 who showed no particle in the cells from epidermal layer, showed the highest particle uptake in HLA-DR^{high} positive cells.

3.3. VLP-Gag-gp140-eGFP

3.3.1. *In vitro* experiments on isolated cells

Uptake of VLP-Gag-gp140-eGFP by isolated epidermal cells detected after *in vitro* incubation with VLPs.

Epidermal cells isolated from adult human skin were used to investigate their general ability to internalize the VLP. LCs were sorted as CD1c positive cells by means of MACS separation from the epidermal cell suspension. The LCs and keratinocytes were incubated for 2 h with VLPs. The epidermal cells were stained with APC-labeled anti-CD-1a, and investigated by flow cytometry. The data showed that the uptake of the VLP could be detected by flow cytometry. Uptake of the eGFP labeled VLP was recognized through a shift in cell population in the FL1-H channel and the CD1a marker (APC: red) was detected in the FL4-H channel. An obvious shift was detected on the histograms from flow cytometry of LCs samples (Fig. 15). The ratio of the relative MFI of the sample group of cells incubated with VLP-Gag-gp140-eGFP to the control group (incubated with PBS) shows the uptake (rel. MFI= 25.77). Epidermal cells isolated from the human foreskin explants were also analyzed, and the LCs population showed a significant VLP uptake (data not shown).

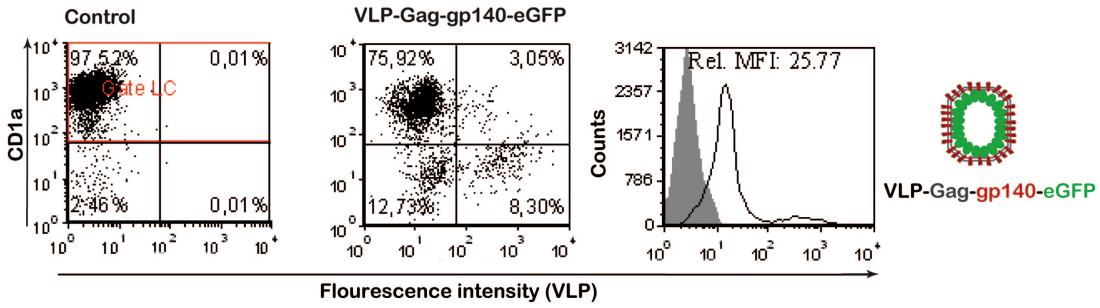

Figure 15. Uptake of VLP by human epidermal cells *in vitro*: Epidermal cells isolated from human abdominal skin explant were incubated for 2 h (50 µg/µl, 2 h, 37 °C, incubator) *in vitro* with VLP-Gag-gp140-eGFP. LCs were enriched by CD1c-MACS separation. In dot plot, the cells incubated with the VLP shows an obvious shift to the right. The histogram analysis shows particle uptake in isolated CD1a-positive LCs (gray = controls; black line = cells incubated with VLP).

3.3.2. Skin penetration

Skin penetration profile of VLP-Gag-gp140-eGFP on the skin explants after a topical application combined with skin pricking was evaluated in the skin organ culture model, which was maintained in cell culture for 40 h. Compared to the previous VLPs, VLP-MLV-based particles contain GFP coupled to the Gag protein within the particle, which was meant to increase specificity of detection compared to secondary labeling with CFDA. Furthermore, gp140 was incorporated in the outer membrane resulting in a more complex virus mimic compared to the previously studied construct. The hypothesis was that this construct even increase uptake and/or cell migration.

The VLP-Gag-gp140-eGFP carrying the HIV-1 envelope protein (gp140) is considered another promising particle-based vaccine candidate for TC immunizations. After 40 h of incubation, the isolated cells from the epidermis and the dermis were investigated for particle uptake using flow cytometry (n=6). Pricking causes disruption of the barrier making particles accessible to skin cells. The particle uptake was variable amongst donors, which was potentially caused by inter-individual differences. In donor 1, the HLA-DR-negative cells of the epidermis and the HLA-DR-positive cells from the dermis contained the VLP. In donor 2, dermal HLA-DR-negative cells and HLA-DR-positive cells from both layers showed the uptake. Donors 3 and 5 showed particle uptake in the epidermal cells (HLA-DR-positive and

negative), but not in the dermis. Donor 4 and 6 showed particle uptake in all cell populations of the different skin layers (Fig. 16).

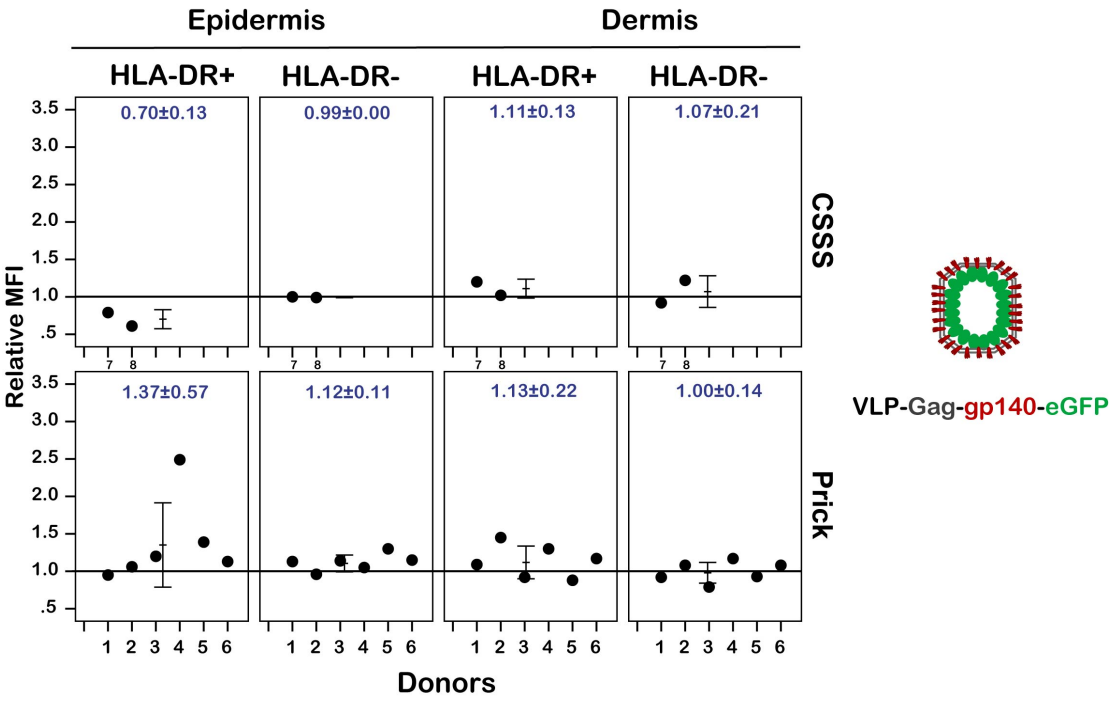

Figure 16. Uptake of VLP-Gag-gp140-eGFP by skin cells after TC administration followed by pricking on a human skin culture model: eGFP labeled VLPs were applied topically followed by pricking. Uptake of particles by HLA-DR-positive and negative cells was analyzed by means of flow cytometry after cells were isolated form the epidermis and dermis. Relative MFI was used to show the uptake compared to control cells. A relative MFI higher than 1.1 indicates particle uptake by cells. The results show that VLPs were taken-up by cells of different skin layers. For each cell population average relative MFI±SD are shown (blue).

3.3.3. Migration of DCs

The effect of TC application of VLP-Gag-gp140-eGFP on activation and migration of the APCs was investigated using the human skin tissue culture model. The particulate form and special design of the VLPs lead to uptake by the cells. In the current study, the activation and migration of cells was investigated using a skin organ culture model (n=6 for pricking samples and n=2 for CSSS). The cells that migrated out of the skin tissue were gathered and stained with HLA-DR marker cells (see material

and method). The presence of the VLP in the cells that migrated out of the skin tissue into the culture medium was evaluated using flow cytometry. Relative MFI has been used to determine the uptake compared to control cells. Relative MFI higher than 1.1 indicates particle uptake by cells. In this study, 5 of the 6 donors showed VLP uptake in migrated cells. The donors showed different uptake patterns. In donor 1, VLPs were present in both HLA-DR-positive and high positive cells with higher uptake in HLA-DR^{high} cell population compared to the HLA-DR-positive one. In donor 2, 3, and 6, the HLA-DR^{high} cells showed high uptake but no VLPs were detected in the HLA-DR-positive cells. Donor 4 showed the opposite pattern i.e. more uptake in HLA-DR-positive cells rather than in the HLA-DR^{high} cells. Donor 5 showed no VLP uptake in both populations of the migrated DCs, but VLP-positive cells were found in the HLA-DR positive population of the epidermis (Fig. 16). Only one donor was negative for VLP presence in migrated cells (donor 5, Fig. 17B), though the particles were present in all cells both, in the epidermal and dermal layers (Fig. 16). Four out of 6 donors showed higher uptake in the migrated HLA-DR^{high} cells. Donor 2 showed no particle uptake in HLA-DR-positive epidermal cells (Fig. 16), but did show VLP uptake by cell populations of the dermis and by migrated cells. Donor 3 showed particle presence in the epidermis and the migrated cells in the medium, but not in the dermis. Donor 4 and 6 had particles present in all types of epidermal and dermal cells. No specific pattern was detected in the migration and distribution of the particle within the isolated cells from different layers. The results show that the model vaccines were taken in 100% of the donors by epidermal or dermal cells within the tissue and could be detected in 80% of the donors in migrated cells. The results suggest, that these vaccine architectures also reached skin cells, yet no superiority to previously studied constructs was observed and further interpretation is limited by the inter-individual variability.

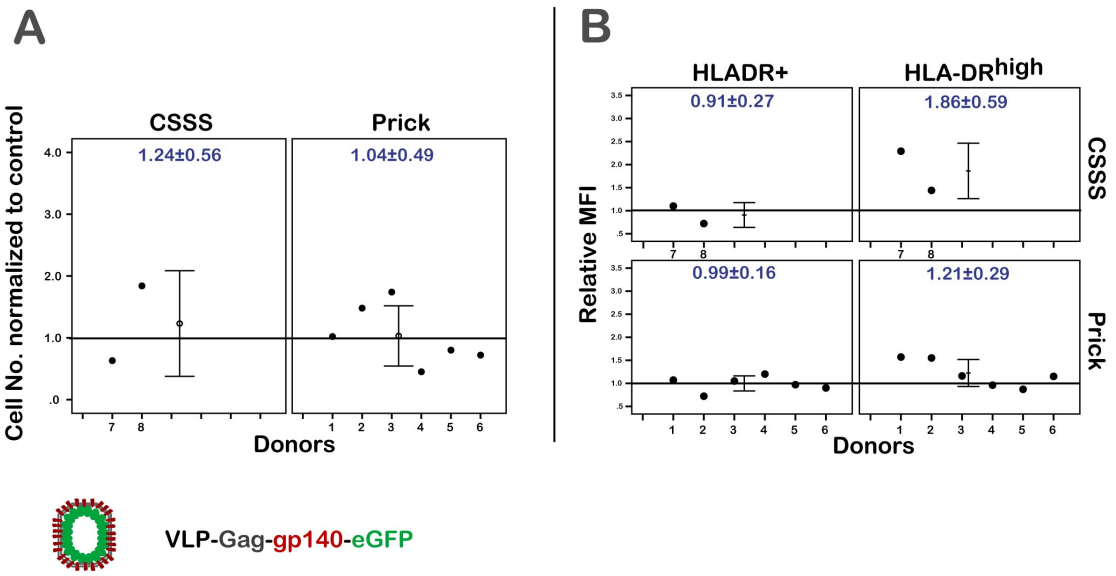

Figure 17. Uptake of VLP-Gag-gp140-eGFP by migrated cells after TC administration by pricking the human skin culture model: Uptake of VLPs by HLA-DR-positive and HLA-DR^{high} cells that migrated out of skin and were collected in the acceptor medium is plotted. The results show that VLPs were present in APCs that migrated out of the skin of 5 out of 6 donors, HLA-DR^{high} migrated cells showed higher particle uptake compared to the HLA-DR positive cells. For each cell population average relative MFI±SD are shown (blue).

3.4. Transcutaneous administration of GTU®-DNA plasmid

Among the experimental vaccine candidates, DNA constructs represent a very advanced type of vaccine, which have reached clinical trials. Since the hypothesis is that CSSS-based vaccine could be useful for TC delivery of particles and large molecules, we assessed skin penetration and cellular uptake of GTU®-DNA. The designed plasmid DNA was administered topically on pretreated skin to evaluate the possible use of the vaccine candidate on the human skin for vaccination. Since the eGFP gene was inserted into the plasmid, a successful transfection of the cells should lead to the expression of the eGFP in the tissue. Yet, in most of experiment performed on GTU®-DNA plasmid, the results were negative, only one experiment pointed towards expression of the eGFP. Possible limitations include low expression of eGFP insufficient for robust detection, and overall lack of proliferative capacity of keratinocytes in the *ex vivo* setting. Following the results of 3 typical experiments are shown.

3.4.1. Detection of eGFP after TC administration

In order to compare the effect of administration method plasmid DNA expression, the plasmid DNA was administered after CSSS, pricking and by ID injection on a skin tissue received from one donor. For each method of application a control (PBS) and a sample treated with plasmid DNA were analyzed. After incubation of the skin tissue with GTU®-DNA plasmid, the cells from the epidermal layer were isolated and evaluated by flow cytometry. No expression of the eGFP was detected in the samples after 16 h. Administration of the plasmid DNA on skin tissue showed no difference between the application methods. The result are demonstrated in the histogram using relative MFI of the samples treated with GTU®-DNA compared to the controls (Fig. 18). A value higher than 1.1 is considered as a successful transfection. None of the methods were successful in transfecting the cells with the plasmid.

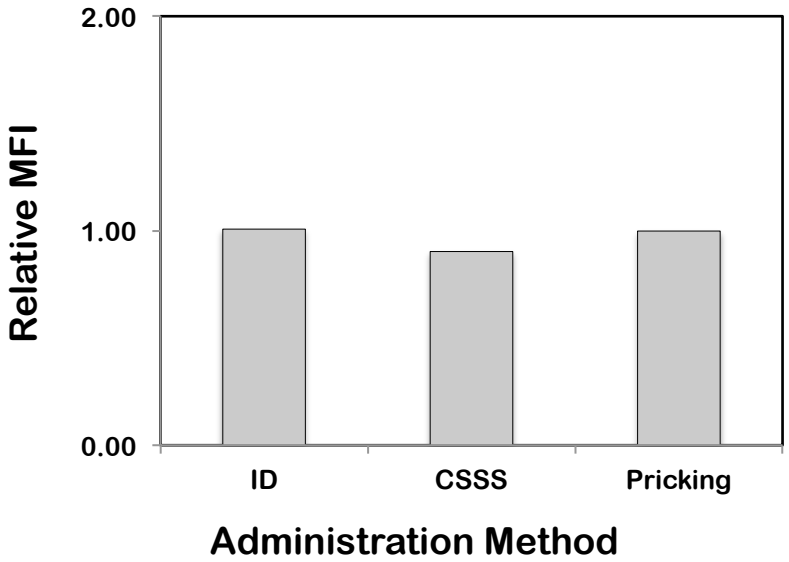

Figure 18. Comparison of three TC application methods on the expression of eGFP after GTU®-DNA application on excised human skin (one donor): Relative MFI has been used to show the eGFP expression compared to the control cells. Relative MFI higher than 1.1 indicates the presence of green fluorescence in the tissue.

3.4.2. Differences by time period after ID administration

The eGFP expression were analyzed in epidermal cells after ID injection. For this reason different incubation time periods were compared in one donor. After injection the skin tissue was incubated in a humidified chamber for 1 or 2 h. Afterwards, the tissues were placed in cell culture medium to ensure the viability for different time periods (16, 24, or 48 h). The best results were obtained after 1 h incubation in the humidified chamber followed by a 24 h incubation in the culture medium (Fig. 19).

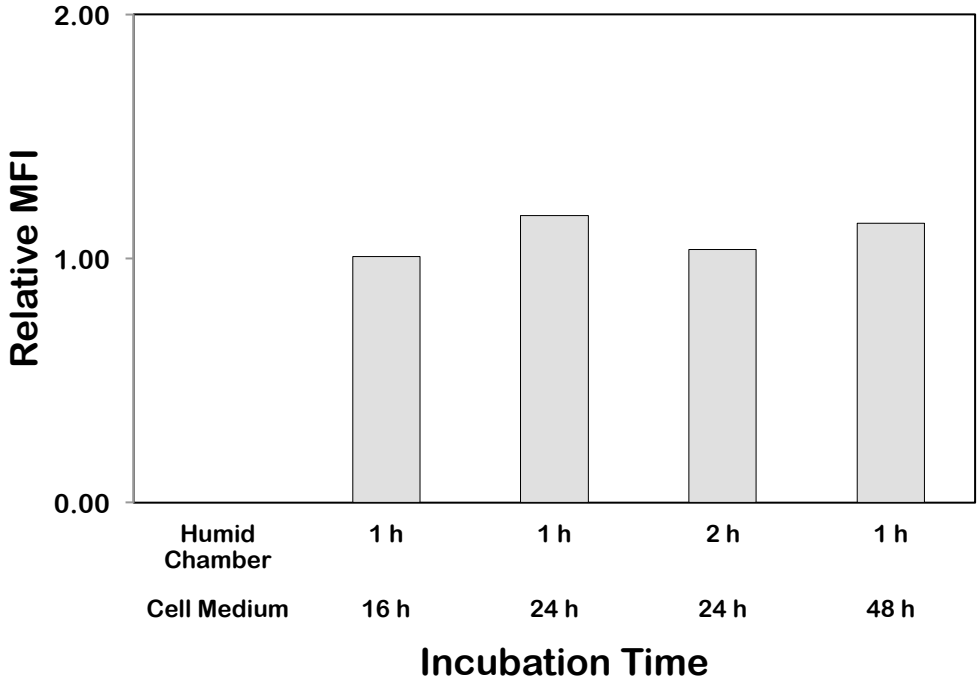

Figure 19. The change of MFI comparing the incubation time after ID application of the GTU®-DNA plasmid: After ID administration and different incubation time, epidermal cells were separated and analyzed by flow cytometry for eGFP expression. The graph shows the relative MFI changes during the incubation time in one donor after ID injection. The incubation time (h) is shown in x-axis (incubation in humidified chamber/incubation in the cell medium).

3.4.3. Expression of eGFP in the epidermal layer comparing different administration methods

Cutaneous administration of the GTU®-DNA was performed comparing three administration methods. The ID (n=5), CSSS (n=5), pricking (n=1), and ID injection plus subsequent negative pressure (n=3, -220 mmHg, 3 min) was performed on the human skin explants. Only a minimal change in the MFI was found among the epidermal cells isolated from the skin (ID (average rel. MFI: 1.11±0.07), CSSS (average rel. MFI: 0.94±0.05), pricking (rel. MFI: 1.00), ID plus negative pressure induced by a suction pump (average rel. MFI: 0.91±0.08)). The dot plot and histogram of different donors are shown in figure 20, 21, and 22.

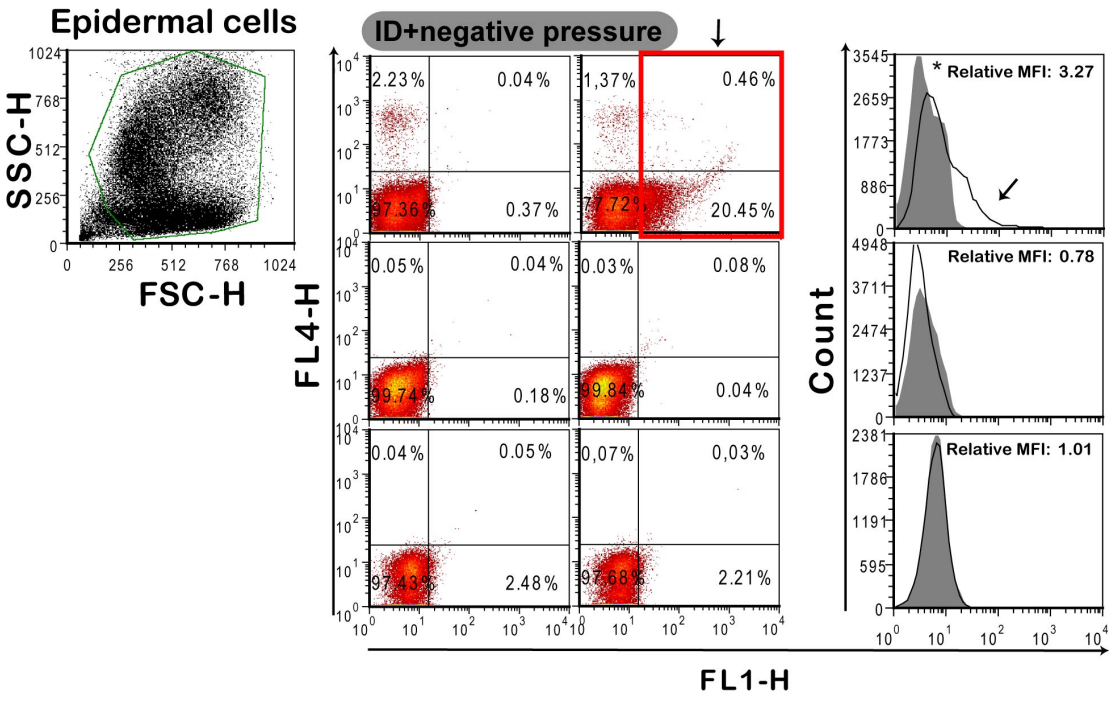

Figure 20. Expression of eGFP in human skin after administration of DNA-plasmid by ID injection plus along with negative pressure (n=3). After 20 h the epidermal cells were isolated and evaluated by flow cytometry. The figure demonstrates in dot plots and histograms of 3 donors. Only one donor (the first row) showed increased MFI (marked with *), with an evident shift in the cell populations in dot plot (red gate). Relative MFI presents the MFI of the samples to the control (PBS). The cells within the red gate showed visible shift in FL1-H channel. The result was nor reproducible by the following experiments.

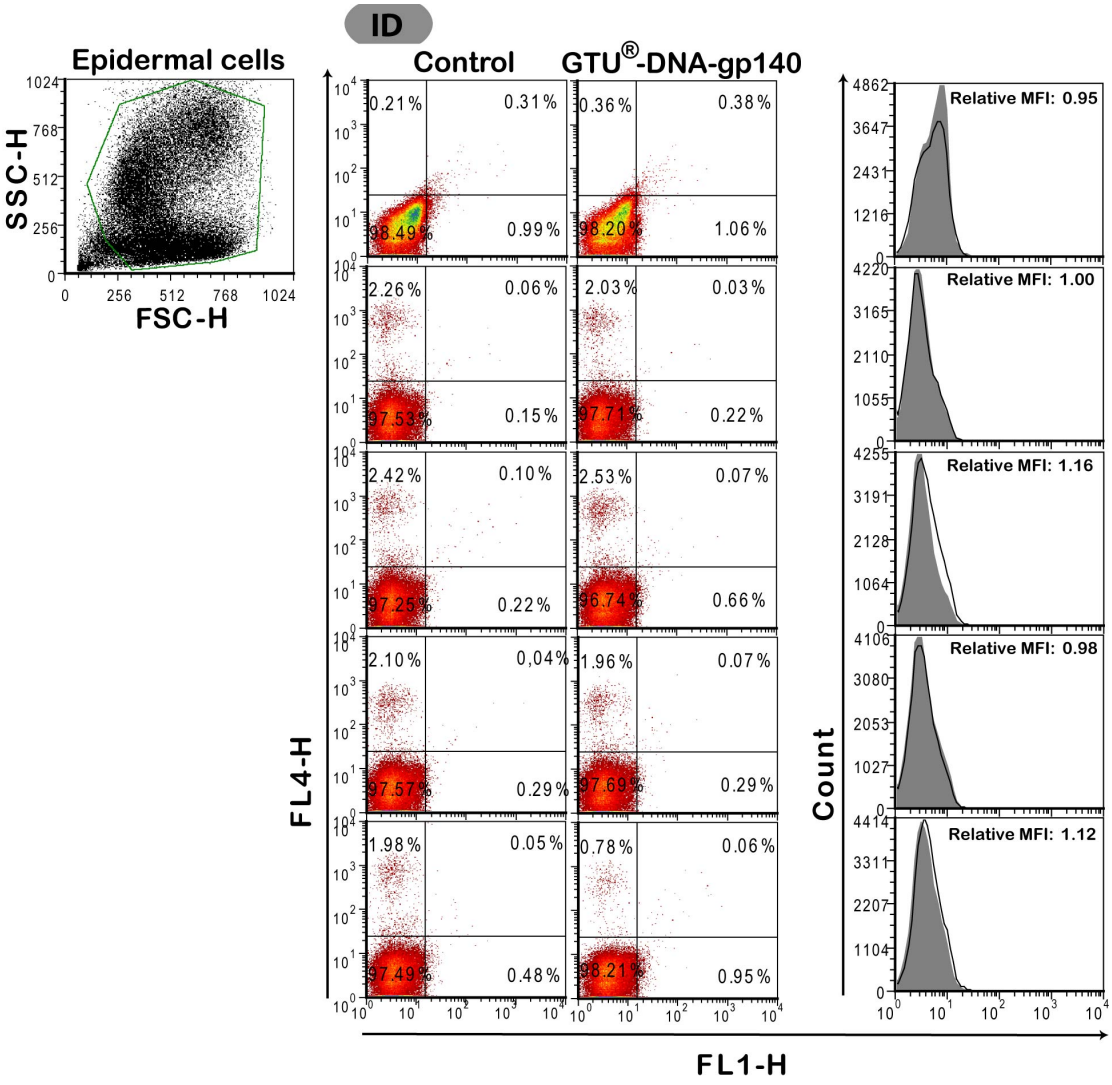

Figure 21. Comparison of expression of eGFP in skin cells after ID administration of DNA-plasmid on human skin explants from different donors. Figure shows dot plot and histogram of the epidermal cells isolated from skin and analyzed by flow cytometry (n=5). The relative MFI of the donors shows minimal change in the fluorescence expression between samples and controls. In dot plots no shift in the cells along the FL1-H channel were detected.

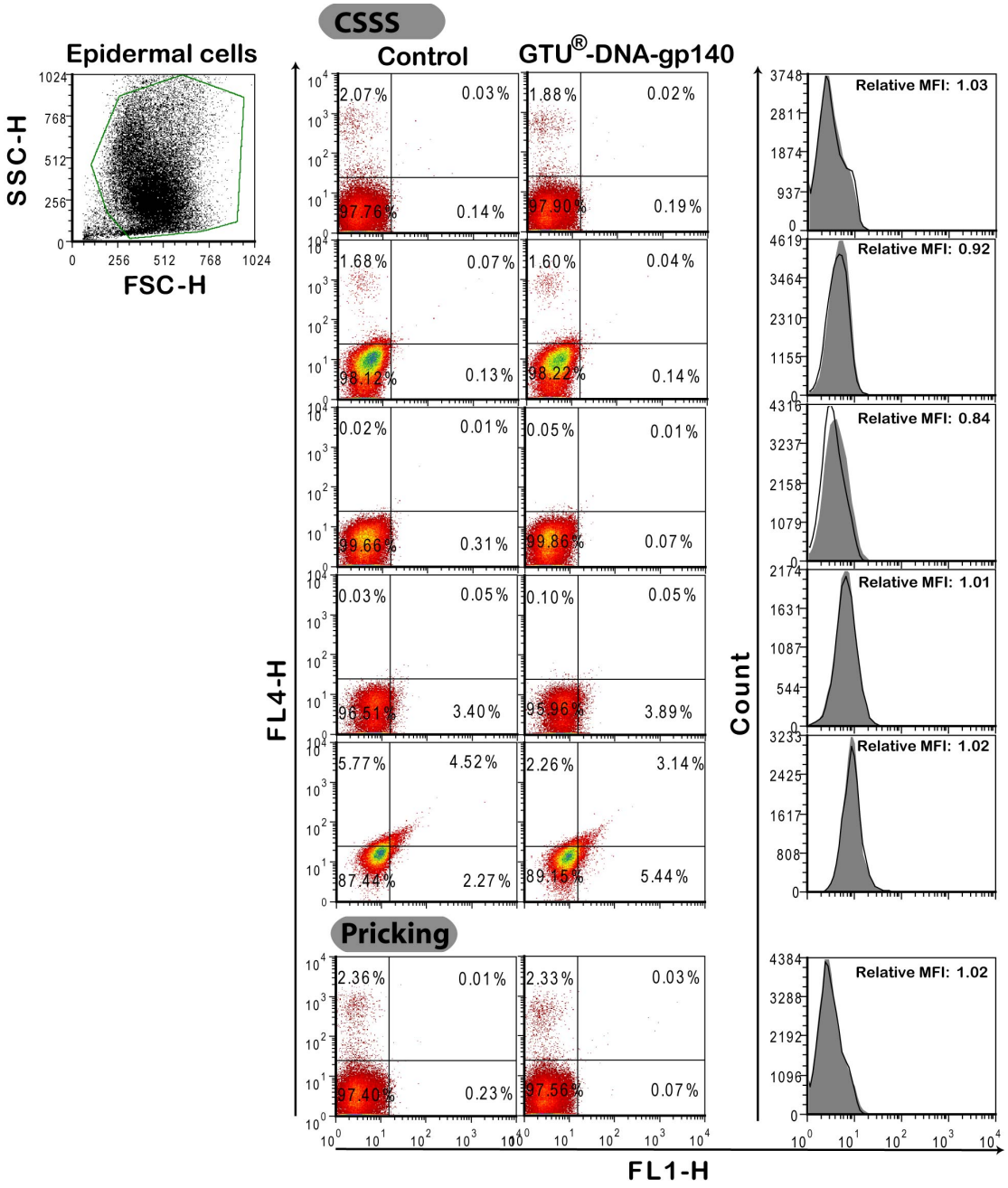

Figure 22. Comparison of different application strategies on the expression of eGFP after DNA-plasmid in skin cells. Dot plots and histograms demonstrate the results of flow cytometry. No eGFP expression was detected after administration of plasmid DNA on samples pretreated with CSSS (n=5) or pricking (n=1).

3.5. PLA-pGTU®-gp140

3.5.1. *In vitro* incubation of the PLA-pGTU®-gp140 with the HaCaT cell-line

Since the application of GTU®-DNA on excised human skin did not lead to sufficient transfection of skin cells, the adsorption to/incorporation in PLA particles were explored as one way to increase DNA delivery. *In vitro* experiments were conducted to assess the capacity of keratinocytes to uptake and express the DNA.

PLA particles were incubated with the human adult low calcium high temperature cultured (HaCaT) cell line *in vitro* (n=2). After 24 h of incubation with the PLA-pGTU®-gp140 the fluorescence intensity of cells was measured by fluorescence spectrometry. An increased fluorescence was detected in the cultured cells (8% increase) compared to the control. However, this low increase is not due to eGFP expression but due to the light scattering properties of particles or an increase in cellular size of samples compared to control. The investigation of the cells using flow cytometry showed no higher fluorescence after incubation with the particle (rel. MFI=1.40) (Fig. 23). Only side scatter increased indicating that some particle uptake had occurred.

Since the DNA plasmid is thought to continue production of the proteins within the proliferating cells, the incubation time of 24 h and 48 h were compared to each other. The data from the flow cytometry and the spectrometer could not confirm an increase of fluorescence over the time. As such, the cells incubated for 24 h showed higher fluorescence intensity (13% increase) by fluorescent spectrometer compared to the cells cultured for 48 h (6% increase). The flow cytometry analysis confirmed no significant increase in eGFP expression after 48 h. Although a higher signal in the side-scatter light channel (SSC) was detected in samples compared to control. The shift in histograms in SSC channel indicates that sample cells had a higher granularity than control ones i.e. uptake of the PLA particles.

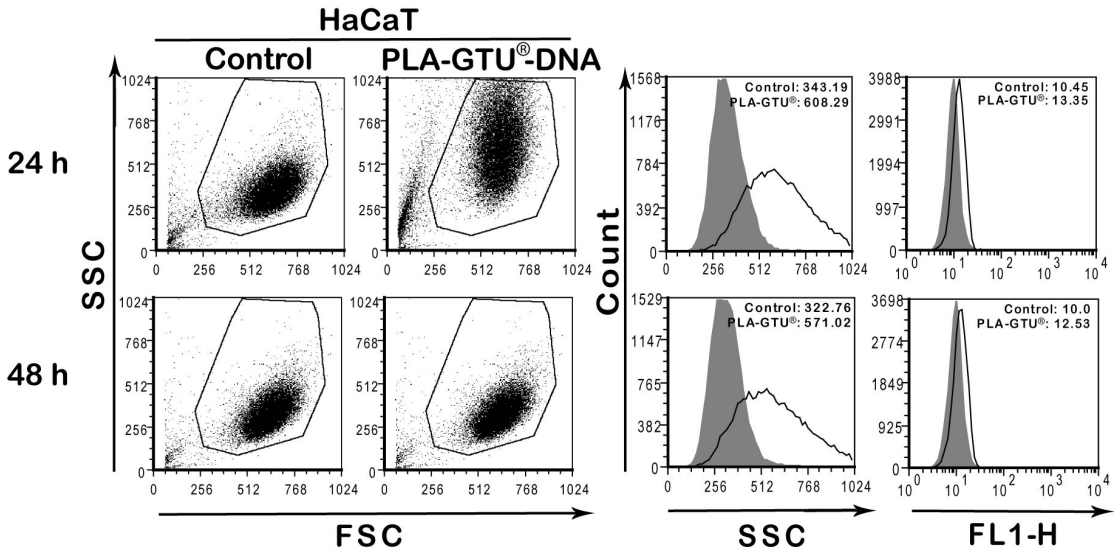

Figure 23. Uptake of the PLA-pGTU®-gp140 by HaCaT cells after 24 and 48 h of incubation: Relative MFI has been used to show the eGFP expression compared to control cells. No fluorescence could be detected, which indicates that the eGFP was not expressed by HaCaT cells. The small shift in histograms was resulted from particle uptake but not the expression of eGFP.

In order to prove that the PLA particles were taken up by the cells, intracellular labeling of gp protein was performed by immunohistochemistry staining. The anti-HIV1 gp120-antibody was diluted at 1:200 and incubated for 1 h with the cells. After washing the cells were incubated with the secondary antibody (anti-rabbit Fluorescein, Vector, diluted 1:50) for another 1 h. As control the PLA particles without gp protein were stained. The cells were then observed by fluorescence microscopy (Fig. 24B). The presence of the gp protein suggests that the particles were captured by the cells and the higher granularity of the cells also likely results from the presence of the PLA within the cells, yet eGFP production could not be identified, which indicated that the uptake of the particle did not result in cellular transfection with the plasmid DNA (141).

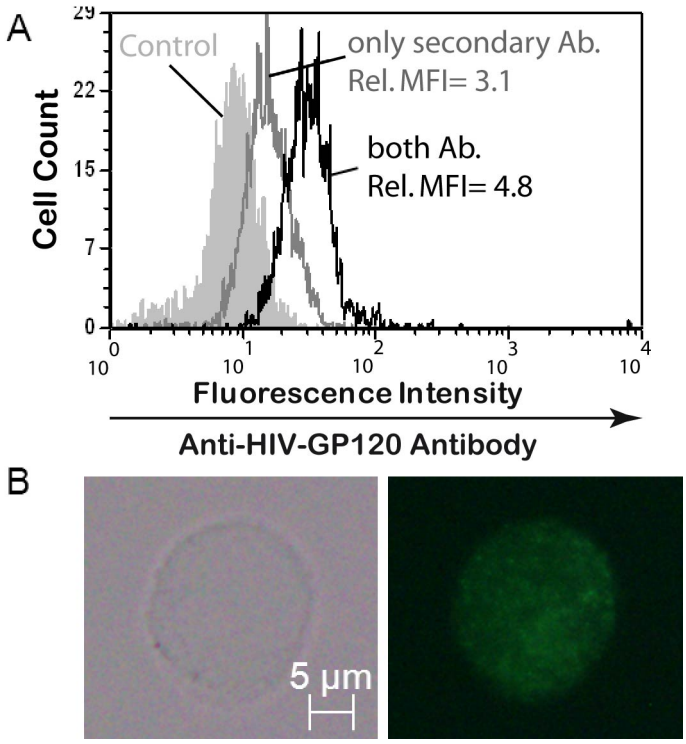

Figure 24. Intracellular staining of the HaCaT cells incubated with PLA-pGTU®-DNA-gp140: Histogram shows the flow cytometry result after immunohistochemistry staining (A). The fluorescence Image shows the presence of the particles after staining of the anti-gp120 antibody (Ab.) (green) (B) (141).

3.5.2. Evaluation of skin sections after administration of PLA-pGTU®-DNA-gp140 nanoparticles on the human skin tissue

After administration of PLA-GTU® nanoparticles on the human skin explants, the tissue sections (5µm) were evaluated by fluorescence microscopy. No expression of the eGFP was detected in the epidermal or dermal layers.

In vitro experiments confirmed the presence of the gp140 protein in HaCaT cells, as indicated by staining with anti-HIV1-gp120-antibody, which binds to a subunit of gp140 (gp120). Hence, tissue sections of the skin treated by pricking were analyzed by immunohistochemical staining to detect the gp140 protein *in situ*. The PLA particles were detected as dark spots on the surface of the SC, and within the pricking

site in contact with the epidermis and the dermis by light microscopy (Fig.25A, D and G).

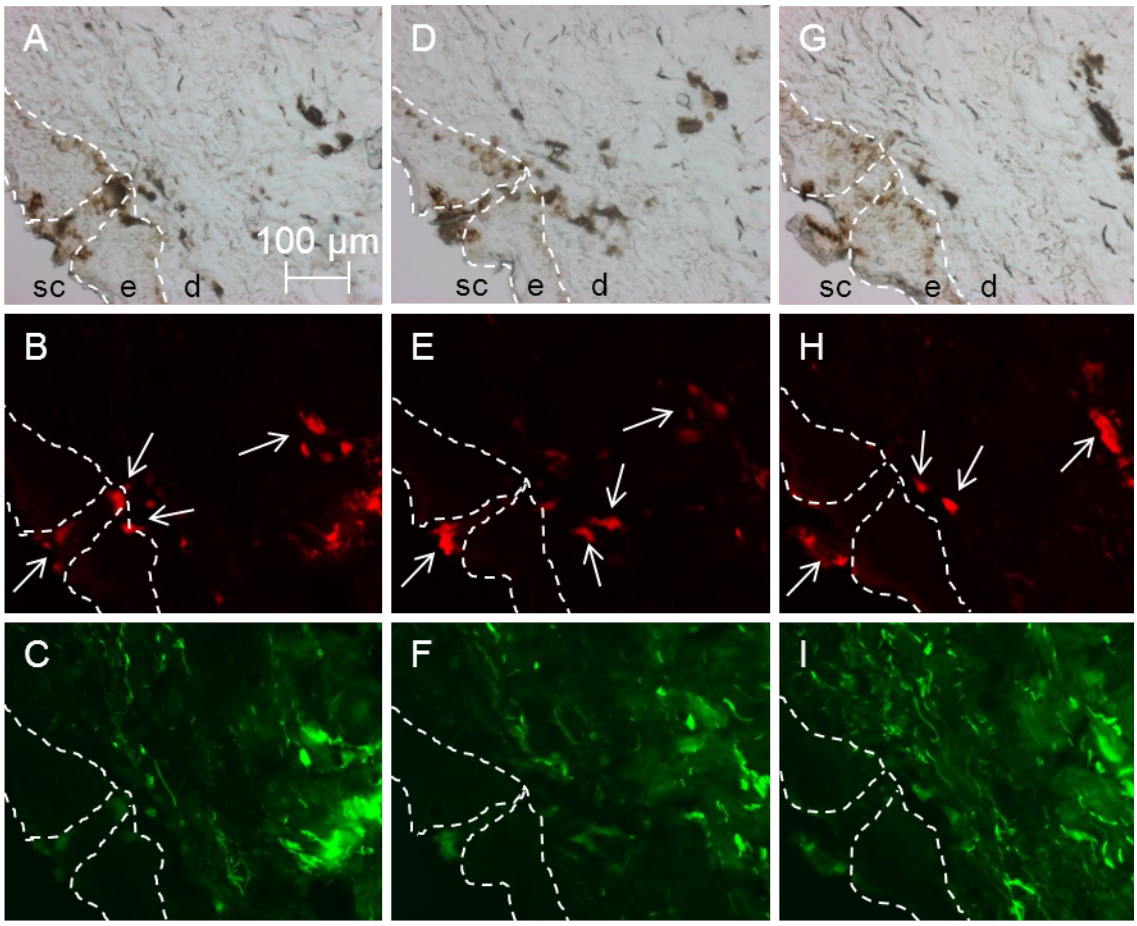

Figure 25. Detection of gp140 in pricking sites after administration of PLA-pGTU®-DNA-gp140 particles on human skin explant (n=1). After incubation of the human skin explants in the humidified chamber (16h), the sections (5 µm) were evaluated by immunohistochemistry staining with anti-HIV-gp120/Alexa Fluor antibodies for presence of gp120 proteins. The light microscopy images show the presence of PLA particles in pricking sites (A, D, G). The particle aggregates were detected as opaque spots of the SC within the pricking site in epidermis (e) and the dermis (d). The fluorescence images under the Texas red filter (red) shows the presence of gp120 in the site of particle accumulation (B, E, H). Under FITC filter (green) the green spots within the dermal tissue and also in the site of particle accumulation are considered the natural immunofluorescence of the dermal skin tissue and the artifact due to the opacity of PLAs (C, F, I) (141).

In the second row of the images shows the section Texas red filter by fluorescence microscopy. The red spots show the location of gp140 protein after immunohistochemical staining, which are matching with the particles location in the light microscopy (Fig. 25B, E and H). The pictures taken by the green filter (FITC)

showed no expression of the eGFP in the epidermal tissue, but green fluorescence were detected within the pricking sites, where the PLA particles are present. The expression of the eGFP should be detected after transfection within the cells, hence these green spots have no relation to the epidermal cells. The green sites in the dermis are the natural immunofluorescence of the dermal tissue. Also a green fluorescence were detected in the sites were the PLAs are present. The green spots present in the dermis also have the same fluorescence intensity compared to the peripheral dermis tissue. Since the particles are opaque when observed under light microscopy, the green fluorescence detected here is due to the increased cellular granularity and is not due to an eGFP expression (141).

3.5.3. Analysis of epidermal sheets for presence of eGFP und gp140 after PLA-pGTU®-DNA-gp140 administration

After administration of PLA particles on the human skin explants by pricking and incubation of in the humidified chambers, the epidermal and dermal layer of the skin detached using dispase solution as explained in material and method section. The epidermal sheets were fixed in PFA 2%. No expression of the eGFP were detected by fluorescence microscopy (Fig. 26 B). The epidermal layers then were stained by anti-HIV1 gp120 (primary antibody9 and fluorescein (secondary antibody) antibody. The gp140 were detected around the pricking site in the epidermis (Fig. 26 C).

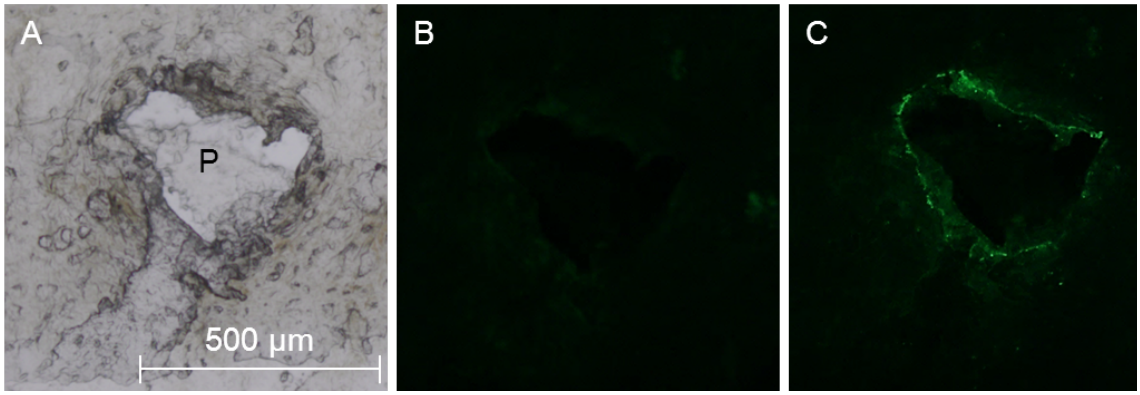

Figure 26. Epidermal layer after administration of the PLA by pricking: The epidermal sheets isolated from the dermis after 16 h of incubation in humidified chamber. A pricking site (p) is shown in the light microscopy image (A). No eGFP expression detected in the epidermis (B) by means of fluorescence microscopy. After immunohistochemical staining the gp140 were detected around the pricking site in the dermis (141).

3.6. Transfection of HaCaT cells with GTU®-DNA plasmid and Lipofectamine®

In order to study the effect of different carriers on plasmid transfection, the HaCaT cells incubated with GTU®-DNA plasmid *in vitro*. As control the HaCaT cells were incubated with PBS or the DNA plasmid without any carrier. To evaluate the effect of the carrier the transfection of the cells was performed by using Lipofectamine® (Invitrogen™) as transfecting agent. An amount of 4.4 µg DNA (1.5 µl GTU®-DNA) was transfected to the cells by means of Lipofectamine®. The Lipofectamine® creates liposome and is a known transfecting agent for plasmid and gene delivery purposes. The fluorescent expression was detected after 24, 29, and 48 h after incubation by fluorescence microplate reader.

The results show the fluorescent expression in the HaCaT cells in controls (PBS or GTU®-DNA) and in the Lipofectamine®-plasmid transfection samples (Fig. 27). The eGFP expression increased depending on the amount of Lipofectamine® used for transfection. No difference with respect to control was detected when only 2 µl Lipofectamine® were used (141).

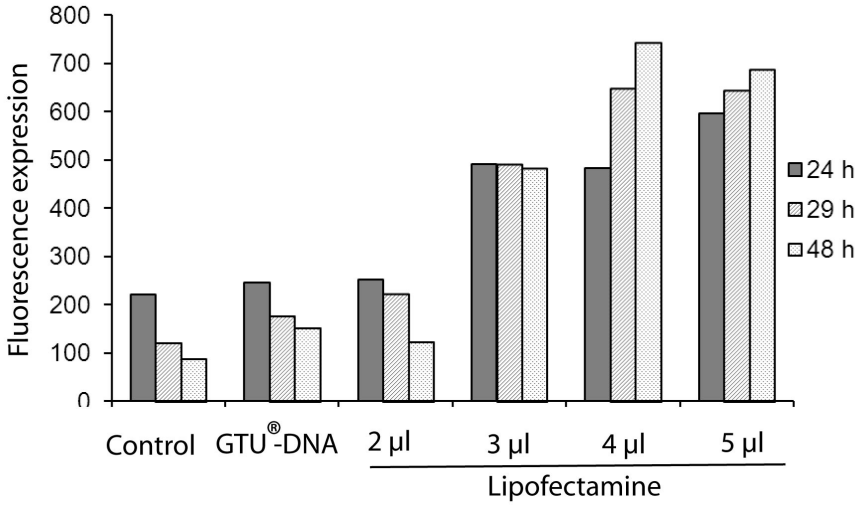

Figure 27. Expression of eGFP by HaCaT cells after incubation with GTU®-DNA plasmid and Lipofectamine®: The cells were incubated with 4.4 µg GTU®-DNA. The fluorescence expression of the cells are shown in control (PBS), after incubation only with plasmid GTU®-DNA, and transfection of GTU®-DNA with Lipofectamine® (2, 3, 4, 5 µl respectively). The eGFP fluorescence was detected when the plasmid was transfected with Lipofectamine® volumes of 3 µl and more. The eGFP-expression increased by the amount of transfecting agent (141).

The expression of the eGFP was also evaluated on HaCaT cells by flow cytometry: Lipofectamine® (0, 3 or 5 µl) was used for transfection of GTU®-DNA (2.3 µl or 6.7 µg), and the eGFP expression analyzed by flow cytometry after 48 h (141).

The flow cytometry showed fluorescent shift in the transfected cells (Fig. 28A). The percentages of positive cells are shown in the histogram (Fig. 28B). The results showed higher expression when more plasmid or Lipofectamine® were used. Increasing the Lipofectamine® also caused higher expression by a similar amount of plamid. The fluorescent picture and the overlap with light microscopy are shown eGFP expression in the HaCaT cells in the cells treated with Lipofectamine® shows (Fig. 28A).

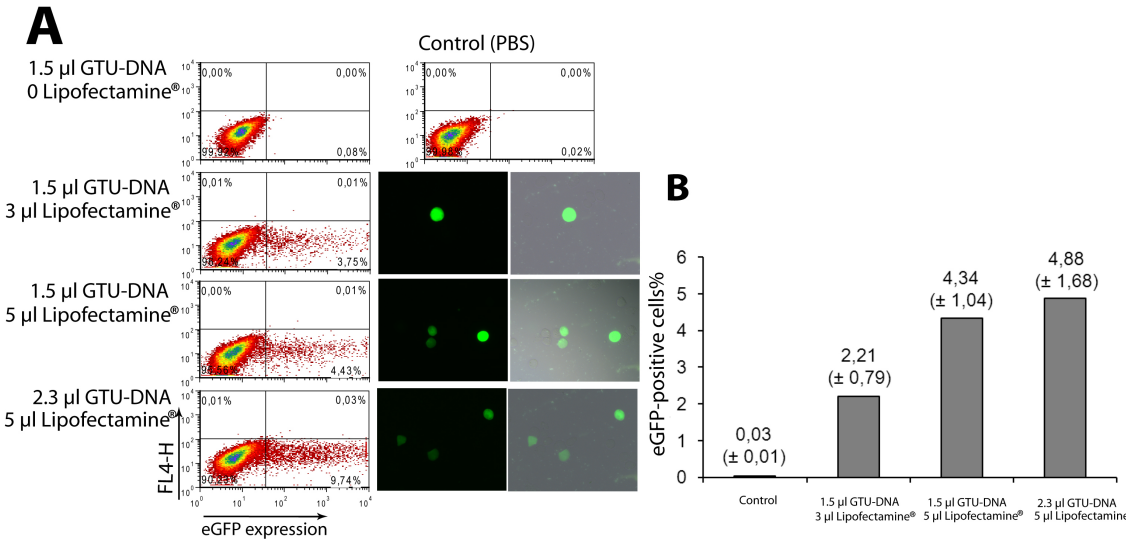

Figure 28. Expression of eGFP by HaCaT cells after *in vitro* incubation with pGTU® and Lipofectamine®: *In vitro* experiment shows successful gene expression after delivery of GTU®-DNA by means of Lipofectamine®. Different amount of Lipofectamine® (3 or 5 µl) and GTU®-DNA (2.3 µl or 6.7 µg) were used for transfection. Dot plots shows the eGFP related fluorescence expression. A visible shift of the cell population could be seen after transfection with Lipofectamine® (A). The histogram shows the percentage of positive cells comparing different experiments setting (n=4) (B). The fluorescence microscopy images confirm the eGFP expression (A) (141).

Discussion

4. Discussion

4.1. The TC delivery of immunogens

The TC targeting of APCs immune cells, already present in the skin tissue, is a promising new vaccination strategy. Previous studies have shown that the immunization via the skin produces immune responses equivalent to or even higher than the IM injection using smaller amount of antigen (29). The skin barrier naturally hinders the penetration of high-molecular weight compounds into the skin and limits vaccination success. Bypassing of the SC seems to be an essential challenge in the development of skin vaccination strategies. Different strategies are therefore created to facilitate the cutaneous penetration and increase vaccine immunogenicity. Skin barrier disruption strategies as well as packaging of antigenic compounds in particles represent two interesting approaches (41). Among noninvasive strategies the targeting of vaccine in the reservoir of HF openings is highly interesting approach. In fact the role of HFs as reservoir and shunt penetration pathway for particles and large molecules has been confirmed in many experimental and clinical studies (73, 75, 77, 92).

Transfollicular delivery is a major pathway to deliver antigens into the skin. The importance of the HF for vaccination goes beyond this strong function, because of the presence of LCs around the HF canal. Physical barrier disruption by CSSS facilitates the delivery into the HF, and enhances penetration of the skin tissue (80). This barrier disruption could also enhance TC immunization since it triggers the release of immunostimulatory cytokines. This could lead to the activation and the migration of APCs present in the tissue and enhance their uptake capacities (142). After migration of the APCs out of the skin, the residing LCs will be replaced within 24 h *in vivo*. Evidence increases that HF plays an essential role in LCs redistribution and skin cell homeostasis (143, 144).

It has been shown that the particulate form can facilitate penetration across the skin barrier as well as the transfollicular pathway. This aspect is especially relevant for liposomes (145). This is interesting because it provides a strong rationale for the use of carrier-based vaccines on the skin. Moreover, binding to particles can increase the immunogenicity. Last but not least, it was found that skin-carrier interactions can

greatly influence the way an antigen is delivered to the skin (e.g., early release from polymeric particles could result in delivery of large quantities of free peptides in the targeted skin layers as well as the desired skin structures) (91). The release depends greatly on the architecture of the carrier. For example, in PLA particles, the early release is particularly beneficial when the SC needs to be repaired or there is a desire to reach high concentrations in HF infundibulum, e.g. to treat acne vulgaris. The size of the particle plays an important role in the penetration through the HF (77). E.g. while larger particles ≥500 nm mainly take advantage of the HF reservoir, where they release their load, smaller particles <200 nm have the potential to translocate across the epithelial barrier and enter immune cells together with the vaccine. A particle that penetrates the HF canal can be captured by the APCs located on the peri-follicular epidermis (75).

In the current study, it was hypothesized that the combination of the TC delivery methods (by barrier disruption) and the particulate form of the antigen could optimize the TC delivery method. In these studies on excised human skin, it was showed for the first time that topically applied VLP particles cross the disrupted skin barrier and are being internalized by skin APCs.

4.2. Human vs. animal skin model

Different skin models have been used to study TC delivery. Animal models are valuable experimental systems, because they allow investigations in living organisms. Although they increase our basic understanding of the underlying mechanisms, the results cannot be extrapolated to the human skin. Porcine skin is the most analogous structure to the human skin since the SC thickness and the structure of the HF is more comparable to human tissue (146, 147). Rodent skin has also been used for *in vivo* and *in vitro* skin permeation studies because the animals are small and widely available. Limitations of this model are higher permeation rates when compared to human skin leading to over prediction of penetration (148, 149). In mice, the overall skin thickness is much lower compared to humans and the high density of HF, which underlines the importance of complimentary studies in humans, especially for studies with focus on transfollicular penetration. Furthermore, investigation of skin delivery methods is very limited due to the higher vulnerability of rodent skin. E.g., the CSSS procedure used in this study leads to major skin damage when applied on murine skin.

In an experiment done by L. Young et al. VLPs (40 nm) were used in conjunction with cholera toxin (CT) on mice. The administration method was TC by rubbing the vaccine on the shaved skin. In this approach no mechanical disruption method was used, but the CT led to chemically induced barrier disruption to allow the penetration of the small particles. The CT acted also as an immunostimulatory material for immune cells. They detected mainly high IF-γ production after TC administration, which is normally related to type-1 immune response (150). The main experiments done with VLP application on the skin tissue of mice is focused on the usage of the microneedles (151, 152). In an experiment Pearton et al. showed that VLP administration by means of microneedles induced antibody response in mice. On the human tissue explants, they further showed that microneedles resulted in morphology change and the LCs migration from epidermal layer (153).

Reconstructed human skin can, to some extent, help to simulate penetration process *in vitro*. However, even though the epidermal layers build up correctly when maintained at the air-liquid-interface these models are known to be hyperpermeable, which leads to overprediction of penetration (154). Also, they lack skin appendages as well as immunocompetent cells and do not possess a developed dermis compartment. Thus, overall their value for skin penetration and cell targeting studies is limited.

The human skin explant is a more relevant model for TC studies. However, physiological characteristics of skin tissue including SC thickness, lipids and appendages can vary significantly among donors, which would affect the studies done on the skin tissue. The TC penetration varies among different body parts mainly due to different SC thickness, lipid composition, and appendages (155). The density of the hair in different parts also affects the TC permeation (156). The influence of blood and lymphatic circulation on the immune activation of the skin cells cannot be evaluated in such excised skin. Also, as indicated by the DNA studies presented in this work, proliferative state of the cells in the excised human skin may not be clearly comparable. Especially on the experiments done with plasmid DNA it could lead to insufficient expression of the eGFP. Such lack of the plasmid expression could be due to the weakness of the model used and not only the efficacy of the plasmid model vaccine. The fact that neonatal foreskin keratinocytes exhibited much higher VLP uptake than adult keratinocytes also points in this direction. To exclude the activation

of the immune cells caused by stress induced by hypoxemia and the surgery, we always compared a skin sample treated with PBS to the skin from the same donor treated only with vaccine vehicle (e.g. VLP). The result of the current study confirms that the human skin model is suitable for evaluation of the topical vaccine model. Viable cells could be recovered even after incubation times for up to 40 h and the migratory cells left the tissue in an active manner and went through the 8 μm mesh.

In conclusion, the organ culture set up using excised human skin is a valuable method to study skin barrier translocation and uptake by different skin cell populations. Moreover, we were able to show that it is suitable to monitor cell migration in response to vaccine application. Investigation of antigen-specific activation would have been of great benefit e.g. by exposure of VLP positive APCs to the T cell clones. However, such studies require tissue and blood from the same donor, which was not feasible in this study. Also, volunteers undergoing plastic surgery cannot be expected to have had previous contact to HIV antigens. So it is unclear whether such T cell clone experiments would give convincing results if the APCs encountered the antigens for the first time in such experiment.

4.3. The VLPs for TC delivery

Delivery of the immunogen in the particulate form improves antigen stability, and can enhance vaccine uptake. VLP have been shown to stimulate the cellular and humoral immune response in the murine model *in vivo* (115). Previous studies confirm that VLPs exhibit the capacity to stimulate the DCs and induce the maturation markers HLA-DR and CD86 *ex vivo* (157). When placed on the human skin tissue (via ID or by means of microneedles), VLP can activate the LCs and induce cellular migration *in vitro* (106). To evaluate the ability of VLP to be captured by cutaneous cells from human skin tissue, in this study isolated epidermal cells were incubated with VLPs *in vitro.* The results show that not only APCs, but also epidermal keratinocytes were able to take up the particles. Since the main focus for immunization is to target the skin APCs, we were able to show that the VLPs were taken up by the epidermal LCs using flow cytometry analysis. The presence of the particle in the cells was then shown using fluorescence microscopy. In subsequent experiments utilizing TEM, the VLPs were detected within the LCs, where VLPs' structures were still preserved. The finding that keratinocytes internalize VLP is interesting because targeting of vaccine

to epidermal cells could help to create vaccine depots and enhance APC-priming. In fact this was also the rational for the use of GTU®-DNA.

The VLP architectures used in this study were previously shown to deliver HIV-1 immunogen and to induce an immune response in mice after IM, intraperitoneal, and subcutaneous injection (113, 125). The current study shows that VLP could also be used for TC antigen delivery. The VLP has been shown to be an optimal carrier due to their biocompatible and biodegradable nature, possession of similar dimensions to a virus, and stable structure. In the case of VLPs, the immunogen is incorporated within the particle structure. It can be carried in the particulate form into the tissue and captured by phagocytic cells like DCs (158). This is important since the induction of the cellular immune response is crucial, especially for the treatment and prevention of intracellular infections (150, 159-161). Labeling VLPs by a fluorescent stain facilitated tracking of particles within the skin tissue, and also enhanced detection of the cell populations that captured the particles. The detection of the VLP in the dermis layer 120 h after inoculation by ID injection confirms the stable structure of the VLP within the human skin environment. In addition, when VLPs were administered by pricking method on the skin tissue, intact VLPs were detected in the SC layers by TEM. This confirms that VLPs are stable even after application on the human skin tissue and even maintain their structure after interaction with the skin barrier. The advantage of VLPs over other drug delivery architectures, such as polymer particles, is that antigens can be directly incorporated into their membrane. Such a structure ensures uptake in particulate form while avoiding delivery of the free antigens, which could increase the chance of immune tolerance. This finding is especially relevant when compared to previous skin penetration experiments conducted with protein antigens loaded on PLA-particles. Here, the particles destabilized upon skin contact and released their load, which result in deposition of free antigen rather than particle bound antigen (91). Also, VLPs can be equipped with mixture of antigens and adjuvants to increase the following immune response.

4.4. The CSSS is an effective administration method for TC delivery of antigens

In this study, noninvasive TC administration was compared with minimal-invasive pricking and invasive ID injection. After ID, VLPs were detected in isolated APCs of the dermis by flow cytometry. In these experiments, the identification of particle-

positive populations was difficult, since the differences in fluorescent intensity between the control cells and the cells associated with particles were small. Although the particle presence within isolated cells was also confirmed by fluorescence microscopy. Therefore, we assume that the small shifts in histograms can be explained by the small size of the particles and also the low number of particles in a single cell.

After CSSS or local barrier disruption by pricking, VLPs were found within the skin cells. Interestingly, the CD1a positive LCs and DCs from the epidermal and dermal layers were able to capture the particles. The results show that VLPs are suitable carriers to target skin APCs. This is also in accordance with the studies done with baboons and mice, which showed that VLPs are able to induce both types of the immunity (162, 163).

After barrier disruption by CSSS, VLPs were found not only in the epidermal cells but also in the dermis layer. The APCs containing VLPs were found in both layers of the skin. The aim of barrier disruption by pricking is to increase the accessibility of particles to immune cells in both layers of the skin. These results showed no major difference between the two administration methods (pricking and CSSS) with regard to the particle uptake by the APCs. In contrast, the ID injection was not able to target the LCs population in the epidermis allowing only dermal cells to access the particles. This is a result of the depots in the dermis. However, previous studies suggest that dermal vaccine depots can attract LCs to migrate downwards (164). In another study using PLA particles, Liard et al. showed migration of LCs into the dermis after ID injection of the particulate PLA model (165). This indicates that the LCs in the epidermal layer do not take up the particle but that the ID injection stimulates the activation and migration of the LCs from the epidermis. While the site of vaccination is the skin, the method of antigen delivery and the particulate form still impact the targeted type of cell population. The capture of the antigen by only dermal APCs after ID injection could lead to a different immune response than the TC method. While the minor removal of the SC layer by CSSS method not only facilitates penetration to the epidermal layer but also surprisingly to the dermal cells. Since overall integrity of the epidermal layer is not manipulated by this method but only the SC, the presence of the VLPs in the dermal layer could be caused by I) increased penetration of the VLP to both the epidermis and dermis via HF or II) migration of activated LCs containing

VLP from epidermis to the dermis. Also a key effect of CSSS compared to skin surface stripping with adhesive tape is the opening of HFs. The cyanoacrylate resin flows into HF openings, where it hardens. The following stripping removes these follicular casts together with cellular debris and dried sebum. This effect makes the HF reservoir accessible for topically applied compounds. Once it penetrates deep into the HF canal it gains access to the APCs in the HF epithelium. Furthermore, lateral diffusion from such depots facilitates direct access to deeper compartments of the dermis (166). With regard to particle uptake by dermal and epidermal APCs, no considerable difference was detected between the low-invasive CSSS model and the invasive pricking. Obviously further immunological studies have to be added. But the results encourage further work on non-invasive ways of TC immune cell targeting. Based on these results it seems that the CSSS method could be more desired for TC strategies.

4.5. The TC application of the VLPs stimulates the migration of APCs

In the current study, the migration of the DCs after topical application of VLP was evaluated using a human skin culture model. The model was specifically designed to gather the cells that migrated out of the skin into the medium. The cells had to migrate out of the skin barrier through a membrane with 8 µm pores in the medium using the skin culture model. The results showed that after 40 h of incubation, HLA-DR positive cells migrated out of the skin. The validity of this experimental set-up is demonstrated by unaltered skin morphology in tissue sections after 40 h of incubation and the viability of the skin cells. Similar to previous results (on the human skin 16 h post-administration), VLPs were detected in the dermal and epidermal layers. After 40 h of incubation the migrated cells contained VLPs were detected in the medium, which means that after taking up the particles from the tissue the cells started to migrate. Since the VLPs were applied topically to the skin, these results shows that after VLPs administration, cells were activated and the cells containing the VLP moved out of skin to reach the lymphatic system.

These findings are in accordance with recently published reports by Pearton et al. who used ID injection of VLPs in the human skin *in vitro* (106). They showed, that after VLP application by pricking and ID injection to human skin in an air-liquid interface culture model, LCs migrate from the epidermis during 24 h of incubation.

4.6. CSSS stimulates cell migration

In the present study the CSSS administration method was used as a low-invasive method. The CSSS partially affects the integrity of the SC by removal of approximately 30% of the skin's SC (73, 77). This mild damage leads to cytokine release and APC activation (77, 167). In previous studies, it has been shown that the CSSS method can enhance the particle penetration in the HF as well as enhance transfollicular delivery (77). After particle accumulation, the HF canal forms a reservoir for the particles (Fig. 29). The particles in the HF canal can then release the antigens into the hair canal or be captured by surrounding APCs (166).

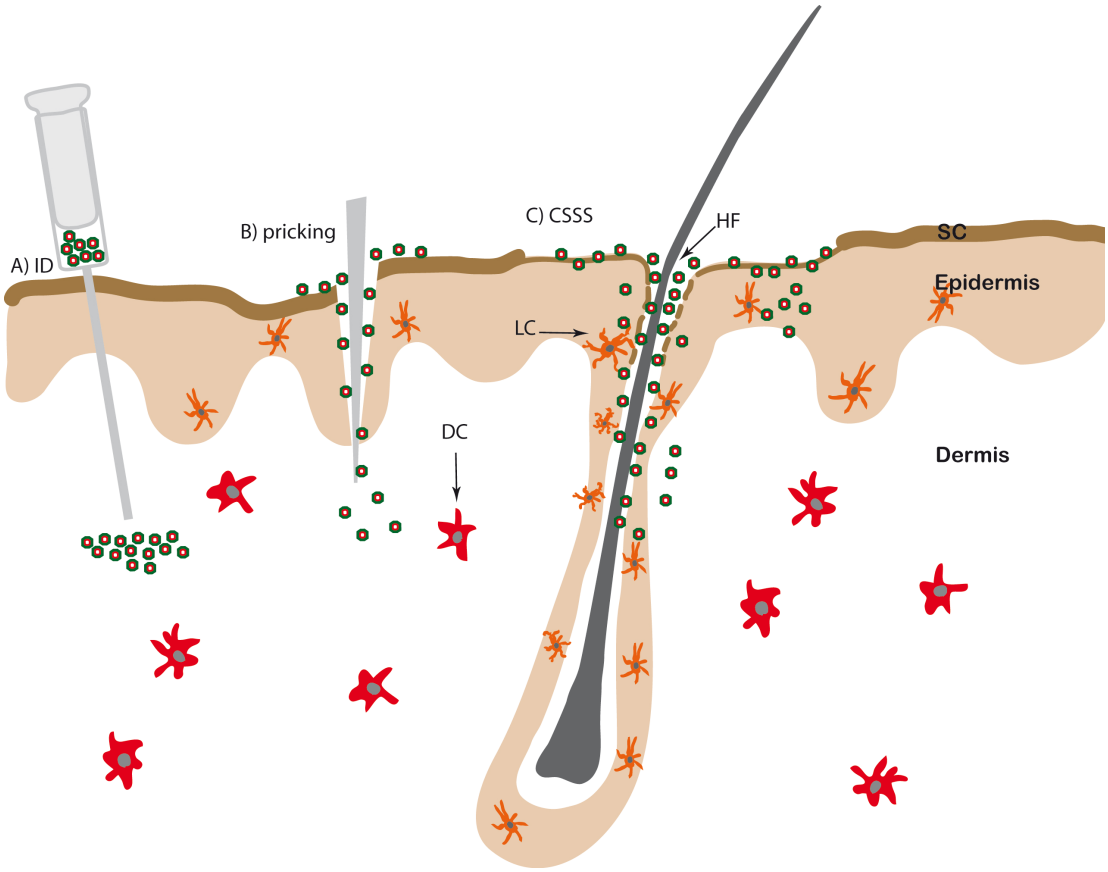

Figure 29. Comparison of the three cutaneous administration methods: The ID injection (A) creates the depot within the dermis that could be reached by the dermal DCs around the injection site, while the pricking method (B) makes the particles accessible for epidermal and dermal APCs in multiple sites of injuries. The CSSS (C) not only enhances the penetration across the mildly disturbed interfollicular SC, but also facilitates VLP follicular penetration, making the particles accessible to abundant LCs around the HF and simultaneously creates depot in HF canal for adsorption and release during an extended period of time.

The CSSS induced higher migration of cells out of the skin when compared to skin barrier disruption with pricking (Fig. 29). One explanation could be the inflammatory stimuli caused by CSSS. Otherwise pricking acts by creating mechanical “holes” in the barrier (91). A study done by Pearton et al. comparing the gene presentation pattern after skin treatment using microneedle demonstrated no significant changes between the untreated skin and the skin treated with microneedles after 24 h skin culture. Hence, an altered gene expression after VLPS was detected compared to the control group (106). In both control and the VLP-treated skin the number of APCs reduced in epidermal sheets show the activation and migration of the DCs after pricking. Although a significant result was only detected when microneedle were used along with the VLPs. Several strategies are being used to overcome skin barrier. Yagi et al. evaluated the removal of 60-80% of SC, by three times application of cyanoacrylate on the human skin, to enhance peptide penetration and immunization via the skin. *In vivo* studies done on the skin of human volunteers showed morphological changes in epidermal LCs after 12 and 48 h. The constant reduction in the epidermal CD1a-postive cells in epidermal sheets in 24 h and 48 h after studies showed the emigration of the LCs. In addition upregulation of HLA-DR and increased number LCs expressing costimulatory molecules (CD80, and CD83) and CD86 activation markers till 48 h after skin pretreatment and topical application of peptide was observed (168). Since the peptide was administered 24 h after SC disruption, therefore the skin pretreatment was essential for activation and maturation of epidermal LCs prior to peptide application. The result of the current study also confirms the migration of activated DCs from epidermis to the dermis after 16 h and also further migration out of the dermis after 48 h. A study done by Vogt et al. showed the morphological changes due to activation of the CD1a-positive LCs in human tissue explants. The number of the LCs in epidermal sheet decreased after one and two times CSSS during 24 h, which shows activation and migration of the LCs even after removing 30% of the SC. The morphological changes did not differ significantly after one or two times skin pretreatment with CSSS (73). Other studies on TC immunization using hydrogel patch containing DT as the antigen and TT also have shown the immune response by producing antibody against the TT and DT. This method mostly benefits from a hydrogel patch staying on the skin for 24 h and the mechanism of penetration of the free protein antigen seems to be barrier disruption do

the occlusion process in the SC layer of the skin. In this method a few percent of the protein antigen will penetrate the skin, which could be minimal in case the antigen concentration used in not much, therefore the use of an adjuvant to ensure the immunization is necessary. Although the detection of immune response is mostly recognized by antibody production, the uptake of the protein by LCs could not be proven (86).

4.7. CSSS vs. microneedle

Despite only partial removal of the skin barrier by CSSS, the cells caught the topically applied particles. The APCs within both layers of the skin tissue as well as the APCs that migrated were able to internalize the VLP. One hypothesis is that the VLPs were taken up by APCs in the epidermis after the CSSS, and then APCs migrated to the dermis layer, and finally out of the skin. Another reason for the presence of the VLP in the dermal DCs and migrated cells could be that the CSSS facilitated the penetration of the VLPs through the SC and the HF to the epidermal and dermal layers (Fig. 29). This process could also be facilitated by the cytokines released after CSSS within the tissue, which stimulate APCs (143).

Pricking the skin induces local disruptions in the epidermis and the dermis. This is the basic reason for designing microneedles. They are being widely evaluated and produced for the purpose of transferring material to the skin tissue. Although local barrier disruption by pricking facilitates direct access of dermis cells to VLPs, results of current study indicate that, in the same donor, pricking did not induce a significantly higher migration of the HLA-DR positive cells compared to CSSS. Never the less, the pricking method creates depot in the dermis, which might induce better results throughout weeks *in vivo*.

The results provide important work with regard to the different methods for currently emerging skin vaccination. While microneedle systems have the advantage to come in one-step application system, the additional pre-activation of immune cells provided by pretreatment of the skin, with CSSS i.e. in a 2-step-patch system could help augment the immune response.

Type of the microneedles, density, length of the microneedle arrays, and the vaccine compound affects the outcome of the microneedle immunization. Pretreatment of the

skin with solid microneedles and then topical application of antigentic compound (DT) by Ding et al. resulted in 1000 fold increase in IgG in mice, hence no significant increase was detected by using the patch formulation without skin pretreatment. Coadministration of adjuvants improved the immune response, although the type of adjuvants affected the immune response type. A significantly higher immune response after coadministration of the CT could reduce the applied dose of the antigen (169). To compare the antigenic compound they also compared DT and influenza subunit vaccine administration on intact skin and also after microneedle pretreatment comparing solid and hollow microneedles. Among microneedles solid ones promoted better results. The influenza vaccine did not induce immune response when administered on the intact skin and also after barrier disruption, although addition of the adjuvant improved the IgG response. The DT was able to produce IgG antibody against the DT after all application methods although barrier disruption and adjuvant coadministration improved the immune response significantly. The bigger size of the subunit influenza vaccine and the lower potency of the compound could be the reason for such differences (170). The posttreatment of the mice skin with solid microneedles for genetic vaccination was evaluated on mice. When 6-12 pass of microneedle injection was done, higher luciferase fluorescence intensity was detected compared to administration on intact skin, subcutaneous, and the ID injection. After 3 administrations with one-month interval, the serum antibody produced by microneedle was higher compared to the other methods (171). This shows improved genetic immunization despite of losing a portion of administered plasmid on the administration site. Microneedles coated with the antigenic compound will reduce the loss of the antigen during administration ensuring the maximum amount enters the skin tissue. The immune response was similar both in microneedles designed with or without condensed arrays (172). Dissolving microneedles are the next generation, which could be made from biocompatible materials. After application the microneedle could stay in the skin and the antigen compound diffuse in the tissue (173).

Furthermore, ID injections were frequently associated with higher pain and reduced local tolerance in previous studies (87). These problems limited their wider use and were the reasons for retraction of some ID vaccines from the market. These observations further underscore that the depth of vaccine deposition is not only crucial for the immune response but also a determinant of tolerability. This aspect

also needs to be considered for the design of microneedle systems. Here the vaccine depots are smaller but are more numerous and control of microneedle insertion depth maybe important to monitor (87, 174).

4.8. Loading of vaccine compounds on particulate carriers for TC delivery

In order to achieve an effective TC immunization, different strategies should be combined. Combination of penetration enhancement strategies, and wise selection of the vaccine compound by using novel vaccine delivery in particulate form will positively affect the outcome. However, different particulate architectures are available, and each comes with specific advantages and disadvantages.

The size of the antigenic compound is critical, when considered to be transported via the cutaneous route. Nanoparticles normally are unable to pass the intact SC barrier. Although quantum dots with different shape, surface coating, and in a size between 4.5-12 nm could penetrate the porcine skin (175). These particles were found in SC, epidermis and even the dermis during a time period between 8-20 h by CLSM. The penetration speed and depth varied among different particles (175). The problem with this kind of carrier and small metal nanoparticles is the possible entry of such particle even into the blood and systemic circulation. A very small size of the particle would reduce the loading capacity and as a result reduce the dosage of the antigenic compound transferred by the carrier for vaccination. Since these particles are non-biodegradable, the possibly systematic toxicity and small carrier capacity make them less attractive for vaccination purposes (175). Generally solid nanoparticles could not penetrate an intact skin barrier, hence lipid vesicles are able to change their shape and pass through the lipid layers of the SC (93). Flexible lipid vesicles could pass through the intercellular space between the corneocytes in SC (96), and could entrap higher amount of antigens (93) compared to more rigid ones. Deformability of their membrane makes liposomes suitable carriers for encapsulation of the protein antigen (93), and DNA plasmid (100) for TC vaccination. However, conventional vesicular particles such as unilamellar liposomes dissolve during the penetration process, which would result in diffuse delivery of free proteins. For vaccination purposes, this is not desirable as repetitive exposure to free protein may result in tolerance. Also particulate vaccines, which enter the cells as particles, were shown to support cross-

presentation and induction of cellular response. Such cellular response could be a special strength of vaccine delivery strategies in skin.

Interactions between the antigenic compound and the skin tissue have to be considered ensuring stability even during the penetration process is a great challenge. I.e., induce an effective immune response the designed antigenic vaccine should be transferred as a whole through the skin and become available to the target immune cells. Especially, in the case of plasmid DNA the whole designed plasmid is needed for an optimal result because cellular uptake, transfection and antigen production have to occur in sufficient amounts. Packaging in a form of particle is beneficial in different ways. Formulation in the form of nanoparticles could stabilize the antigenic compound, and induce a controlled release of the antigen in the tissue. The immunostimulatory properties of the particulate formulation could also increase the antigenic property of the vaccine and improve the immune response (77, 80, 103).

Major types of the vaccine compounds, e.g. antigens, proteins, DNA, are unstable compounds. Encapsulation not only increases the stability in a biological environment. The particulate form also could mimic the pathogen organism (e.g. viruses) and facilitate the cellular uptake.

The delivery system also enables the design of vaccine composed of different antigens. The possibility of addition of the adjuvants and the cell-specific targeting ligands increases the immunogenic capacity of the designed vaccine. This could decrease the antigenic dose needed for inducing the immune response. The high value of the dose-sparing is understandable when a limited amount of an antigen can be produced especially in critical pandemic situations (176).

The penetration of the particles through the skin barrier could be facilitated by previously mentioned strategies. However the safety issue of nanoparticle use in humans should be considered for TC delivery. Injection via ID or the use of pricking ensures the passage of higher amount of particles into the tissue. On the other hand, it facilitates the transfer of these particles into the blood circulation.

The type of the particles also affects the permeation via the skin barrier. Stiff particles are mainly unable to permeate through the intact skin.

The liposomes are able to enter the skin tissue through the lipid layer within the SC. The flexible structure of the liposomes is positively affecting this process.

Solid nanoparticle need skin pretreatment techniques to penetrate into the skin barrier. Although a minor barrier disruption by CSSS could increase the particle penetration. The penetration depth into the skin SC and even the particle uptake by skin cells are influenced by the size of the particles. The particle type also affects the size range, which could penetrate the skin tissue (177). Several studies have evaluated the penetration of polymeric nanoparticles across the SC. Evaluation of the porcine skin after topical administration of non-biodegradable polystyrene particles with CLSM showed 20 or 200 nm fluorescently labeled particles were unable to bypass the intact SC layer after 0.5 to 2 h. Hence, these particles gathered preferentially in the HF orifices which increased by the time of incubation. The follicular penetration also was also more prominent in smaller particles (178). In another study performed on human skin tissue Vogt et al. analyzed the penetration and cellular uptake of the polystyrene particles with the size between 45 to 1500 nm after one CSSS. They could show deep accumulation of the small-size particles in the HF and also their uptake by epidermal LCs. The presence of these particles in the LCs proved that the particles could cross the SC barrier (77). The 40 and 200 nm sized polystyrene particles were also able to penetrate to the HF and even the perifollicular tissue around the HF in murine model after 10 times skin surface tape striping. In this study Mahe and Vogt could show the uptake of the particles by APCs and their transport to draining LN (41). They could also show that vaccinia vector particle in a size of 290 nm could penetrate the epithelium of murine HF, and were detected in skin DCs (41). The polystyrene particles are considered to be more stable in the skin tissue environment that makes it possible to track them within the tissue and the cells. In other experiments Rancan et al. analyzed TC application of PLA particles in a size of 228 and 365 on human skin explants after one CSSS. The study showed that the particles upon application on the skin release their fluorescent content in the HF and the fluorescent dye could be detected in the epidermal layer and the isolated skin cells. The particles were not detected after TC application in the cells, after *in vitro* incubation of the skin cells particle were detected inside the cells by fluorescence microscopy. These experiments show unstable nature of biodegradable PLA in contact with the lipophilic skin environment (179). Further studies confirmed that the affinity of the incorporated material affects the PLA stability in the lipophilic skin environment. Particles carrying hydrophilic compounds showed to be more stable after topical application

and could penetrate the HF and slowly release the incorporated die. On the other hand, PLA particles carrying a lipophilic dye were more unstable and release their compound within the 4h after administration (180). On a study using silica particles after CSSS they showed only particle penetration and their entrapment in the cells only in 42 nm sized particle but not bigger (177).

The antigen compound could be also loaded onto the surface of the particles. In case of PLA particles positively charged materials like antigens and DNA plasmid could passively be adsorbed to the surface (135). In experiments done on human skin explants Rancan et al. showed that these particle could release the bounded antigens with in the HF canal (91). The mechanism of detachment of loaded antigen is not well understood. But hypothetical factors include the reaction of the hydrophobic HF environment with particle, pH gradient, or reaction of the PLA with other proteins present in the HF canal. The uptake of released antigens further were detected by skin APCs shows diffusion of the antigen after minor barrier disruption by CSSS.

The mechanism of particle uptake by the cells and further processing of the particle is also affected by particle characteristics. The biodegradable particles are known to degrade in the environment to small nontoxic compound (104). These biodegradable polymers have been already been tested in animal models and approved to be used in forms of implants and resolvable sutures in humans (105). The size of the particle affects the uptake (higher uptake by cells in smaller particles) (91). The uptake of the particle by the cells depends on the concentration of the particle and is time – dependent (181, 182). The mechanism of uptake and the interaction of the particle with the cell affect the delivery of loaded compound into different compartments. After internalization of the particle the interaction particle with lysosomal membrane affects the intracellular delivery. In an interesting study Panyam et al. showed endosomal scape of the particles into the cytoplasm within 10 min (181). The surface charge change of the particles upon contact with liposomal membrane and the pH change were suggested as the reasons (181). The particles in the intracellular space act as depots for sustained release of the compounds. These results are interesting considering the current experiments on the PLA-GTU® particles. In the HaCaT cells we could show increase of cellular granularity after particle uptake and the gp protein was detected within the cells. After particle uptake the interaction of the particle with the lysosomal environment could lead to the damage of the plasmid DNA. Since the

plasmid DNA in loaded on the surface of the PLA the particle charge change upon particle-vesicle interaction could deactivate the pGTU® or prevent its release along with the particles. In the current study the internalization of the particles was detected indirectly with the immunohistochemical staining of the gp protein and the change in cellular granularity. Utilization of the fluorescently labeled particles and using TEM could elucidate the intracellular interaction of the particles.

On the other hand if the PLA could enter the cytoplasm but not target the pGTU® into the nucleus no transfection could be seen. The successful transfection of the HaCaT by means of Lipofectamine® could be the result of encapsulation of the pGTU® within the liposomes. The encapsulation of plasmid in these liposomes allows for the delivery of the DNA to the cytoplasm without sequestration in endosomes. Since the on the PLA model the plasmid was bounded on the surface it could cause the digestion of the pGTU® before it is transported into the nucleus. Otherwise the gp antigen shows that the PLA were able to deliver the antigen to the skin tissue and also within the cells.

For TC vaccination purposes, biodegradable polymeric particles as well as the lipid nanoparticle are interesting options especially for the aim of follicular targeting (183, 184). Non-flexible particles could enter the HF and therefore improve the follicular targeting compared to the free antigen. The key role in this regard is the size of the particles. The nanoparticles not only improve the HF targeting, and protect the antigen from aggregation or cleavage but also showed the immune stimulatory effect. The immunostimulatory effect is also determined by the material used for particle production.

As one example, which has already been assessed in clinical trials, polyethylene imine nanoparticles have been used for delivery of the plasmid DNA for HIV vaccination as a DermaVir patch vaccine formulation. The vaccine formulation also showed increase of CD8+ cells in combination with antiretroviral therapy in phase II clinical trials (185, 186).

5. Conclusion

Based on these results, we can conclude that the TC application of VLPs is able to target immune cells in the epidermis as well as the dermis layer. VLPs are suitable carrier models for TC vaccination, since they are stable in the human skin environment and can penetrate the skin's layers. For such a vaccination, the transfollicular pathway seems to play a critical role. The skin acts as a barrier that hinders the penetration of organisms and immunogens into the tissue and thus impedes access of the resident immune cells to the antigen. As such, strategies to pass this barrier are necessary. Tape stripping and barrier disruption methods can independently activate the innate immunity cascade through proinflammatory cytokine release in the tissue. Consequently, they could act as unspecific adjuvants. Activation of APCs results in enhanced uptake capacities, expression of maturation markers, and improved immune response. Comparing non-invasive and invasive methods of application, it appears that non-invasive CSSS tends to target more superficial skin APCs, while vaccine compounds, which penetrate along the pricking disruptions, reach both epidermal and dermal APCs. However, both approaches resulted in migration of VLP-positive cells. Here, no obvious differences were detected between the two methods for TC delivery (Table 1). The cells that migrated out of the skin contained VLPs in both TC application methods. This shows that even when using the CSSS method, immune cell activation and targeting is achievable without disrupting the integrity of the skin barrier. Although DNA vaccines have shown promising result in the mice, the plasmid vaccine models have not been as effective in the human experiments. Studies have shown induction of humoral and cellular immune response in murine studies. But penetration, cellular uptake and effective expression of the DNA were hard to recapitulate in excised human skin. The results of the current study showed that the transcutaneous application of the naked DNA plasmid were not able to transfect the cells. Among the carrier used loading of the DNA plasmid also was not effective. Although, the PLA carrier successfully delivered the loaded antigen to the skin tissue and into the cells but was not able to transfer the DNA plasmid into the nucleus. Maybe loading the plasmid on the surface of the particle and its contact with the skin environment cause damage to the DNA plasmid. The results of transfection of the HaCaT cell line by means of Lipofectamine® show that the plasmid originally is able to transfect the cells when

delivered effectively to the site of action. Lipofectamine® creates liposome-like particles, that shows that the particulate delivery is suitable for gene delivery when chosen wisely. The different results comparing the PLA and Lipofectamine®-mediated delivery to the HaCaT cells confirms that the particle type can affect the site of the action of the delivered compound.

Table 1. Presence of the VLP in the different skin cell population. Table shows different cell populations, which were found to have taken up VLPs. TC application (CSSS and pricking) is compared to conventional ID injection.

VLP UPTAKE	CSSS	PRICKING	ID
Epidermal LCs	+	+	-
Epidermal cells	+	+	+
Dermal cells	+	+	+
Dermal DCs	+	+	+
Migrated HLA-DR +	+	+	not done
Migrated HLA-DR^{high}	+	+	not done

Abstract

6. Abstract

Novel routes of immunization could help improve immune response, spare vaccine and could help increase the vaccination coverage, especially when they allow for non-invasive vaccine delivery. Due to the presence of different immunocompetent cells, the skin is a highly interesting target organ for novel vaccination routes. Previous studies showed that targeting the hair follicle is a promising strategy for the delivery of large molecules and particles. However, the principal challenge is to find a vaccine compound, which is stable in the skin environment, penetrates sufficiently across the skin barrier and effectively targets resident immune cells.

In this study, novel experimental vaccine compounds against HIV-1 antigens were investigated for their ability to penetrate human skin. Three types of fluorescently labeled virus-like particles (VLP), including VLP-Gag-eGFP, VLP-Gag-gp140-eGFP, and VLP-Pr55Gag-CFDA, plasmid DNA, and polylactic acid particles carrying plasmid DNA and the HIV-1 capsid protein gp140, were studied in excised human skin *ex vivo*. Short-term incubation in a humidified chamber for 16 hours as well as a newly established tissue culture model for incubation and cell migration studies for up to 40 hours were used as experimental models. Skin penetration and cellular uptake were studied by fluorescence microscopy, transmission electron microscopy, and flow cytometry of cells isolated from the skin as well as migratory cells. Invasive and non-invasive techniques were used for administration of the model vaccines on the skin. Cyanoacrylate skin surface stripping, a non-invasive technique performed prior to vaccine administration, which targets hair follicles by opening follicular orifices and improving particle entry into the hair follicle canal, was compared to skin pricking or intradermal injection, respectively.

Although each VLP type exhibited a different uptake pattern, VLPs in general could be detected in almost all skin samples, and VLP-positive cells were found in epidermal and dermal cell suspensions in excised human skin and in the cells isolated from the human skin *ex vivo* and *in vitro*, respectively. In the human skin culture model VLPs were found in the cells isolated from skin tissue and in cells that migrated out of the skin. The VLP uptake by epidermal and dermal cells was slightly higher after pricking than after cyanoacrylate skin surface stripping. Interestingly, even a non-invasive administration method appeared to promote VLP translocation to

the dermal layer. Also, the non-invasive method induced similar migration of cells out of the skin and similar particle uptake by activated cells when compared to pricking. Expression of plasmid DNA in human skin *ex vivo* was not observed, neither after application of naked DNA nor after administration of the PLA-protein-bound plasmid. Cell culture studies suggested PLA-particle uptake and successful gp140 delivery but still no expression of plasmid encoded green-fluorescent protein.

These results suggest that VLPs are suitable carriers for skin vaccination, because they appear to stay intact during skin barrier penetration and cellular uptake. Both, non-invasive and invasive methods of application were effectively targeting the immune cells in skin. Even non-invasive administration induced cellular uptake and migration of activated immune cells. The results are in line with previous studies showing that cyanoacrylate skin surface stripping enhances penetration and activates skin immunoactive cells. The lack of plasmid DNA expression might be due to insufficient transfection in this model because cellular uptake of the co-delivered protein could be shown. Hence, although biodegradable nanoparticles offer great potential for co-delivery of different forms of vaccine, further research is required to ensure effective skin penetration and cellular uptake of such complex architectures.

Zusammenfassung

7. Zusammenfassung

Einbringen von Impfstoffen über andere Wege als klassische Injektionen könnte helfen Immunantworten zu verbessern, Vakzin einzusparen und Impfraten zu verbessern, besonders wenn die Impfung auf nicht invasivem Weg erfolgen kann. Aufgrund der Anwesenheit von verschiedenen immunkompetenten Zellen ist die Haut ein sehr interessantes Zielorgan für neuartige Impfstrategien. Frühere Studien haben gezeigt, dass das Targeting von Haarfollikeln eine vielversprechende Strategie für das effektive Einbringen von großen Molekülen und Partikeln ist. Allerdings ist die wichtigste Herausforderung einen Impfstoff zu finden, der in der Haut stabil ist, ausreichend durch die Hautbarriere penetriert und residente Immunzellen effektiv erreicht.

In dieser Studie wurden neuartige Arten von experimentellen Impfstoffen gegen HIV-1-Antigene auf ihre Fähigkeit die menschliche Haut zu penetrieren untersucht. Drei Arten von Fluoreszenz-markierten, Virus-ähnlichen Partikeln (VLP), (VLP-Gag-eGFP, VLP-Gag-gp140-eGFP und VLP-Pr55Gag-CFDA), sowie Plasmid-DNA, Polymilchsäure-Partikel mit Plasmid-DNA und dem HIV-1 Capsid-Protein gp140 wurden in exzidierten menschlichen Haut *ex vivo* erforscht. Als experimentelle Modelle wurden die Kurzzeit-Inkubation in einer feuchten Kammer für 16 Stunden, ein neu entwickeltes Gewebekulturmodell und Zellmigrationsstudien über bis zu 40 Stunden etabliert und verwendet. Hautpenetration und Zellaufnahme wurden mittels Fluoreszenzmikroskopie, Transmissionselektronenmikroskopie, Durchflusszytometrie isolierter Hautzellen sowie migrierten Zellen untersucht. Invasive und nicht-invasive Verfahren wurden für die Applikation der Impfstoffe auf die Haut verwendet. Als eine nicht-invasive Methode für die Verabreichung von Partikeln wurde die Cyanacrylat Abtragung der Hautoberfläche verwendet, dadurch wird das Haarfollikel-Targeting verbessert. Diese Methode wurde mit der Pricking Technik oder der intradermalen Injektion vergleichen.

Obwohl jeder VLP-Typ ein anderes Aufnahme-Muster zeigte, konnten VLPs allgemein in fast allen Hautproben nachgewiesen werden. Außerdem wurden VLP-positive Zellen in epidermalen und dermalen Zellsuspensionen aus exzidierter Humanhaut *ex vivo* und in den isolierten Zellen der menschlichen Haut *in vitro* gefunden. Im Gewebekulturmodell wurden VLPs in den aus der Haut migrierten

Zellen gefunden. Die VLP-Aufnahme durch epidermale und dermale Zellen war etwas höher als nach Pricking. Interessanterweise schien sogar die nicht-invasive Methode zur Verabreichung der VLPs die Translokation in der Haut zu fördern. Auch diese nicht-invasive Methode induzierte eine ähnlich hohe Migration von Zellen aus der Haut und eine ähnlich hohe Partikelaufnahme von aktivierten Zellen im Vergleich zum Pricking. Im Gegensatz dazu wurde die Expression von Plasmid-DNA in der menschlichen Haut *ex vivo* nicht beobachtet, weder nach der Applikation purer DNA oder nach der Verabreichung des PLA-Protein-gebundenen Plasmids. Zellkulturstudien zeigten eine PLA-Partikelaufnahme und erfolgreiches Einbringen von gp140 Protein, aber keine Expression der Plasmid-kodierten grün-fluoreszierenden Proteins.

Unsere Ergebnisse deuten darauf, dass VLPs geeignete Träger für die Hautimpfung sind, weil sie während der Penetration der Hautbarriere und der zellulären Aufnahme scheinbar intakt bleiben. Die beiden nicht-invasiven und invasiven Methoden erreichten effektiv die Immunzellen in der Haut. Auch nicht-invasive Verabreichung induzierte eine zelluläre Aufnahme und die Migration von aktivierten Immunzellen. Die Ergebnisse stimmen mit früheren Studien überein, die zeigten, dass Cyanacrylat-Abrisse die Penetration verbessern und die Immunität aktivieren. Das Fehlen von Plasmid-DNA-Expression ist möglicherweise erklärbar aufgrund unzureichender Transfektion in diesem Modell, da die zelluläre Aufnahme des co-gelieferten-Proteins nachgewiesen wurde. Obwohl biologisch abbaubare Nanopartikel ein großes Potenzial der Applikation der verschiedenen Formen der Impfstoff bieten, ist weitere Forschungen erforderlich, um die Hautpenetration und zelluläre Aufnahme von solchen komplexen Architekturen zu gewährleisten.

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9. List of publications

Book Chapter

- Rancan F, **Afraz Z**, Combadiere B, Blume-Peytavi U, Vogt A. 2013. In: Nanotechnology in Dermatology: Hair Follicle Targeting with Nanoparticles. Springer. ISBN 978-1-6414-5033-7.

Articles

- Vogt A, Hadam S, Deckert I, Schmidt J, Stroux A, **Afraz Z**, Rancan F, Lademann J, Combadire B, Blume-Peytavi U. 2014. Hair follicle targeting, penetration enhancement and Langerhans cell activation make Cyanoacrylate Skin Surface Stripping a promising delivery technique for transcutaneous immunization with large molecules and particle-based vaccines. Experimental Dermatology, 24 (1): 73-75.

- Volz P, Boreham A, Wolf A, Kim TY, Balke J, Frombach J, Hadam S, **Afraz Z**, Rancan F, Blume-Peytavi U, Vogt A, Alexiev U. 2015. Structures and dynamic in the stratum corneum of human skin as revealed by single molecule fluorescence microscopy and penetration of a large amphiphilic molecules. International Journal of Molecular Science, 16(4): 6960-6977.

Poster presentations

-High serum homocysteine levels accompany poor cardiac function in coronary artery disease patients. Afraz Z et al. **18th European Students Conference**, 7-11. October 2007, Berlin (Germany)

- CD1a^{high} positive Langerhans cells preferentially internalize HIV-1 virus-like particles after transcutaneous administration. Afraz Z, Rancan F, Hadam S, Wagner R, Schäfer-Korting M, Blume-Peytavi U, Vogt A. **Arbeitsgemeinschaft Dermatologische Forschung** (ADF) Annual meeting, 14-16. March 2013, Dessau (Germany)

-Targeting of antigen presenting cells by HIV-1 virus-like particles for transcutaneous vaccination. Afraz Z, Rancan F, Hadam S, Wagner R, Schäfer-Korting M, Blume-Peytavi U, Vogt A. **International Hair Conference**, 4-6. May 2013, Edinburgh (Scotland)

- Targeting of antigen presenting cells by HIV-1 virus-like particles for transcutaneous vaccination. Afraz Z, Rancan F, Hadam S, Wagner R, Schäfer-Korting M, Blume-Peytavi U, Vogt A. **Gesellschaft für Dermopharmazie** (GD) Annual meeting, 21-23. March 2014, Berlin (Germany)

Oral presentations

- Workshop: Tissue Sampling, Techniques for Skin Cell Separation. **Cutaneous and Mucosal HIV vaccination (CUT'HIVAC)** Annual meeting, 7-9. November 2012, Berlin (Germany)

- High serum homocysteine levels accompany poor cardiac function in coronary artery disease patients. Afraz Z et al. **3rd Young Medic's International Conference,** 19-21. November 2005, Yerevan (Armenia)

10. Curriculum Vitae

For data security reasons the curriculum vitae is not included in the online version.