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Habilitationsschrift

Targeting of Skin Antigen-Presenting Cells

A Rationale for Nanoparticle-Based Transcutaneous Vaccination

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"…the most important aspect of my career is that it has been fun – the joy of predictive results materializing, the even more rewarding experience of serendipitously discovering an entirely unexpected phenomenon, and the special gratification of having the results applied to medical problems."

Mildred Cohn, Biochemist, Member of the National Academy of Sciences, In: "The Door in the Dream" by Elga Wasserman. National Academy of Sciences, 2000, Joseph Henry Press, Washington, D.C.

Table of Contents

Abbreviations

Antigen-Presenting Cell	APC
Anti-Rejection Drug	ARD
Basal Cell Carcinoma	BCC
Basal Cell Nevus Syndrome	BCNS
-Cytosine-Phosphate-Guanine-	CPG
Cyanoacrylate Skin Surface Stripping	CSSS
Dendritic Cell	DC
Enhanced Green Fluorescent Protein	eGFP
Enzyme Linked Immuno Spot Technique	ELISPOT
Glioma-Associated Oncogene Homolog	Gli
Human Immunodeficiency Virus	HIV
Human Leukocyte Antigen	HLA
Human Papilloma Virus	HPV
Hedgehog Interacting Protein	HIP
Intradermal	i.d.
Intramuscular	i.m.
Ionizing Radiation	IR
β-Galactosidase Encoding Structural Gene	LacZ

Langerhans Cell	LC
Major Histocompatibility Complex	MHC
Modified-Vaccinia Ankara Virus	MVA
Non-Melanoma Skin Cancer	NMSC
Oligodeoxynucleotide	ODN
Patched	Ptch
Scanning Transmission X-Ray Microscopy	STXM
Skin Associated Lymphoid System	SALT
Smoothened	Smo
Sonic Hedgehog	Shh
Squamous Cell Carcinoma	SCC
Subcutaneous	s.c.
T Helper 1	TH1
T Helper 2	TH2
Transcutaneous	t.c.
Tumor-Associated Antigen	TAA
Ultraviolet Light	UV Light

Introduction

1.1. Rationale for the Targeting of Skin Antigen-Presenting Cells

The skin is the largest organ covering 1.6-1.9 m^2 in human adults. Located at the interface to the environment, it is continuously exposed to environmental hazards, including (UV-) radiation, mechanical, thermal, chemical and biological stress factors. The epidermis, especially its outermost layer formed by the cornified keratinocytes of the stratum corneum, provides a sophisticated physical and biochemical barrier to prevent putative pathogens from penetrating. In addition to this anatomical barrier, the human skin has resident and migrant cells with immunological properties. Epidermal and dermal antigen-presenting cells (APCs), lymphocyte subpopulations, monocytes/macrophages, neutrophils and mast cells interact through a myriad of mediators with keratinocytes and endothelial cells to provide a finely balanced second line of defence against pathogen entry. Skin APCs are among the key players of this skin associated lymphoid tissue (SALT) [1]. They are differentially located in the skin layers: dendritic cells (DCs) can be found at high density in the dermis, whereas Langerhans cells (LCs) are preferentially localized in the epidermis [2]. Although LCs account for only 1% of the total skin cells their dendrites form a close network which covers approximately 25% of the skin's total surface area [3-5]. According to a current paradigm, skin APCs internalize antigen, differentiate and migrate to local skin-draining lympoid tissue, where they encounter naïve T and B cells and initiate adaptive immune responses. There is also evidence that skin APCs are capable of presenting self, keratinocyte-expressed antigens to naïve T cells [6;7], and they seem to be tolerogenic under steady state and non-inflammatory conditions [8-10]. The abundance and localization of skin APCs, however, and their potent capacity to induce immune responses make the skin an attractive tissue for APC targeting, e.g. by transcutaneous (t.c.) vaccine delivery. The rationale for skin APC targeting is even stronger in the light of recent reports on the capacity of local lymph node DCs to process exogenous particulate and cell-associated antigens via MHC Class I pathway known as cross-presentation [11;12]. The concept of cross-presentation provides a mechanism for the immune system to induce CD8 T cell responses against viruses and tumors and suggests that targeting of vaccine compounds to skin would be a valuable strategy to induce cellular immune responses [12]. In fact, vaccines have generated major successes in the control of infectious diseases, but several obstacles remain in their development against chronic viral diseases, such as HIV, hepatitis C or against cancer, where cellular immune responses are critically involved in the disease control.

1.2. Vaccination

1.2.1. Vaccination Against Infectious Diseases

Classical prophylactic vaccines aim at preventing infection by inducing in naïve individuals a strong memory that will control pathogen dissemination at entry in the organism *("prophylactic vaccination"*). Another concept is to use vaccines as therapeutic tools during an established infection by reinforcing or broadening defences when specific immune responses are unable to do so during the natural course of disease, and when conventional antimicrobial therapy is not available or efficacious enough *("therapeutic vaccination").* This strategy is currently being evaluated in HIV infection, where therapeutic vaccines aim at limiting costs and toxicity of a lifelong antiretroviral therapy and at preventing disease progression in the absence of treatment by reinforcing immunity to HIV [13]. Currently, however, only live attenuated vaccines, e.g. vaccines against tuberculosis, measles and rubeola, have been shown to induce strong CD8 T cells, and their applicability in immunosuppressed individuals is limited by the risk of uncontrolled virus dissemination. Thus, efforts have to be directed towards an improvement of T cell immune responses also in immunosuppressed, e.g. HIV infected individuals [14;15].

1.2.2. Vaccination Against Tumors

Recently, the classic concept of vaccination against infectious diseases has been extended to immunization against cancer by preventing infections with cancer-causing viruses, or as tool to treat existing cancer. Prevention of tumors which form as a result of virus infection, e.g. cervix cancer caused by human papillomavirus infection or liver cancer caused by chronic hepatits B infection, has been shown to be feasible with conventional vaccination strategies against those viruses, which lead to virus elimination long before the tumor forms. When challenged before the cancer has developed, the host's immune system has not been impaired by tumor-induced immunosuppression and the immune system may eliminate subclinical tumors much more effectively than it can eliminate macroscopic tumors. This concept provided the basis for the idea that tumor rejection can be obtained by immunizing with tumor-associated antigens (TAAs) [16]. Indeed, shared antigens appear to be predominant targets of tumor-specific immunity [17], and the use of overexpressed or mutated oncogenic growth-factor-receptors as TAAs has yielded rational targets for specific immunoprevention and attempts of immunotherapy [18]. Animal studies have shown that cancer vaccines are most effective in protection against subsequent tumor challenge. And there is also evidence that vaccination can prevent tumor occurrence in genetically predis-

posed animals. For example, vaccination with dendritic-tumor fusion cells prevented the development of tumors in up to 43% of transgenic mice predisposed to develop spontaneous mammary carcinomas [19]. Besides class I/II expression and intratumoral cellular infiltrates, tumor homogeneity is among the key prognostic features that may help to select tumor types and patients who are more likely to benefit from antigen-specific cancer vaccines than others [20]. So far, however, vaccines have shown limited potential in curing established tumors. Clinical trials of therapeutic cancer vaccines recapitulate the observation in animals, i.e. the trials show the ability of the vaccine to stimulate, to widely varied degrees, specific antibodies and T cells, but with few exceptions rarely result in an objective clinical antitumor response [21].

1.2.3. Rationale for Vaccination Against Basal Cell Carcinomas (BCCs)

Due to its exposed position, the skin is highly susceptible to the accumulation of genetic damage and retention of carcinogens, and, indeed, non-melanoma skin cancers (NMSCs), first and foremost squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs), are the most common human neoplasias. Studies with biopsy proven NMSC cancer patients and immunosuppressed organ transplant recipients suggest that impaired immune surveillance as a result of ultraviolet (UV) light, chemotherapeutic agents or other immunosuppressants appears to be a major risk factor for skin cancer formation. In turn, therapeutic strategies to enhance immune control mechanisms by topical application of immune response modifiers or by anti-tumor vaccination have increasingly gained interest over the past years. As outlined in the following, several aspects of the molecular biology and the immunology of BCCs encouraged us to explore the possibility to vaccinate against BCCs. BCCs are comparatively homogenous tumors. Genetic instability of BCCs appears to be much less than that of most tumors. Thus, one study found aneuploidy in 19% of 509 BCCs compared with 75% in other solid tumors [22]. Allelic deletions on chromosome 9 at the patched gene locus are characteristic for BCCs, but deletions are uncommon at other chromosomal sites. Accordingly, Quinn et al. found loss of heterozygosity of one or more 9q markers in 33 of 44 BCCs [23]. Mutational activation of hedgehog signalling with overexpression of hedgehog target genes is the pivotal step in the development of BCCs. This activation most commonly is effected by mutations in the tumor suppressor gene PTCH1, which encodes a protein which normally inhibits hedgehog signalling [24;25]. Among the target genes uniformly overexpressed in BCCs is the putative transmembrane protein hedgehog-interacting protein (Hip1). In animal models, mutational activation of hedegehog signalling is sufficient to induce BCC-like lesions, and mutations in PTCH1 and/or SMO, both components of the hedge-

hog signalling cascade, have been found in >90% of human BCCs [26-28]. This characteristic defect of BCCs led us to investigate further the expression of the hedgehog target gene Hip1 as a possible candidate for future immunization studies. Interestingly, there are some hints that the immune system may help control human BCCs. Thus, Curson and Weedon first described the infiltration of BCCs by immune cells together with possible signs of regression in BCCs, e.g. disruption of the palisaded architecture of the tumor cells at the periphery, occurrence of apoptotic cells, and dermal deposition of collagen [29]. Using these criteria, they found that 81 out of 400 tumors examined had evidence of previous or continuing regression. Several groups have found significantly increased numbers of CD3 and CD4 cells in the tumors, and the finding of increased levels of IFN-gamma mRNA in tumors suggests a possible role for CD4 cells and Th1 cytokines in the control of BCCs [30-32]. Furthermore, non-specific stimulation of the immune system by local injection of IFN-alpha or by application directly to BCC tumors of the immune response modifier imiquimod can reduce tumor size or even eliminate BCCs [33-37]. Postulated mechanisms for the efficacy of IFN-alpha include the stimulation of the CD95 (Fas) receptor in the BCCs, which in untreated patients constitutively express CD95 ligand but not the receptor. The peritumoral infiltrate could support the resulting apoptotic suicide by the secretion of IFN-gamma or IL-2, which may trigger further up-regulation of CD95 on BCC cells. The immune response modifier imiquimod activates the immune system through localized induction of cytokines such as IFN-alpha, IFN-gamma and IL-12, and thus its mechanism of action may be similar to that of direct cytokine treatment with IFN-alpha. Although cytokine therapy of BCCs is still experimental and its long-term efficacy has been questioned [38], the clinical success of local immunostimulation with IFN-alpha and imiquimod and the putative role of infiltrating T cells in regressing BCCs encouraged us to assess the possibility of BCC immunoprevention using TAAs. Such non-invasive, ideally preventive therapy against BCCs would substantially improve life quality and the management of individuals at high risk of developing BCCs. E.g. in the rare autosomal dominant basal cell nevus syndrome (BCNS, Gorlin syndrome, MIM 109400) the patients develop dozens to hundreds of BCCs starting in early childhood or adolescence [8;9;39]. Local growth and treatment of the multiple tumors in these patients inevitably cause significant scarring and disfigurement. Since BCNS patients develop such large numbers of BCCs, even partial prevention of tumors would provide great clinical benefit for these patients.

1.2.4. Alternative Vaccination Routes and Drug Delivery Systems May Improve Current Vaccination Strategies

Over the past years, numerous vaccination concepts have successfully been developed in animal models and subsequently brought to clinical trials. The overall outcome, however, has not met the expectation and is still challenging the field of immunology and vaccine design. Facilitating vaccine compound penetration into immunization sites that are rich in APCs as well as specific migration and activation of APCs would benefit to the efficacy of new vaccines in the induction of protective immune responses. A future goal for vaccine design is therefore to increase vaccination efficiency by reaching the highest number of APCs possible and to achieve the high local concentrations required to induce a potent immune response. Promising approaches for innovation include modifications of the mode of vaccine application, for example, mucosal or cutaneous administration, better targeting of APCs, and mechanisms of improved antigen processing and presentation [40]. Among the various approaches, two different strategies have gained our interest and will be discussed in the following: (i) skin applied vaccines and (ii) the use of nanoparticle compounds as vaccine delivery systems.

1.3. Transcutaneous Vaccination

1.3.1. Crossing the Skin Barrier

Protection against pathogen entry is an important function of the skin. On the outer skin surface, multiple layers of corneocytes, embedded in lipid layers made of ceramides, cholesterol and their derivatives, form the stratum corneum, which represents an effective physical barrier especially to the penetration of compounds larger than 500 Da [41]. Passage through the stratum corneum occurs at low diffusion rates across the lipid layers. In fact, the diffusion pathway of water in the stratum corneum is fifty times longer than suggested by the thickness of this skin layer, which lead to the assumption that corneocytes play a limiting role in penetration and that the penetration pathway of topically applied compounds follows the winding route along the intercellular space. Transcellular penetration if existent at all, probably accounts only for a small amount of compounds, but cannot be excluded since some groups detected topically applied compounds in corneocytes. Complementary to these findings, Weigmann et al. reported that substances are mainly located in the uppermost cell layers of the stratum corneum, where they are continually depleted due to the physiological process of desquamation [42]. In conclusion, the stratum corneum significantly reduces the penetration rates of topically applied compounds, and as a consequence, ac-

cessibility of skin APCs. Recent investigations, however, have demonstrated the important role of skin appendages, above all hair follicles, as entry point and reservoir for topically applied compounds [43].

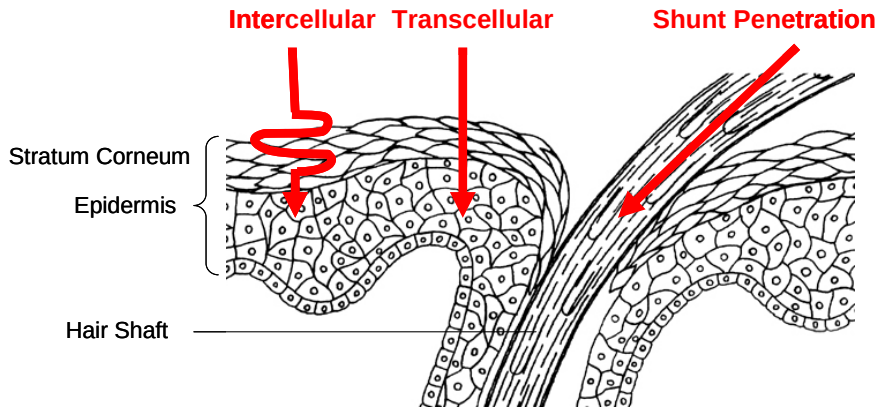

Fig. 1: Penetration Pathways in Human Skin.

Penetration may occur through the corneocytes or through the intercellular space of the stratum corneum. Recent studies also demonstrated the importance of shunt penetration pathways through skin appendages, most importantly hair follicles, which represent physiological breaks in the skin barrier.

1.3.2. The Hair Follicle: Reservoir and Penetration Pathway

Initially, skin appendages were not considered to be significant penetration routes, as evidence suggested that they accounted for only approximately 0.1% of the skin surface area [44]. These calculations, however, did not take into the account, that the hair follicles represent invaginations, which extent deep into the dermis with a significant increase of the actual surface area available for penetration. With a rich perifollicular vascularization and changes in the differentiation pattern along the follicular duct, the hair follicle possesses distinct characteristics which favour penetration. Multiple studies suggest, that the follicular penetration route may be especially relevant for hydrophilic and high molecular weight molecules as well as particle-based drug delivery systems [43;45-47]. It has long been known that the penetration rates of topically applied compounds vary considerably in the different regions of the human body, and multiple studies reveal that the presence of hair follicles significantly contributes to this effect. E.g. the penetration of corticosteroids is considerably lower in hairless skin, as compared to haired skin [48;49]. Similar observations have been made in tissue-engineered skin where the insertion of

hair follicles significantly increased the penetration rate of hydrocortisone [50]. Due to its unique anatomy, the hair follicle infundibulum is the key compartment for such shunt penetration. While an intact and relatively impermeable horny layer similar to that of the interfollicular epidermis covers the acroinfundibulum, this barrier is interrupted in the lower follicular infundibulum.

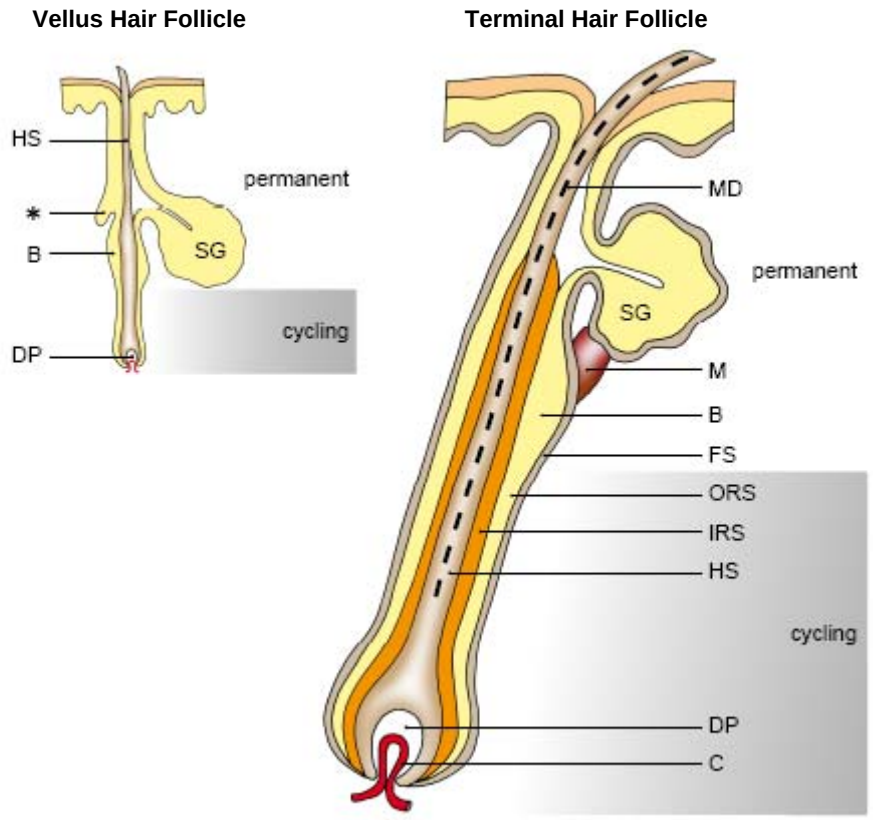

Fig. 2: Anatomy of the Pilosebaceous Unit (modified from Vogt *et al.* 2008) [54]**.**
All hair follicles follow a common architecture. Together with the sebaceous gland (SG) and the arrector pili muscle (M), the hair follicle is part of the so-called pilosebaceous unit. The fibrous sheath (FS) and the epithelial outer and inner root sheaths (ORS, IRS) form concentric layers which ensheath the hair shaft (HS). Hair growth results from the proliferative activity of matrix keratinocytes in the bulb, which sit on the dermal papilla (DP). The dermal papilla is a condensate of specialized mesenchymal cells with important inductive properties. It also provides nutrition via a capillary loop (C), which is especially prominent in terminal hair follicles. The permanent, superficial component, which includes the infundibulum, has to be differentiated from the transient cycling component of the hair follicles. The morphological dividing line between those components lies below the bulge (B) region and the insertion of the arrector pili muscle (M). The infundibulum is the key compartment for shunt penetration.

The corneocytes in this area appear smaller and crumbly, suggesting that the skin barrier is incomplete and permeable in this region [51;52]. This incomplete barrier provides the opportunity for intense interactions between topically applied compounds, the hair follicle epithelium and associated cell populations such as APCs, mast cells, melanocytes or stem cells. Drug delivery systems and formulations designed to selectively target the human hair follicle may allow the conveyance of relevant doses of active compounds into the follicular duct. Possible applications include the treatment of hair growth abnormalities, as well as hair follicle-associated diseases and general skin disorders [53].

1.3.3. Immunology of the Hair Follicle

The hair follicle, as a physiological break in the skin barrier, is a conduit for intensive interactions with the environment [54]. Interestingly, only low numbers of immune cells are found in the transitory hair follicle compartments [55]. The restricted distribution of intraepithelial T cells and LCs, accompanied by a suppressed MHC II dependent antigen presentation, the virtual absence of MHC class I expression, and the concomitant high expression of immunosuppressive mediators and immunomodulators, including alpha-MSH, TGF-beta1 and ACTH, suggest that the hair follicle, between the bulb and the lower bulge region, constitutes an area of transient immune privilege [56]. Just like other immunoprivileged tissues, such as the anterior eye chamber, the hair bulb is devoid of lymphatics and is ensheathed by a special extracellular matrix barrier, both of which might hinder immune cell trafficking. In contrast, however, APCs can be found at particularly high densities in hair follicle-bearing skin [57]. They are especially concentrated around the upper portion of the hair follicles, where they are found not only in the suprabasal layer but also in the basal layer of the outer root sheath [55;58]. The loss of the stratum corneum toward the lower part of the hair follicles implicates that APCs, which reside in and around the hair follicle, are more accessible for topically applied vaccines than APCs in the interfollicular epidermis, which are shielded by the stratum corneum. Targeting of vaccine to the hair follicle and to follicular APCs, may, hence. improve the efficacy of t.c. vaccination [59].

1.3.4. Rationale for Transcutaneous Vaccination

Vaccines are classically injected into muscles or subcutaneous tissues, where local depots of vaccine compounds have to be captured by immature DCs, promote DC maturation and migration to the draining lymph nodes, where they prime the naive vaccine-specific T cells. Those tissues are

however very poor in DCs. In addition, part of the vaccine is bound unspecifically to connective tissue or undergoes degradation. To achieve the high local concentrations required to induce a potent immune response, targeting of vaccines toward DCs would help at enhancing their immunogenicity. Conventional intramuscular (i.m). or subcutaneous.(s.c) injections also raise some psychological or cultural difficulties. Indeed, injection procedures present several disadvantages, such as the risk of blood transmission, infection caused by unsterile equipment or unsterile reconstitution, instability of the vaccine preparation and injury due to improper injection techniques [60]. Among future administration routes, e.g. transnasal, transvaginal and others, the t.c. route appears to be highly promising for the following reasons: The skin is one of the largest immune organs in the first line of contact with pathogens and is rich in potent APCs such as LCs in the epidermis and DCs in the dermis [61]. The use of the skin as a target organ for vaccine design has been spurred by the fact, that immature DCs can be found at high densities in the epidermis and the dermis of human skin [3]. These cells can efficiently initiate primary immune responses both *in vitro* and *in vivo* [62], i.e. the skin appears as a prime target organ to generate cellular and humoral immune responses [63]. Many studies have found that intradermal (i.d.) immunization allows using quite smaller doses of antigen than i.m. immunization, probably because of the better availability of APCs at the site of inoculation [64-66]. For example, the i.d. injection of one-fifth of the dose of a conventional influenza vaccine is sufficient to induce better or equal immune responses than conventional i.m. vaccinations [67]. Glenn et al. (2000) elegantly demonstrated that t.c. vaccination is safe and efficient. His pioneer studies were followed within the past few years, by different methods for t.c. immunization such as depilation cream [68,69], or shaving [70;71]. However, those methods are to some extent invasive, require the treatment of large skin surfaces and promote a random penetration through the skin. Also, the focus of these former studies was on the induction of humoral immune responses, and the relevance of the results for therapeutic vaccination approaches remains unclear. Only recent implications of epithelial DCs in cross-priming suggest that vaccination via the t.c. route may be relevant in the induction of cellular immune responses [11]. In fact, the identification and targeting of the DC subset able to induce cytotoxic CD8 T cells against antigens delivered via mucosa or skin, is a major issue for the development of efficient anti-infectious and anti-tumoral vaccines [72]. The literature has also explored the expression of tissue-specific homing molecules that can direct antigen-experienced T cells to particular peripheral tissues. By focusing the targeting of vaccine compounds to the skin and mucosal APCs within regional lymphoid tissues, one can favor a mucosa imprinting homing phenotypes [73]. This aspect of re-directing the immune system remains unknown in vaccination strategies. Indeed, differential targeting of epidermal or dermal APCs could also lead to

differential quality of immune responses [74;75]. Overall the emerging evidence for the great potential of the skin as target organ for vaccine delivery, encouraged us to make use of our own expertise in the field of skin penetration in order to develop t.c. vaccination protocols. Our aim was to utilize the reservoir function of the hair follicle to preferentially target hair follicle-associated APCs.

1.4. Particle-Based Drug Delivery Systems

1.4.1. Skin Penetration of Particle-Based Drug Delivery Systems

As outlined above, drug delivery systems on the nanometer scale have gained great attention in the last years because of their biodistribution and loading-release features that allow selective drug delivery and release of active compounds over a prolonged time course to the specific site of action. In the fields of dermatology and cosmetics, micro- and nanoparticles have been studied since decades and some formulations are already commercially available. Several kinds of particles are being investigated as drug delivery systems for topical applications, including solid lipid nanoparticles and polyester nanoparticles like poly-lactic acid, poly-lactic-co-glycolic acid and poly-ε-caprolactone and semi-solid particles such as liposomes. Interestingly, particle-based drug delivery systems, when applied topically on the skin surface, show a clear tendency to aggregate and remain in hair follicle openings. Rolland et al. reported that microspheres of 3–10 µm after application on human skin aggregated in the follicular orifices whereas particles larger than 10 µm remained on the skin surface [47]. Particles of 1 µm spread widely on intact skin and also penetrated into the upper layers of the stratum corneum, but no penetration into viable epidermis was observed. Specific delivery and controlled release of adapalene incorporated in 5 µm microspheres into the hair follicles were demonstrated *in vitro* and *in vivo* on hairless rats and on human skin. Similarly, rhodamine-6G-loaded 5 µm microspheres dispersed into silicone entered into follicular duct without penetration within the stratum corneum [76]. Methylene blue-loaded 5 µm microspheres penetrated into the follicular duct and in sebaceous gland structures of hairless rat skin without penetration into the stratum corneum [77]. Building on these findings our group performed a large series of investigations on the penetration profiles of microparticles from 6.0 µm down to 0.75 µm on freshly excised human scalp skin. 6.0 µm particles aggregated in the infundibulum of terminal hair follicles and penetrated down to approximately 500 µm [45]. Smaller particles with a diameter of 1.5 µm or 0.75 µm penetrated deeper with 40% of terminal hair follicles targeted down to a depth of approx. 800 µm. These data suggest, that microparticles of different sizes allow for targeting of different hair follicle compartments e.g. the infun-

dibulum or the bulge region. In accordance with the work on solid particles, follicular aggregation and penetration along the hair follicle duct was also confirmed for liposomal preparations [78;79]. Du Plessis et al. evaluated the effect of the particle size of liposomes carrying cyclosporine A on the deposition of drugs into the skin strata of hairless mice, hamsters and onto porcine skin. They found that the intermediate particle size of 300 nm resulted in both the highest reservoir in the deeper skin with the exception of the porcine ear, as well as in the highest drug concentration [80]. In recent studies, the focus shifted to the direct delivery of biological compounds as well as vectors for DNA expression. For example, topically applied melanin entrapped in phosphatidylcholine liposomes induced hair shaft pigmentation in white-haired mice [81]. Several researchers have shown that the envelopment of a vaccine in liposomes elicited a clearly increased humoral or cellular immune response, compared to the non-enveloped vaccine [82-85]. Balsari et al. reported that liposomes can deliver monoclonal antibodies into the hair follicles of rats for protection against doxorubicin-induced alopecia [86]. Liposomes loaded with DNA have been used to target high molecular weight DNA to hair follicles in histocultured skin, as a model for gene therapy of hair growth processes. The successful targeting of liposomes loaded with lacZ reporter gene or the repair gene T4 endonuclease V demonstrates the potential of particulate preparation for drug delivery in the context of gentherapeutic approaches [87;88]. In summary, encapsulation using nano- and microparticulate systems is an increasingly implemented strategy in drug delivery and may provide the base for a new generation of transcutaneous, more precisely, "transfollicular" delivery of bioactive compounds [89]. Compared to conventional preparations, such systems may enable sustained release, resulting in an extended activity or enhanced uptake [90], and the possible reduction of adverse effects [91]. Furthermore, encapsulated substances are shielded from degradation in the particles [92]. Studies by multiple groups demonstrated that functional coatings can further improve targeting of anatomical sites [93;94], such as the eye [95;96], nose [97;98], brain [99;100] and intestine with particles [101;102].

1.4.2. Particles in Immunotherapy

At present, several projects in the fields of nanotechnology and biomaterial research aim at using nanosystems as vaccine carriers. Several studies suggest, that antigen adsorption to nanoparticles may help improve the efficacy of current vaccination strategies. According to Scheicher et al. the uptake of particle-bound antigen by bone marrow-derived DCs triggers their activation and increases their antigen presentation capacity compared to free, that is, not particle-bound, antigen [103]. Shen et al. found in cloned DCs that the presentation of exogenous ovalbumin was markedly

enhanced when the antigen was particulate [104]. Similarly, particle-mediated immunization of pigs with plasmid DNA expressing the influenza A hemagglutinin resulted in protection against the following challenge, which was equivalent to or better than a commercial swine influenza vaccine [105]. Results show that not only the colloidal properties but also the polymer composition and macromolecular architecture are critical to induce an effective immune response.

1.5. Project Plan

As outlined above, current vaccination strategies face different challenges. Frequently, transfer of results obtained *in vitro* or in animal models to humans is complicated, which underlines the importance of robust experimental models which yield reliable preclinical data and which help choosing the most promising approaches for translation into pilot studies in humans. The presented work focuses on two different aspects of this complex field: The studies which were conducted in the time period of 08/2001-11/2002 in the Department of Dermatology, University of California San Francisco, focused on the investigation of immunosurveillance and immunotherapeutic strategies against BCCs. Haired Ptch1+/- mutant mice were characterized in the laboratory of Prof. E.H. Epstein as the first mouse model of UV radiation and ionizing radiation (IR)-induced BCC-like tumor formation, and also demonstrated that Ptch inactivation and Hedgehog target gene activation are essential for BCC tumorigenesis [106].

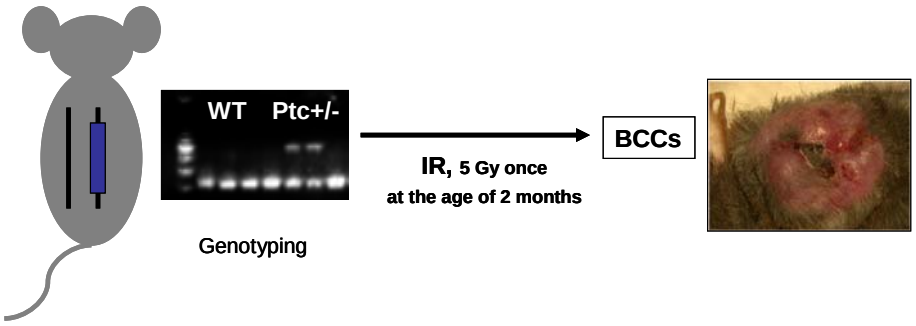

Fig. 2: Ptch1+/- Mice Closely Mimic Human BCC Development.
Haired Ptch1+/- mice, in which the lacZ and neo genes where substituted for part of exons 1 and 2, develop BCC-like tumors after chronic exposure to UV or after single treatment with IR [106]. E.g. after treatment with 5 Gy IR at the age of 2 months, all develop microscopic BCC-like tumors by the age of 7 months, and 50% of surviving individuals develop visible BCCs by age 15 months, making this a reliable and practical tumor model.

The tumors form endogenously and provide the unique opportunity to study novel therapeutic approaches against this highly common skin cancer. The aim of our studies was to further validate this experimental model. In order to mimic the course of disease observed in organ transplant recipients, we studied the effects of long-term immunosuppression on tumor burden in ptch1+/- mice. In the next step, we explored the possibility to reduce BCC formation in those mice by immunotherapeutic and immunpreventive strategies directed against hedgehog target gene products. Mutational activation of the hedgehog signalling cascade is a key event in BCC formation and overexpressed gene products represent promising targets for tumor vaccination. The hedgehog signalling pathway, however, is a highly conserved developmental signalling pathway, which is essential during embryogenesis, i.e. potential TAAs represent self-antigens. Therefore, the project also included the investigation of hedgehog target gene expression in mouse tissues. This work on tumor vaccination fostered further questions regarding possibilities to optimize conventional vaccination strategies. As discussed earlier, modifications of the mode of vaccine application and modified vaccine preparations are promising approaches for innovation. Therefore, after changing to the Clinical Research Center for Hair and Skin Science, Department of Dermatology and Allergy, Charité – Universitätsmedizin Berlin in 12/2002, I pursued projects in the fields of t.c. drug delivery and the use of particle-based drug delivery systems. Work by different groups had demonstrated that particles, when applied topically on human skin, aggregate in hair follicle openings and penetrate along the follicular duct. Small particles in the nanometer-range had not been studied at that time. Translocation of particles into the viable tissue, however, was widely considered unlikely. Indeed, even at present, there is little evidence that nanoparticles at a size exceeding 100 nm penetrate into intact skin [107] and if particles < 100 nm translocate through the intact skin barrier remains unclear. Building up on these findings the aim of this project was to investigate the penetration of solid nanoparticles in human skin, possible translocation pathways into the viable tissue and cellular uptake. Because antigen adsorbed to particles had been reported to be more effective at inducing immune responses, we were especially interested in the application of nanoparticles in t.c. vaccination strategies. To study all these aspects, different experimental models had to be developed and established in the laboratory. Our hypotheses challenged the fields of skin penetration as well as vaccination immunology and encouraged us to explore and evaluate new technologies, such as *in vivo* confocal laser scanning microscopy and Scanning Transmission X-ray Microscopy (STXM). Translation of important findings into clinical applications was key to this project. The goal was to use the body of data generated in the experimental models to perform proof-of-concept studies in human volunteers.

3. Discussion

3.1. Ptch1+/- Mice are a Suitable Model to Study Effects of Long-Term Immunosuppression on BCC Formation

In our studies on anti-rejection drug (ARD) administration in ptch1+/- mice, long-term administration of ARD caused immunosuppression and significantly increased the formation of endogenous BCCs. We were able to prosecute this study using cyclosporine A and prednisolone, a combination of immunosuppressive drugs used widely in organ transplant recipients, at doses that caused substantial suppression of antibody formation and of cell-mediated immunity but were well tolerated. This study demonstrates that ptch1+/- mice are a robust model for ARD enhancement of NMSC carcinogenesis. The mouse model facilitates greatly the study of the mechanisms of enhancement, the screening of different drug regimens for their relative potential to produce this side-effect, and the study of interactions of ARDs with environmental and genetic factors. The latter is of particular interest, because not all immunosuppressed patients develop NMSCs, and in those who do, the numbers of lesions vary considerably. Our studies demonstrate that the ptch1+/- mouse model should be useful for the investigation not only of the mechanism of ARD enhancement of BCC formation, admittedly with the proviso that mechanisms of enhancement could differ depending on the environmental insult (i.e. IR vs. UV radiation) but also of genetic factors that may help determine which organ transplant recipients develops BCC. That such genetic factors are important is suggested not only by the marked differences in development of NMSCs, both SCCs and BCCs, in organ transplant recipients within the population and between populations (e.g. the incidence of NMSC is very low among organ transplant recipients of Asian or Hispanic ancestry [129;130], R, Hirose, UCSF. Personal communication), but also by the association to BCCs with specific HLA phenotypes in non-organ transplant recipients [129;131]. In our model, enhancement of BCC formation occurred in the absence of UV exposure, suggesting that continued UV exposure is not essential for drug-enhanced BCC formation and that the effects of ARD in this model were at a post-initiation stage of BCC development. A small number of publications have reported that ARD can enhance experimental mouse (mostly hairless) squamous cell photocarcinogenesis but the reported enhancement is far less impressive than is the increased incidence of SCCs observed in human organ transplant recipients [129;132-137]. These reported studies used UV photocarcinogenesis and mostly used mutant hairless mice, in which UV radiation primarily causes papillomas and carcinomas of the squamous cell lineage. In these models, the immunosuppressive effects of UV radiation and

ARDs overlap, and the mice are genetically immuno-impaired [129;138]. By contrast, we have studied formation of BCCs rather than of SCCs and have used ptch1+/- mice of normal follicular structure and without known immuno-impairment, and these mice develop BCC-like tumors in response to chronic UV radiation or, as in this study, to a single treatment with IR. Inhibition of immunosurveillance is an assumed mechanism for enhanced skin tumor formation in organ transplant recipients. Evidence favoring this view includes the increased incidence of NMSCs in patients who have other immunosuppressive disorders [129;138;139]. But ARDs also affect processes beyond immunosuppression. For example, cyclosporine A alone has been reported to stimulate angiogenesis, to inhibit multidrug resistance pump activity, to inhibit DNA repair, and to stimulate tumor cell activity directly [129;138;140-145].

3.2. Preventive Anti-Tumor Vaccination with Tumor-Associated Antigens May Help Control BCC Formation in Predisposed Individuals

The identification of mutations causing BCNS allows the early identification of affected individuals. In a study of 90 Caucasian BCNS patients, 80% had at least one BCC, and the number of BCCs ranged from 1 to >1000 [146]. The continued accumulation of BCCs makes assessment of preventive agents highly feasible and clinically important. Vaccination with a tumor antigen could be an ideal method to reduce, even prevent tumor formation in these individuals. The recently published reduction of the incidence of HPV-16 infection and of HPV-16-related cervical intraepithelial neoplasia in a study of 2,392 young women receiving HPV-16-virus-like-particle vaccine demonstrates the great potential of anti-tumor vaccines, although the target in this study was an infectious agent which causes tumors rather than a TAA [147]. Direct vaccination with TAAs, as performed in our study, extends this traditional concept of vaccination to endogenously forming tumors. Ptch1+/-mice present a valuable model for the investigation of therapies against BCCs because the mice develop endogenous BCCs, closely mimicking the course of diseases in high-risk individuals such as BCNS patients. We report the use of Hip1, as a TAA for immunoprevention of BCCs in ptch1+/- mice treated with IR. The injections of Hip1 polypeptides were well tolerated in the mice and induced B and T cell responses. The number of BCCs was significantly reduced. These results suggest that immunization with proteins specifically up-regulated by hedgehog signalling may hold promise as a preventive option for patients such as those with BCNS, who are destined to develop large numbers of BCCs. As reviewed by Finn et al. the rationale for preventive immunization is strong [21]. Most identified human TAAs, however, are self-antigens with some expression in normal adult tissue. Carcinoembryonic antigen, for exam-

ple, is a TAA which is overexpressed in various carcinomas including gastrointestinal carcinomas but is also expressed in normal colonic mucosa [148].

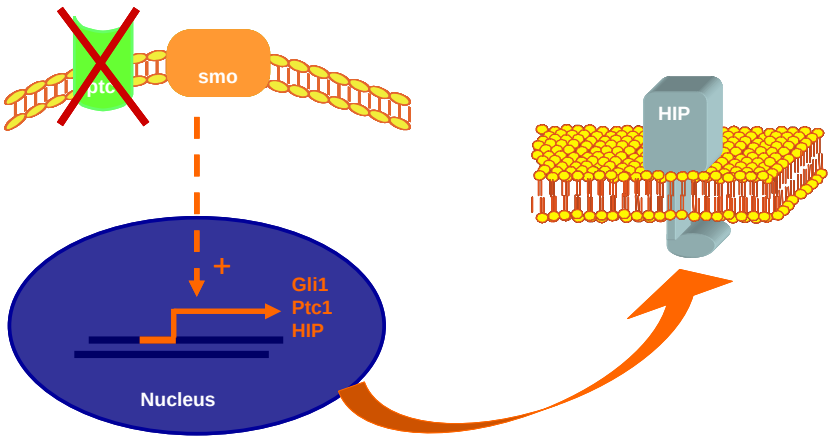

Fig. 3: Mutational Activation of Hedgehog Signalling is the Pivotal Step in BCC Development. Hedgehog signalling is physiologically active during early development and plays an important role in morphogenesis. Mutational inactivation of ptch has been found in 85% of BCCs [27]. This activation most commonly is effected by mutations in PTCH1, which encodes a protein which normally inhibits hedgehog signalling. Among the hedgehog target genes uniformly overexpressed in BCCs is the recently identified putative transmembrane protein hedgehog-interacting protein (Hip1;hHip) [149]. This consistent overexpression potentially provides a target for immunization. (Ptch=Patched, Gli1=Glioma-associated oncogene homolog1, ptc1=patched1 gene, Hip= Hedgehog-interacting protein, smo=smoothened)

Similarly, tyrosinase-related protein 2 [150] and Her-2/neu antigen are expressed not only on cancerous cells but also on normal tissue, and in some cases successful rejection of established tumors could be achieved only in the presence of severe autoimmune responses [151;152]. However, antitumor responses against other normal differentiation antigens can be generated with little evidence of autoimmunity [153-155]. We have defined Hip1 as TAA and present evidence, that immunization against BCCs with recombinant fragments of Hip1 induced immune responses despite the "self-nature" of this protein. In our study of various tissues of ptch1+/-mice, quantitative real-time RT-PCR identified basal expression of Hip1 mRNA in all tissues examined except heart tissue. These results are in accordance with reports by Bak et al. who found expression of human Hip1 in fetal and adult human tissue except ovary [156]. Because hedgehog signalling is so important in embryonic development, we assumed that the antigenicity of Hip1 would require the co-administration of adjuvants. In rodents and humans, CPG-ODNs stimulate the expression of Th1-like cytokines and of costimulatory molecules and lead to a general increase in antigen-

presenting function [157]. If used as adjuvant, CPG-ODNs give potent antibody and cytotoxic T cell responses. CPG-ODNs are not immunogenic and unlike Freund's adjuvant do not cause granulomatous reactions. Therefore they can be injected repetitively without severe side effects. [158;159]. In our study, the repetitive administration of the peptide PT27 in combination with CpG-ODNs was sufficient to overcome the self-tolerance expected due to high level expression of Hip1 during development and low-level expression in adult tissue. We found strong PT27-specific antibody responses in PT27-injected mice, and this peptide also induces anti-Hip1 antibodies in rabbits (unpublished data). The exact localization of Hip1 within the cells remains unclear, and its immunogenic properties so far have not been investigated in detail. Yet, as a putative transmembrane protein, Hip1 might well be accessible to antibody binding. In other cancer models antitumor antibodies have been shown to mediate anti-tumor effects effectively. Antitumor activities of the recently approved therapeutic antibodies Trastuzumab, specific for the protooncogene p185HER-2/neu in breast cancer, and Rituximab, a monoclonal. IgG1 specific for the B cell marker CD20, prove that antitumor antibodies can be a powerful tool in cancer therapy. Spleen cells from PT27-injected ptch1+/- mice produced significantly more IFN-gamma in response to *in vitro* stimulation with the recombinant Hip1 peptide PT27 than did spleen cells from control mice receiving saline or CpG injections. Moreover, 19 out of 23 peptide-injected mice developed delayed type hypersensitivity. Detailed immunology-focused studies will help to elucidate further the immunological properties of Hip1 and to optimise future immunization strategies. Central to our study was the more general observation that Hip1 injections were well tolerated and induced detectable B cell and T cell responses and that these findings may be of clinical relevance because Hip1-injected mice developed significantly fewer microscopic tumors than did control mice.

3.3. Particle-Based Vaccination in C57Bl6 Mice: Building a Rationale for Nanoparticle-Based Transcutaneous Vaccination

We investigated the percutaneous penetration, the cellular uptake and the trafficking of 40 nm and 200 nm solid polystyrene particles as well as MVA-eGFP particles in C57Bl6 mice using *in vivo* fibre-based confocal microscopy, fluorescence microscopy on cryosections and cell separation techniques. We demonstrated *in vivo*, that topically applied particles and particulate vaccines penetrated by human hair follicles into the perifollicular tissue, and we identified nanoparticle-positive APCs n the skin. Our studies demonstrate that the hair follicle penetration pathway is a suitable route for vaccine penetration for nanoparticles, protein, DNA, and virus-like parti-

cles. Interestingly, Fan et al. found that topical vaccination requires intact hair follicles, pointing to the operation of efficient mechanisms for induction of immune responses to protein encountered within the follicle [160]. Having been previously identified as a portal for both, protein and DNA entry to the skin [81], hair follicles are candidates for targeted delivery of therapeutic agents [161]. We were able to demonstrate clearly, that within hours, nanoparticles migrated to the proximal lymph nodes, most likely in association with migrating APCs. Further experiments need to be performed to study the process of nanoparticle internalization by LCs and DCs and the basis of APC migration and nanoparticle transport. Our data however, suggest that t.c. vaccination with nanoparticles might be a promising approach for future vaccination strategies. It takes advantage of two important particle characteristics: (i) improved immunogenicity of particle-bound antigens and (ii) preferred penetration by hair follicles.

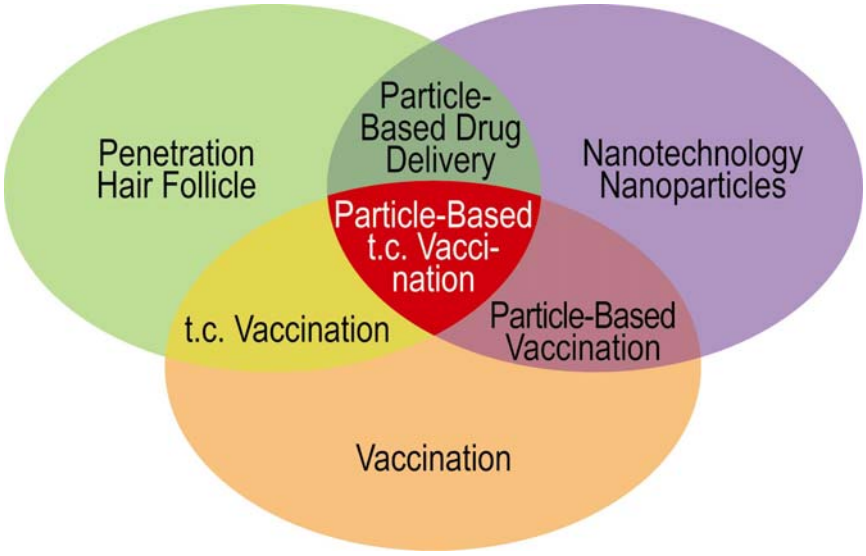

Fig. 8: Rationale for Transcutaneous Vaccination with Nanoparticle-Bound Vaccine.
Our data reinforce the concept of t.c. vaccination with nanoparticle-based vaccines. It takes advantage of two important particle characteristics: (i) improved immunogenicity of particle-bound antigens and (ii) preferred skin penetration by hair follicles, which provide an important interface for nanoparticle interactions with the surrounding tissue and APCs and subsequent shuttling to lymphoid tissues.

To further validate our concept of t.c. targeting of skin APCs with particle-based vaccines, the immunogenicity of model antigens including ovalbumin expressing plasmid and MVA-eGFP particles, was assessed by immune assays and revealed the efficacy of our t.c. vaccination protocol to generate protective immune responses. We concluded, that nanoparticle-based vaccine

preparations may allow an improved targeting of APCs in the skin compared to nonparticulate systems. This hypothesis is supported by former studies, which suggested that adsorption of vaccine compounds to nanoparticles trigger the activation of APCs and improve their antigen-presenting capacity resulting in better immune responses [103-105]. The possibility to target skin APCs, however, had not been explored at that point, and was an entirely novel approach. Because nanoparticle translocation across human skin was generally assumed to be highly unlikely, proof-of-concept studies with precise analysis of particle penetration properties in human skin was key to this project and provided the base for the following experiments on human skin explants.

3.4. Particle-Based Drug Delivery Through Human Skin

3.4.1. Rationale for the Concept of "Hair Follicle Targeting"

In our study on the penetration of polystyrene particles in human explants, we were able to demonstrate that, after pre-treatment of the skin samples with CSSS, all polystyrene particles, i.e. 40 nm, 750 nm or 1,500 nm particles, aggregated in vellus hair follicle openings and penetrated along the hair follicle duct. Only 40 nm particles penetrated deeply into the fine hair follicles, while 750 nm and 1,500 nm remained in the superficial parts of the infundibulum. As outlined in the introduction, various authors already reported deposition of different types of particles in the hair follicles. While Rolland et al. were the first group to demonstrate the delivery of adapalene-loaded 5 µm microspheres into facial vellus hair follicles [47], other authors studied 5 µm rhodamine-6 G-loaded microspheres or methylene blue loaded 5 µm nylon microspheres, respectively [76;77]. Similarly crystallized drugs, solid lipid particles as well as flexible particle types have been used in the past. In our department, detailed studies on the penetration depth of different particle sizes ranging from 6 µm down to 750 nm were performed and revealed that the penetration depth of the particles in human scalp terminal hair follicles strongly depended on the particle size [45]. In our studies, we were able to show, that the particle size is also a major determinant of the penetration depth in vellus hair follicles. According to the significant size differences between terminal and vellus hair follicles, 750 nm and 1,500 nm particles penetrated deeply into terminal, but not into vellus hair follicles, i.e. smaller particle sizes are required to target deeper parts of vellus hair follicles compared to terminal hair follicles. Hence, hair follicle targeting by particle-based drug delivery is possible not only in the large terminal hair follicles of the scalp but also on other body regions covered with fine vellus hair follicles. Our results extend the concept of hair follicle targeting to vellus hair follicles and to smaller particle sizes, which offers opportunities for new developments, not only in hair therapy and in the treatment of hair follicles-associated dis-

eases but also for gene therapy and immunotherapy. This is especially relevant for the design of patch systems, because the application of drug delivery patches on vellus-hair bearing skin, e.g. the upper arm or the back, is much more comfortable than application on scalp skin which requires shaving or other skin pre-treatment techniques. The possibility to target specific hair follicle types also opens new perspectives for hair therapies: While targeting of the bulge region in terminal hair follicles, for example, may allow to inactivate stem cells in patients suffering from hypertrichosis, activation of stem cells in miniaturized hair follicles affected by androgenetic alopecia may help reconvert these hair follicles into strong, pigmented terminal hair follicles.

3.4.2. Possible Applications in Dermatotherapy

The presented work provides the base for the design of advanced drug delivery systems, which, in future applications, may allow specific targeting of bioactive molecules to hair follicle compartments located at different depths along the hair follicle and to hair follicle-associated cell populations. Targeting of sebaceous glands to treat acne vulgaris was the first concept, which was evaluated in clinical applications. Beyond, the high density of APCs around the upper parts of the hair follicle as well as the identification of epithelial stem cells and multiple precursor cell populations in and around the bulge region opens up interesting perspectives for immunotherapy and gene therapeutic approaches. Based on these considerations, we defined the ***hair follicle infundibulum***, the ***sebaceous glands*** and the ***bulge region*** as major targets of interest in both, terminal and vellus hair follicles [53]. Pecularities of the ***hair follicle infundibulum*** anatomy have already been described in the introduction. Although the acroinfundibulum is covered by an intact, rather impermeable stratum corneum, this barrier is interrupted in the lower infundibulum, where the differentiation pattern switches from epidermal differentiation to a tricholemmal differentiation pattern. Only few to little corneocytes remain, which renders this part of the infundibulum highly permeable. This means that epithelial cells and associated cell populations such as APCs, mast cells and others are readily accessible for topically applied compounds. The ***sebaceous gland*** is interconnected with the human hair follicles via the sebaceous duct, which opens into the follicular duct in the lower infundibulum. There is evidence that topically applied compounds entrapped in liposomes accumulate not only in the hair follicle itself but also in the sebaceous gland. Bernard et al. studied the penetration of the anti-androgen RU 58841 in normal hairless rat skin and scarred hairless skin without sebaceous glands. They found that the solution was localized in the stratum corneum, whereas liposome-entrapped RU 58841 was mainly located in the sebaceous glands [162], and concluded that adsorption of acne therapeutics to particles

may help increase their efficiency. The ***bulge region*** in the outer root sheath, at the insertion level of the M. arrector pili, is well recognized as the reservoir of epithelial stem cells, which are capable of repopulating the hair follicle as well as the interfollicular epidermis [163]. Therapeutic manipulation of stem cells may allow for the treatment of hair loss and hair overgrowth, as well as skin diseases. The recent localization of melanocyte stem cells in this lower part of the permanent hair follicle further offers the opportunity to treat pigmentation disorders [164].

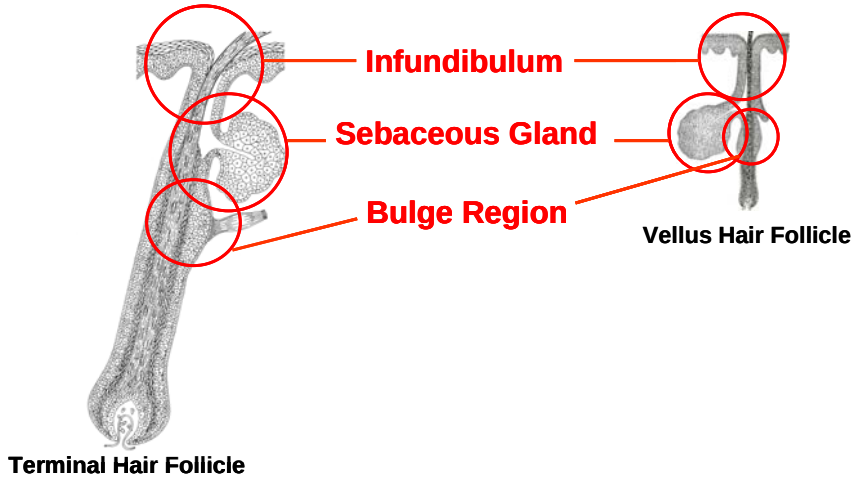

Fig. 4: Follicular Targeting Aims at Selective Delivery of Active Compounds into Human Hair Follicles (modified from Vogt *et al* 2005) [53].
The hair follicle infundibulum, the sebaceous gland and the bulge region are important target structures both, in terminal and in vellus hair follicles. Possible applications include the therapy of hair diseases and hair follicle-associated disorders as well as drug delivery [53].

The bulge region is of particular interest for gene therapeutic approaches. Hair follicles are easily accessible, and Li et al. demonstrated *in vitro* that hair follicles can be targeted by liposomes loaded with DNA [81]. Building on these findings Domashenko et al. successfully introduced plasmid DNA encoding for lacZ reporter gene into human hair follicles in a xenograft model [165]. The introduction of genes into follicular stem cells offers wide opportunities for novel treatments or hair and skin diseases, especially genodermatoses.

3.4.3. Impact of Hair Follicle Variations in Different Body Regions

Size and dimensions of the hair follicles differ enormously among the different hair follicle types and body regions. While lanugo hair is produced *in utero* and generally shed before or shortly

after birth, vellus hair follicles of different size and density cover most of the skin surface area in adults. Vellus hair fibres are usually thin with a diameter <30 µm and a length <2 mm. Terminal hair is characterized by hair fibres >60 µm diameter and >2 mm length. It is mainly found on the scalp, as well as in hormone-dependent body regions such as the beard, the axilla and the pubic region [54;166]. Size and position of key target structures differ significantly among the different hair follicle types. The fact that 750 nm particles penetrated deeply into the large terminal hair follicles of the scalp, but not into fine vellus hair follicles from the retroauricular region is a results of those variations. With our morphometric measurements in human terminal and vellus hair follicles, we were able to generate precise data on the size and the position of key target structures, such as the hair follicle infundibulum, the entry level of the sebaceous duct and the bulge region. We were able to clearly demonstrate minor intra- and interindividual variations, suggesting that specific hair follicle types within one skin area are mostly homogeneous [113]. The fact that we determined those parameters is especially relevant, because the hair follicles were measured in routine specimen obtained from a large variety of patients, and, still, revealed that there was only minor intra- and interfollicular hair follicle variability. The results suggest that our protocols for particle-based hair follicle targeting, which were developed on a limited number of volunteers, can be extended to larger numbers of patients. Yet, the measurements were performed on normal, otherwise unaffected hair follicles, and further studies will be required to validate this approach also for hair disorders and hair follicle-associated diseases, e.g. androgenetic alopecia or acne vulgaris, where the course and severity of the diseases may lead to an increased inhomogeneity of affected hair follicles. At the time, however, this is the first study, which addresses this issue in such detail. Only the position of the bulge region in scalp terminal hair follicles had been investigated before by Viragh and Meuli, who were interested in the changes which occur during childhood and adolescence [167]. In their study on young patients, the bulge region in human terminal hair follicles of the scalp was located at a depth of 500–800 µm, whereas the entry level of the sebaceous duct was at approx. 100–500 µm. In contrast, only few information was available on vellus hair follicles. Although structurally similar to terminal hair follicles there is evidence for a characteristic histomorphology and histochemistry of vellus hair follicles. They are smaller than terminal hair follicles and produce fine, silky hair with an average diameter of 30 µm, which is generally unmedullated and unpigmented. Blume et al. first determined the density of vellus hair follicles on different body sites by phototrichogramm [168]. Otberg et al. measured the infundibular volume of vellus hair follicles from different body sites by cyanoacrylate stripping and reported that vellus hair follicles on the forehead are among the smallest hair follicles of the human body, whereas the largest vellus hair follicles are found on

the calf. Due to the high density of approximatel 290 to more than 400 vellus hair follicles per cm^2 on the forehead as compared approximately 14 vellus hair follicles per cm^2 on the calf. However, the forehead had the highest overall infundibular volume [112]. Because our focus was to investigate hair follicle penetration, we also measured the diameter of hair follicle openings and the thickness of the epithelium. Besides surface area and accessibility, the thickness of the epithelium is an important determinant of the penetration rate of topically applied compounds. The fact, that the epithelium of the vellus and the terminal hair follicle infundibulum was thinner than the interfollicular epidermis further supported the favourable properties of the hair follicle epithelium for penetration processes.

3.5. Investigation of Hair Follicle Penetration with Novel Technologies

3.5.1. *In Vivo* Confocal Laser Scanning Miroscopy

During the course of this project we generated results, which challenged the fields of particle penetration and immunology. To further substantiate our findings, we were forced to develop new experimental models and to employ novel technologies. Whether or not translocation of nanoparticles into the viable tissue occurs, for example, was intensively discussed at that time. First studies in this field were based on the microscopic evaluation of tissue sections only. In diffusion cell models, such as the Franz diffusion cell, authors quantified the diffusion of active compounds through the skin samples into a receptor medium [169]. Different stripping techniques were introduced including conventional tape stripping and CSSS [170]. The number of non-invasive techniques applicable *in vivo*, however, was, and still is, limited. Differential tape stripping, for example, removes the entire stratum corneum followed by a CSSS procedure and is clearly limited to *in vitro* models. CSSS alone is well tolerated but, when used as diagnostic tool, yields static data such as the volume of the infundibulum [112]. In contrast, advanced optical systems, such as *in vivo* confocal laser scanning microscopy and *in vivo* Raman spectroscopy, represent promising new approaches, which, in future, may allow to determine the kinetics of fluorescent dye penetration in the epidermis and into hair follicle openings and the effect of different stimuli or stress factors on the penetration rates [171;172]. In our studies in C57Bl6 mice, *in vivo* investigation of the distribution of topically applied nanoparticles on the skin surface became possible by the use of non-invasive "Fibered Confocal Fluorescence Microscopy" technology. The technique produces virtual cross-sections of the epidermis *in vivo* down to a depth of 80 ± 5 µm from the skin surface. In our studies, the two optical probes used for acquisition of the images had respective diameters of 1.5 mm (ref. S-1500-5.0) or 1.8 mm (ref. HD-1800-2.5). The S-

1500 probe provides images at 80 µm form the skin surface with a slice thickness of 15 µm and a lateral resolution of 5 µm. The HD-1800 probe provides images at 80 µm from the skin surface with a slice thickness of 20 µm and a lateral resolution of 2.5 µm. With this techniques we were able to monitor the penetration of fluorescent particles into hair follicles and the diffusion into the perifollicular tissue *"real-time" in vivo*. Moreover, we detected aggregations of fluorescent particles as early as four hours after application in draining lymph nodes, but not in non-draining lymph nodes. We were even able to track MVA-eGFP *in vivo* and to detect the presence of fluorescent infected cells or infected cell debris in the draining lymph nodes. These experiments helped to extent our findings on the penetration and cellular uptake of fluorescent model particles to "functional" particles, in this case MVA, with a diameter of approx. 290 nm. According to our own measurements, the thickness of the interfollicular epidermis in humans varied between 64 µm and 99 µm in vellus hair-bearing skin of the retroauricular regions and between 72 µm and 136 µm in scalp skin [113]. The vellus hair infundibulum extended down to 225 µm, the terminal hair infundibulum to 580 µm. Therefore, the confocal laser scanning system with a depth of 80 µm is not suitable for hair follicle studies in humans. Lademann et al, however, recently reported the *in vivo* use of a fibre-based confocal microscope in humans with a field vision of 200 x 200 µm, which reached down to a depth of up to 250 µm. This device covers the length of the vellus hair infundibulum and although less precise than measurements on histological sections, *in vivo* laser scanning microscopy may become an important tool to monitor follicular penetration non-invasively in human volunteers.

3.5.2. Scanning Transmission X-Ray Microscopy

To further elucidate if at all, and via which penetration route, particles of different sizes and properties penetrate human skin, we applied for the first time STXM, which yielded high resolution images of the distribution of single small particles on the skin surface. STXM offers the opportunity to study not only nanoparticle penetration, but also interactions of the particles with biological tissue. In STXM, high-brilliance synchrotron radiation is tightly focused (40 nm), and the sample is raster-scanned while recording the intensity of transmitted x-rays in order to produce a two-dimensional image. X-ray microscopy also provides, besides spatial resolution, chemical contrast, which is a result of strong variations of the absorption cross-section in core level absorption. This is due to the distinct near edge X-ray absorption fine structure (NEXAFS) [119]. In this way, the combination of spectroscopic and ultrastructural data provides at about 40 nm spatial resolution information of the chemical composition of the sample. Therefore, x-ray

microscopy has significant advantages compared to fluorescence microscopy techniques besides chemical contrast and sub-40 nm spatial resolution, since even wet samples without staining or using markers can be studied [120]. In the future, STXM may provide detailed ultrastructural and functional studies with emphasis on both, cellular uptake and tissue alteration. The investigation of conformational or chemical changes of nanoparticles in response to skin and tissue exposure will also be of importance to further understand the interactions of nanoscale materials and human tissue on a molecular level. In such studies, STXM may be a key instrument to study the penetration of single nanoparticles with a high ultrastructural resolution. Moreover, the chemical selectivity of STXM is advantageous for the identification of changes on the nanoparticle surface, which are caused by uptake and translocation in skin, allowing. Further standardization of the procedure and larger series of investigations may allow us to study nanoparticles of different shapes, chemical composition, and surface modification and their interaction with human skin. In contrast to the existing techniques and models, this approach could help to study both, consequences of particle penetration in the biological tissue and alterations of the particle structure as a result of such particle-tissue interactions.

3.6. Nanoparticles Penetrate Barrier-Disrupted Human Skin

3.6.1. Impact on Drug Delivery Strategies

The key finding in our studies on particle penetration in human skin explants and in C57Bl6 mice was, that solid 40 nm polystyrene particles, after pre-treatment of the skin with CSSS (human skin explants) or tape stripping (murine skin), did not only penetrate deeply into hair follicle openings, but also through the hair follicle epithelium into the perifollicular tissue. We demonstrated this clearly in both experimental models using advanced microscopy techniques such as laser scanning microscopy of human and murine tissue sections as well as *in vivo* confocal laser scanning microscopy in mice. We further provided, for the first time, functional evidence for nanoparticle uptake by living cells after topical application of particle preparations on the skin surface. Fluorescent 40 nm particles were identified in human LCs and in C57Bl6 mice. These functional studies further substantiate the fact that such translocation occurred. Although deposition [43] and even retention [115] of solid particles in the follicular duct has been well documented, there are only a few reports on the penetration of solid particles into the viable tissue. The vast majority of previous studies in this field were performed on particles sized > 750 nm, and translocation could be excluded for those sizes. Only recently, research shifted towards smaller particle sizes. At present, there is little evidence that nanoparticles at a size exceeding 100 nm penetrate intact skin [107]. If this is the case for smaller particles is still controversially discussed and,

within the past few years, has led to the initiation of multiple studies in this field. Cross et al., for example, reported rather minimal penetration of 26-30 nm micronized zinc oxide into the upper layers of the stratum corneum of human skin [173], while Baroli et al. suggest that metallic nanoparticles as small as 5.9 nm penetrated intact stratum corneum and hair follicles in a diffusion cell model of excised human skin [174]. These groups, however, used microscopic techniques and diffusion cell models and some focused on penetration across the intact stratum corneum of interfollicular epidermis not taking into account shunt penetration along skin appendages. Internalization by cells was not studied, which underlines the novelty of our approach. The relevance of nanoparticle translocation across the skin barrier becomes even more apparent by investigations on particle penetration in barrier-disrupted or otherwise damaged skin as well as the possibility of shunt penetration via skin appendages.

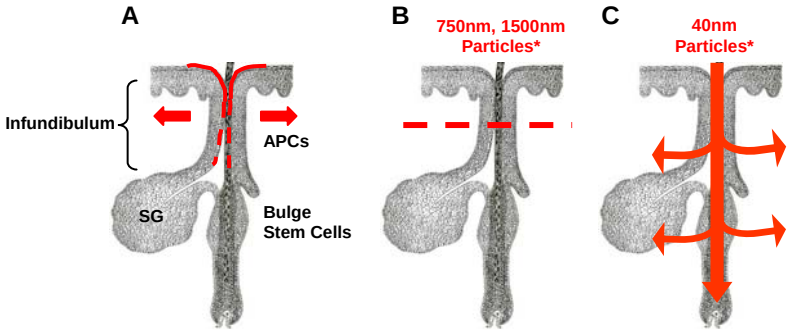

Fig. 5: Its Unique Anatomy and Strategic Position Within the Skin Makes the Hair Follicle Infundibulum an Ideal Long-Term Reservoir and Site of Penetration (modified from Knorr *et al.* 2008) [44].

Cell populations in and around the hair follicle epithelium such as APCs are easily accessible via the porous barrier in the lower parts of the infundibulum (A). The sebaceous gland (SG) as well as the bulge region as site of epithelial stem cells and multiple other precursor cell populations are further important target structures. The follicular route is the preferred penetration pathway for micro- and nanoparticles. Penetration depths and the capacity to penetrate the epithelium depend on the particle size, suggesting that larger particles may be used to deposit high concentrations of compounds in the follicular duct from where they can be released (B), while small particles in the size range of 40 nm, especially in barrier-disrupted skin, may be used to directly deliver particles loaded with active compounds to specific cell populations (C) [44].

Because our interest was to explore the possibility to use nanoparticles as antigen carriers in vaccination, we intended to achieve such translocation and decided to use CSSS as skin pretreatment procedure. CSSS is non-invasive, easy to perform and facilitates follicular penetration of topically applied particles by removing cellular debris and sebum from hair follicles [45]. CSSS also

removes approx. 30% of the stratum corneum and induces mild barrier disruption in humans (Vogt *et* al., unpublished data). Thus, from our data we can only conclude that translocation of 40 nm polystyrene particles occurred in barrier-disrupted skin. Such increase of particle penetration rates after barrier disruption was also reported by Borelli et al., who reported significantly increased penetration of 3.5 nm fullerene amino acid-derivatized peptide nanoparticles after flexing of porcine skin [175]. Our first assumption that particles may be used to deposit high concentrations of active compounds in the hair follicles and to reach those high concentrations in different compartments of the hair follicles was based on the first reports on particle penetration and the fact that the penetration depth of particles in the hair follicles depends on the particle size. The finding that larger particles in the size range of several hundred nanometres aggregated in the hair follicle openings but did not penetrate into hair follicle epithelium supports this concept. The chances that complete particles in this size range enter the circulation are minimal, which mitigates concerns regarding harmful effects caused by particle translocation. However, the physicochemical properties of each particle type and possible degradation products have to be considered. In particulate drug delivery systems, active compounds may be encapsulated in microspheres or loaded onto the particles. They may as well be incorporated into lipid or wax particles or matrices, that release the drugs in response to internal or external stimuli (e.g. heat). According to Lademann et al, particles entrapped in follicles are protected from the regular shedding of the epidermis suggesting, that particle-based drug delivery systems may even allow a controlled release of compounds over prolonged periods of time, e.g. by slow destabilization or degradation of the particles. For the treatment of skin disorders, such controlled delivery could help reduce side effects caused by repetitive applications of high dosages, which with conventional therapies are required to maintain effective drug concentrations within the skin. For systemic drug delivery, such delivery systems could help reduce application frequencies and/or the surface area required for the delivery of relevant dosages. The finding that solid nanoparticles in the size range of 40 nm reached skin APCs after skin pretreatment with CSSS, however, opens up a completely new aspect of particle-based drug delivery. Particles may not only be used as carriers, which release active compounds once deposited in the hair follicles. We found that they also have the capacity to penetrate with the drug. Because vaccination efficacy has been shown to increase with particle-bound antigen, this aspect of drug delivery is especially interesting for the design of t.c. vaccination systems. While flexible particles such as liposomes destabilize during the penetration process, solid particles of different physicochemical composition could be loaded with antigen and functionalized with surface modification that affect charge and/or lipophilicity or that selectively target cell populations with receptor ligands or antibodies against cell

surface antigens. In this context, the term "antigen" could mean proteins, glycoproteins, peptides or peptide fractions, polysaccharides, lipopolysaccharides, toxoids, conjugated carrier proteins,

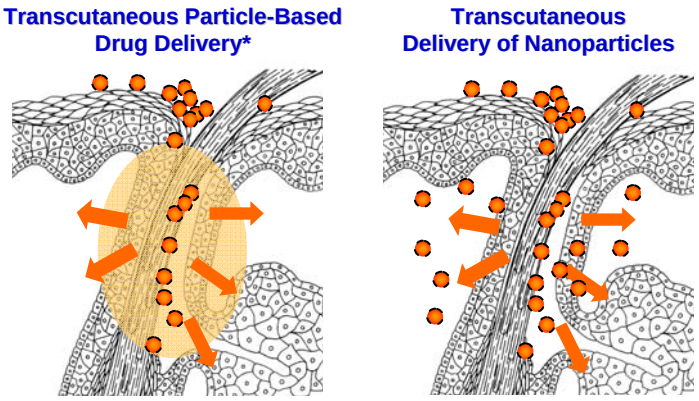

Fig. 6: **Particle-Based Drug Delivery versus Delivery of Nanoparticles by Hair Follicle Targeting**.

The penetration of particles along the hair follicle is size-dependent. Based on the observations by our group and others that larger particles did not penetrate the hair follicle epithelium, we concluded that such particles could be deposited in the hair follicle infundibulum and retained for prolonged time periods. There, release of incorporated active compounds and maximum concentrations in and around the hair follicle could be achieved e.g. by controlled destabilization of the particles. In contrast we found that 40nm polystyrene particles, after application on barrier-disrupted skin translocated into the perifollicular tissue suggesting, that solid nanoparticles in this size range could be used as carrier systems to directly target cell populations in and around hair follicles (* modified from Vogt *et al.*, 2005) [53].

cell extracts, viral extracts, life attenutated, killed,m inactivated or recombinant viral, bacterial or parasitic particles, DNA, polynucleotides, recombinant nuclei acids or others. Moreover, one could think of pathogens and molecules involved in orphan diseases and cancer cell extracts, which links this part of the project to our previous studies on anti-skin cancer vaccination in predisposed individuals.

3.6.2. Impact on Transcutaneous Vaccination Strategies

Taken together, we were able to combine two complementary experimental models, the human skin explants to study the translocation process directly in human skin, albeit *ex vivo*, and the animal model for *in vivo* imaging of particle trafficking and immunogenicity. We identified the hair follicle as a major penetration pathway for nanoparticles and particulate model vaccines such

as MVA-eGFP. All studied particles (40 nm, 200 nm polystyrene particles and MVA-eGFP) penetrated along the follicular duct, translocated into the perifollicular tissue and were taken up by epidermal and dermal APCs, which migrated to the proximal lymph nodes. We traced *"naked"* fluorescent model particles from the hair follicles to the draining lymph nodes. We were even able to detect topically applied MVA-eGFP in murine skin and in draining lymph nodes, and we demonstrated the capacity of this t.c. immunization method to generate immune responses after topical application of DNA, protein or virus particles. The application of small immunogenic compounds such as ovalbumin coding DNA or MVA expressing the eGFP showed that particle-based targeting on hair follicles induced both humoral and cellular immune responses. T.c. applied MVA also induced protection against challenge with Vaccinia Virus. Thus, we were able to build up a strong and well documented rationale for the use of nanoparticles in t.c. vaccination. The concept of t.c. vaccination with particles is based on two important particle characteristics: (i) improved immunogenicity of particle-bound antigens and (ii) preferred penetration via hair follicles, which provide an important interface for nanoparticle interactions with the surrounding tissue and APCs and subsequent shuttling to lymphoid tissues. In addition, these new vaccination strategies take advantage of the properties of particulate compounds, e.g. microspheres and nanoparticles or virus-like particles, respectively, to better target APCs, which subsequently reach the secondary lymphoid organs as sites of the immune response. Our results further show, that the size of the particles needs careful consideration. DCs are capable of internalizing particles up to several micrometers. Yet, for t.c. applications particle size limitations are clearly imposed by the skin barrier and the hair follicles. Our own studies suggest that the maximum size of particles capable of penetrating skin is probably around 200nm [59;176]; Vogt and Combadiere, unpublished data). Moreover, immunological studies by other groups revealed that the particle size affects the quality of the immune responses and may even influence the Th1/Th2 cytokine balance. Mottram *et al*. investigated particle-induced immunity in mice and found that particles in the size range of 40-50 nm were taken up preferentially by DCs and induced particularly strong immunity. Interestingly, the induction of IFN-gamma secreting CD8 T cells in response to the administration of 40-50 nm particles was significantly higher compared to larger particles in the size range of 93-123 nm coated with ovalbumine, which preferentially induced CD4 T-cell activation and IL-4 production [59;177]. Consequently, the quality of humoral and cellular responses induced will depend on the appropriate targeting of APCs, i.e. choice of appropriate vaccine carriers, vaccine doses, route of administration and also the use of adjuvant. Further studies using functionalized nanoparticles will help to further validate this route of immunization and its use in clinical applications.

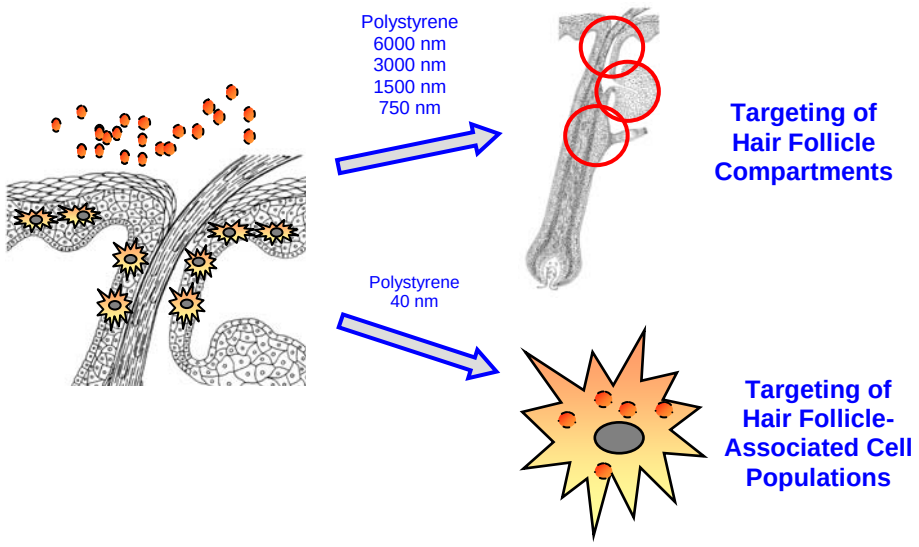

Fig. 7: Consequences of Nanoparticle Penetration on t.c. Vaccination Strategies.
Fluorescent polystyrene particles ≥750 nm remained in the infundibulum with no further penetration into the viable tissue. In contrast, we found that 40 nm polystyrene particles where internalized by LCs after topical application on barrier-disrupted skin, suggesting, that nanoparticles in this size range could be used as carrier systems to target particle-bound vaccine to skin APCs. Highly effective targeting of maximum numbers of skin APCs could be achieved by loading of nanoparticles with multiple antigens, adjuvants and surface modification for improved APC targeting, e.g. cell surface receptors ligands.

Today, research efforts in the nanotechnology of biomaterials aim at designing nanosystems as vaccine carriers, which provide optimal targeting of DCs, for example by allowing transport through mucosal or cutaneous epithelial barriers. Results show that not only the colloidal properties but also the polymer composition and macromolecular architecture are critical to induce an effective immune response. Several kinds of particles are actually investigated as drug delivery systems including semi-solid particles such as liposomes, as well as a wide range of solid particles with different physicochemical properties, e.g. solid lipid nanoparticles, polyester-particles based on poly-lactic acid (PLA), poly-lactic-co-glycolic acid or poly-ε-caprolactone and others. Because the reports on particle translocation in barrier-disrupted and otherwise pre-damaged skin raised significant safety concerns, particles with biodegradable properties, such as PLA-particles, are especially interesting candidates. PLA is a linear, lipophilic, biodegradable polymer. Lactic acid, the start monomer, is easily derived from renewable resources like corn starch or sugarcanes. The fact that lactic acid is the only degradation product following the polymer hydrolysis, makes PLA polymers of interest for several applications. PLA micro- and nanoparticles

have widely been studied as delivery systems for systemic and topical applications [59;178]. A number of different drugs have already been successfully encapsulated in PLA nanoparticles [59;179;180], including DNA, proteins and peptides. The particles are already being used to produce bioabsorbable implants for orthopaedic surgery [59;181], for the treatment of facial lipoatrophy in HIV patients [59;182] as well as for treatment of scars and for esthetic rejuvenation [59;183]. The PLA particles are biodegradable, which minimizes hazardous effects. Particle production is easy and feasible also in larger batches. Both characteristics facilitate translation into clinical studies.

3.6.3. Impact on Nanoparticle Toxicity

In our studies, we clearly demonstrated particle uptake by APCs and keratinocytes. The great potential of these findings for the design of particle-based therapeutic systems is obvious. With regard to unintended exposure to nanoparticles in the environment or cosmetic products, however, the results also underline the importance of further risk assessment, especially in the light of the increasing number of individuals suffering from skin disorders and diseases with significant barrier disruption, e.g. eczema. Particle-associated toxicity is relevant for environmental exposure as well as for exposure to engineered particles. According to a commonly used working definition, the term nanoparticles refers to nanoscale material with a diameter of < 100 nm. However, hazardous effects may also occur after exposure to larger particles and agglomerates, where destabilization and disintegration can cause release of smaller fragments and certain components. Accumulation and prolonged retention of nanoparticles, albeit useful for drug delivery purposes, further increase the risk of destabilization. Changes of surface coating, surface charge and others as a result of particle-tissue interactions. Engineered particles may also be contaminated with solvents, side products, organics, endotoxins etc. The insight that nanoparticle translocation occurs via human skin is rather new and was significantly promoted by our own results. In contrast, nanoparticle deposition and translocation in the respiratory tract and possible consequences for other organ systems has been studied for many years [184]. A large variety of acute and chronic effects including inflammatory reactions, exacerbation of asthma, genotoxicity and carcinogenesis have been attributed to particle inhalation. Besides the physicochemical composition, particle surface area, particle number, charge and surface coatings appear to be major determinants of particle-associated toxicity [185]. In pulmonary toxicity studies for example, ultrafine particles enhanced inflammatory responses when compared to larger-sized particles of identical chemical composition at equivalent mass concentrations [186 187]. Particle size may also be relevant for cardiovascular effects [188]. Cardiovascular effects in response to inhaled nanoparticles

have been described in animal models and in humans, but in most cases the exact component causing those effects was not identified. Kainthan et al. reported that cationic, but not anionic nanoparticles, including gold and polystyrene particles, may cause haemoylsis and blood clotting [189]. Multiple groups reported that nanoparticles are even capable of crossing the blood brain barrier. Transsynaptic transport through the olfactory epithelium after inhalation was suggested as well as uptake through the blood-brain barrier. Kreuter et al proposed uptake via receptor-mediated endocytosis as one possible delivery mechanism. In this case, nanoparticles could mimic lipoprotein particles [190;191]. Oxidative stress-related inflammatory reactions have been observed for various types of nanoparticles [192-195]. On the cellular level, dose-dependent cyotoxicity and oxidative stress-related reactions have already been observed for a number of nanoparticles in different cell culture systems [196;197]. Further studies are required to elucidate the effects of penetration and long-term deposition of nanoparticles in the skin on cell membrane integrity and biological functions. With regard to the large number of newly emerging applications of nanomaterials investigations which study both, particle-associated effects on the organism and particle alterations as a result of particle-tissue contact, are crucial. In fact those considerations provided one important rationale for the employment of STXM in this context.

3.7. Possible Impact of Particle Uptake by Keratinocytes on Transcutaneous Vaccination Strategies

No matter which carrier system will turn out to be most effective, targeting of APCs is key to success of any vaccination strategy. It has been shown, however, that the predominant transfected cell types after abrasion are keratinocytes [69]. Keratinocyte uptake clearly decreases the antigenic load available for the actual immunization and APC targeting. The expression of DNA in LCs and DCs after t.c. vaccination remains unknown. Although the evidence for LCs as the targeted APCs used in topical immunization is indirect, the observations that immunization can be conducted in entirely uninflammed skin and that skin hydration has been involved in this process are highly suggestive that skin penetration and LC targeting occurs. LCs are a specific subtype of DCs localized in the epidermis. Functional, *in vitro* studies have demonstrated that antigen-pulsed LCs that have been isolated from epidermis can induce T-cell proliferation comparable in magnitude to those induced by similarly pulsed macrophages [69;198]. In addition, in our studies, MVA application induced an enhanced immune response, which was protective. Other studies investigated the involvement of keratinocytes in immune responses in murine models. Sugitak K et al. recently published, that TLR9 ligand was able to enhance the hapten-presenting

capability of LCs in epidermal cell suspensions, but not purified LCs, suggesting that bystander keratinocytes play a role in the enhancement of LC function [69;199]. Gaffal et al. reported that particle bombardment of the epidermis with plasmid DNA using gene gun resulted in antigen expression in keratinocytes of the epidermis leading to antigen-presentation in the draining lymph nodes by migratory DCs [69;200]. All of these studies were conducted in animal models. Thus, further *in vivo* analysis on human skin explants and clinical trials are necessary to understand the role of keratinocytes in the induction of T cell immune responses and to optimize targeting of skin APCs.

3.8. The Role of Cyanoacrylate Skin Surface Stripping in Transcutaneous Vaccination

In order to facilitate follicular penetration, CSSS was applied in all our studies on human skin. CSSS facilitates follicular penetration by removing cellular debris and sebum from the hair follicle openings [201]. In fact, earlier studies by our group revealed that the use of CSSS increased the penetration of topically applied nanoparticles into human terminal hair follicles [45]. Because the density and dendricity of APCs is especially high around the upper part of the hair follicle and because the stratum corneum in the lower parts of the hair follicle openings is getting increasingly permeable, we hypothesized that targeting of hair-follicle associated DCs may be especially relevant for t.c. vaccination strategies. The use of CSSS allowed us to detect, to our knowledge for the first time, relevant translocation of topically applied nanoparticles across the skin barrier. In the mouse model, where CSSS could not be applied due to the thin and vulnerable epidermis, we also found that tape stripping significantly increased the efficiency to t.c. vaccination and concluded that inclusion of CSSS into t.c. vaccination protocols, which aim to target nanoparticles into the viable tissue, is probably crucial. We successfully translated our experimental protocol into a standard operating procedure ready to be applied in human volunteers. The following pilot study on human volunteers confirmed safety and efficacy of this newly developed protocol for t.c. vaccination on skin surface areas as small as 16 cm^2. CSSS is not only another method to abrade the skin surface. Other than conventional adhesive tape stripping or abrasion techniques (scotch pads or chemical abrasion etc), which only increase the permeability of the interfollicular epidermis, CSSS also removes debris and sebum from the hair follicle openings [45]. As a result, the number of hair follicles available for shunt penetration increases and topically applied compounds are deposited in the hair follicle openings for prolonged periods of time. There they are protected from the regular shedding process, which occurs in the interfollicular epidermis as a result of the ongoing renewal of the epidermis [43]. CSSS also removes approx.

30% of the stratum corneum and induces mild barrier disruption in humans (Vogt, unpublished data).

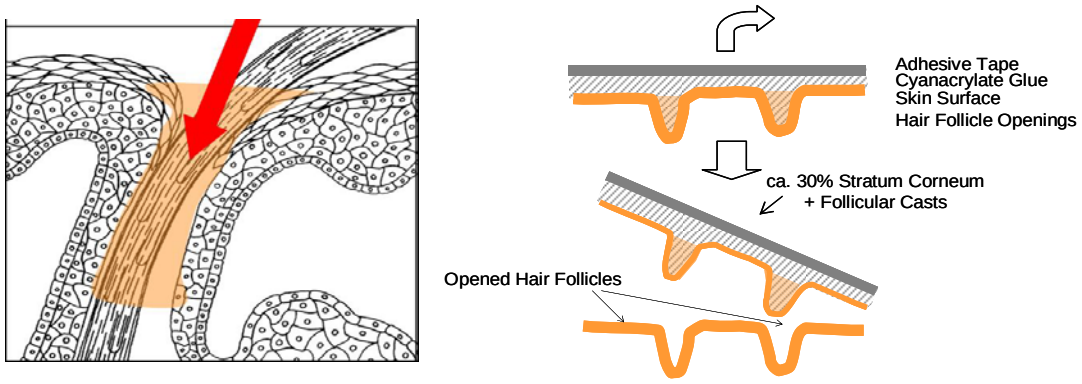

Fig. 9: Cyanoacrylate Skin Surface Stripping (CSSS) Facilitates Follicular Penetration.
CSSS removes keratinized material, lipids and other cell debris from the follicular openings and approx. 30% of the stratum corneum. Occasionally hair fibres, especially vellus hairs, are removed as well. The rest of the stratum corneum and the viable epidermis are left intact. This technique is routinely used in our laboratory and also by other groups to improve the percutaneous penetration of topically applied compounds and to determine the amount of substance, which enters the follicular reservoir.

Our results further reinforce earlier studies on tape-stripping immunization in mouse models, which demonstrated that the application of immunogenic compounds onto tape-stripped skin could elicit strong and protective immune responses against pathogens and cancer [202;203]. Other groups further demonstrated that the removal of the stratum corneum increased expression of MHC class II, CD86, CD40, CD54, and CD11c on LCs, but did not cause them to migrate [204;205]. Rapid migration from epidermis to draining lymph nodes was obtained, however, by exposure to antigen after removal of the stratum corneum, suggesting that maturation and migration of LCs are independently regulated events. Indeed, addition of an adjuvant such as cholera toxin increased the immunogenicity of antigen. Similarly, the use of DNA that can activate Toll-like receptors can participate to the maturation of APCs [206]. Up-regulation of IL-8 and E-selectin gene expression has been reported even after minimal trauma of otherwise normal human skin, such as gentle rubbing [207]. In summary, CSSS may represent a valuable tool in t.c. vaccination providing both enhanced penetration and immunostimulatory effects [208].

3.9. Transcutaneous Vaccination in Human Volunteers

In our pilot study on 11 healthy human volunteers, we evaluated the safety and the immunogenicity of the newly developed t.c. vaccination protocol based on only one CSSS procedure before the vaccine application. We demonstrated that t.c. immunization with 0.5 ml anti-influenza vaccine was safe and immunogenic in healthy individuals on small skin surfaces (16 cm^2 and 32 cm^2). We report the induction of T cell responses after t.c. vaccination of human volunteers, and we provide strong evidence that, in contrast to conventional i.m. injections, t.c. vaccination may help to induce not only CD4 but also CD8 T cell immune response. This is the first investigation on the application of a conventional vaccine by t.c. route that might be critical in the quality of cellular immune responses. The overall efficacy of different procedures for t.c. immunization has been demonstrated in numerous animal models (mice, rats, macaques and others). Only few concepts have been translated into clinical applications, i.e. pilot studies and Phase I clinical trials. As described in the introduction section, Glenn and his group [70;209] were the first group to immunize human volunteers via t.c. vaccination using patch systems. Our approach differs from previous studies with regard to the vaccination protocol, i.e. the pre-treatment of skin with CSSS, and the focus on the induction of cellular immune responses.

3.9.1. Cyanoacrylate Skin Surface Stripping is Safe and Effective

As described previously inclusion of CSSS is the integral part of our vaccination protocol, a method which we demonstrated to facilitate follicular penetration and which probably allows for improved targeting of skin APCs via hair follicles. Interestingly, Fan et al found that topical vaccination requires intact hair follicles, pointing to the operation of efficient mechanisms for induction of immune responses to proteins encountered with the follicle [160]. Having been previously identified as a portal for both protein and DNA entry to the skin [81], hair follicles are candidates for targeted delivery of therapeutic agents [161]. Targeting of perifollicular skin DCs might improve *in vivo* loading of vaccine antigens into these DCs and subsequent induction of T cell responses, e.g. via cross-presentation. In our study, vaccine application of one or two 4x4 cm skin surface areas, which were pre-treated with only one CSSS procedure, was sufficient to induce cellular immune responses, respectively. Considering the fact that a significant portion of the vaccine was probably lost by unspecific binding to the skin surface, resulting in a reduced effective dose of vaccine in t.c. vaccinated compared with i.m. vaccinated volunteers, these results are especially encouraging. In contrast to previous studies by other groups, we did not per-

form boost applications and no chemical or membrane permeabilizing molecules were used as proposed by others. Lisziewicz et al. recently showed that skin abrasion and vaccination using DNA induced an immune response to HIV in monkeys. However, this approach was partially invasive using large skin surface areas of four locations of 40 cm^2 in monkeys combined with several applications of large quantities of vaccine material [210]. While our studies were conducted and the manuscript was being prepared, Yagi et al. demonstrated that five percutaneous peptide immunizations induced cytotoxic T cell responses specific for melanoma and HIV peptides [211]. In their study, however, the authors performed multiple subsequent CSSS procedures leading to a complete removal of the stratum corneum, which is not only time consuming but also abrasive and painful, especially when applied on skin surface areas as large as 100 cm^2. This surface used for immunization is extremely large compared with 16 cm^2 or 32 cm^2 in our study. Also, after such abrasive treatment, LCs have upregulated HLA expression and costimulatory molecules. Moreover, Yagi et al. used a total of percutaneous peptide immunizations compared to only one in our study.

3.9.2. The Route of Vaccination May Impact the Quality of the Immune Response

The evaluation of effector/memory T cell frequency observed after t.c. vaccination was very similar to the one observed after i.m. vaccination and statistically not different. This result reinforces the fact that vaccination with Agrippal® vaccine was immunogenic by both, t.c. and i.m. route of vaccine administration. We found that t.c. vaccination with Agrippal® induced a three-fold increase of influenza-specific effector T lymphocytes at days 14 and 28 as measured by IFN-gamma-ELISPOT assay. This is in accordance with previous studies in mouse models showing the privileged role of tissue DCs to induce cellular immune responses. In addition, studies have shown that tape stripping induces activation of APCs in skin [208;212], which is an important step in the induction of efficient cellular immune responses. Our study shows that this pathway of immunization might be of importance in the induction of cellular immune responses in humans. We also show that only t.c. vaccination induced both CD4 and CD8 cellular responses, whereas i.m. vaccination induced stronger effector Th1 CD4 but not CD8 T cell responses. Indeed, the seasonal influenza vaccine preparation is selected for its high safety and efficacy in the induction of humoral immune responses by i.m. route. This could explain the high level of CD4 cells observed in the i.m. group. Influenza-specific CD4 cells function mainly to promote high-quality antibody responses but have not been shown to operate directly as chief effectors of virus control [213]. We assessed the nature of the cellular immune responses by using IFN-gamma-ELISPOT

assays, cell surface marker staining against CD4 and CD8, specification of IFN-gamma and IL-2 production, as well as pentamer staining. Frequencies and absolute numbers of IFN-gamma-producing CD4 cells were increased in both, t.c. and i.m. vaccinated individuals. In contrast, frequency and absolute numbers of IFN-gamma-producing CD8 cells remained below the detectable level in all individuals of the i.m. group (0 of 4 responders at days 14 and 28). We also analyzed the production of both IL-2 and IFN-gamma Type I cytokines by influenza-specific CD4 and CD8 cells in four individuals using multiparametric analysis. Vaccine-induced antigen-specific T cells were producing both, IFN-gamma and IL-2, confirming a Th1 profile. Again, influenza-specific CD8 responses remained undetectable in the i.m. individuals. This is the first demonstration of such differences in the quality of the cellular immune responses by different route of vaccination in humans and remains a crucial step in the development of vaccine strategies.

4. Summary

The skin is one of the largest immune organs in the first line of contact with pathogens and is rich in potent antigen-presenting cells (APCs), such as Langerhans cells in the epidermis and dendritic cells (DCs) in the dermis. The abundance and localization of skin APCs and their potent capacity to induce immune responses make the skin an attractive tissue for APC targeting, e.g. by transcutaneous (t.c.) vaccine delivery. In fact, vaccines have been very successful in controlling infectious diseases, but several obstacles remain in their development against pandemic chronic diseases, such as HIV, hepatitis C, or cancer, where cellular immune responses play a crucial role in disease control.

The aim of this work was to study novel approaches for APC targeting and vaccination. The first series of investigations focused on proof-of-concept studies for vaccination against basal cell carcinomas (BCCs), the most common type of skin cancer. Genetically predisposed individuals as well as organ transplant recipients under long-term anti-rejection drug therapy are especially prone to BCC development. Non-invasive therapy would substantially improve life quality and disease management for those patients. Because BCCs are characterized by a striking homogeneity and low genetic instability, these tumors present an interesting experimental model for the investigation of tumor vaccination strategies. Haired ptch1+/- mice develop endogenous BCC-like tumors after exposure to ultraviolet or ionizing radiation. In our studies we demonstrated that long-term anti-rejection drug administration significantly increased the BCC burden in ptch1+/- mice, which suggests that ptch1+/- mice represent a suitable model to study BCC formation in high risk individuals. Mutational activation of Hedgehog signalling with overexpression of Hedgehog target genes such as Hedgehog-Interacting Protein (Hip) is the pivotal step in BCC development. We defined Hip1 as a tumor-associated protein, and designed recombinant Hip1 peptides for immunization to prevent the development of BCCs in ptch1+/- mice. Immunization with either of two large recombinant Hip polypeptides was well tolerated, induced B and T cell responses, and reduced the number of BCCs despite the self-nature of this protein. We concluded that immunization with proteins specifically upregulated by hedgehog signalling may hold promise as a preventive option for patients destined to develop large numbers of BCCs.

The work on tumor vaccination fostered further questions regarding possibilities to optimize conventional vaccination strategies. Facilitating vaccine compound penetration into immunization sites rich in APCs and improved APC targeting would benefit the efficacy of new vaccines in the induction of protective immune responses. Implications of epithelial DCs in CD8 cell cross-priming suggested that vaccination via the t.c. route may be especially relevant in the induction of cellular immune responses. Meanwhile, experimental evidence emerged that antigen

adsorption to nanoparticles may further help improve the efficacy of current vaccination strategies. These findings encouraged us to explore the t.c. route of vaccination using nanoparticles and particulate vaccines. We established two complementary experimental models: human skin explants to study particle penetration and translocation directly in human skin, and mouse studies for *in vivo* imaging of particle trafficking and immunogenicity. We identified the hair follicle as a major penetration pathway for nanoparticles and particulate model vaccines in both model systems and found that the penetration depth into murine hair follicles and human vellus hair follicles depends on the particle size. The key finding in our studies was that solid 40 nm polystyrene particles, after pre-treatment of the skin with cyanoacrylate skin surface stripping (CSSS, human skin explants) or tape stripping (murine skin), not only penetrated deeply into hair follicle openings, but also through the hair follicle epithelium into the perifollicular tissue. We were even able to detect topically applied Modified Vaccinia Ankara Virus expressing enhanced Green Fluorescent Protein (MVA-eGFP) in barrier-disrupted murine skin and in draining lymph nodes using *in vivo* confocal laser scanning microscopy, and to demonstrate the capacity of this t.c. immunization method to generate immune responses after topical application of DNA, protein or virus particles. In a large series of investigations, we further generated important data on the size and proportions of different hair follicle types. We found that hair follicle types in different body regions represent rather homogenous groups, suggesting that hair follicle targeting is feasible also in larger numbers of individuals. Because direct imaging of single nanoparticles was not possible with conventionally used microscopic techniques, we developed protocols for the detection of single particles in human skin by scanning transmission X-ray microscopy. In the future, this technology may also allow for the investigation of particle-tissue interactions. Based on these experimental findings we were able to build a strong rationale for nanoparticle-based t.c. vaccination strategies, and we concluded that inclusion of CSSS, a method which opens hair follicles for penetration without disrupting the epidermis, into such t.c. vaccination protocols is probably crucial.

Translation of our ideas into clinical applications was key to the success of this project. We developed a standard operating procedure for t.c. vaccination based on CSSS, and also conducted a pilot study on healthy human volunteers to assess its safety and efficacy. Cyanoacrylate Skin Surface Stripping was well tolerated in all individuals. Transcutaneous vaccination according to our newly established protocol induced both effector CD4 and CD8 T cell responses, whereas i.m. injection induced strong effector CD4 in the absence of CD8 T cells, as assessed by intracellular cytokine staining and tetramer analyses. This is the first demonstration of such differences

in quality of the cellular immune responses by different routes of vaccination in humans, and it remains a crucial step in the development of vaccination strategies.

5. Zusammenfassung

Die Haut gehört zu den größten Immunorganen des Menschen und steht in direktem Kontakt mit der Umwelt und möglichen Pathogenen. Sie ist reich an Antigen-präsentierenden Zellen (APZ) wie den Langerhanszellen in der Epidermis und den dermalen dendritischen Zellen. Die Dichte und die Lokalisation dieser APZ sowie ihre Fähigkeit, effektiv an der Induktion von Immunantworten mitzuwirken, machen die Haut zu einem attraktiven Organ für das gezielte Heranbringen von Wirkstoffen, z.B. von Impftstoffen, an das Immunsystem. Tatsächlich hat die Entwicklung von Impfstoffen in wesentlichem Maße zur Bekämpfung infektiöser Erkrankungen beigetragen. Es bestehen jedoch weiterhin Herausforderungen bei der Entwicklung von Therapien gegen weltweit häufige chronische Erkrankungen wie HIV, Hepatitis C oder Tumorerkrankungen, bei deren Kontrolle zelluläre Immunantworten eine wichtige Rolle spielen. Das Ziel dieser Arbeit war die Untersuchung neuartiger Strategien für das Heranbringen von Wirkstoffen an APZ der Haut.

In den ersten Versuchsreihen untersuchten wir am Beispiel von Basalzellkarzinomen (BCC) in ptch1+/-Mäusen Möglichkeiten, Tumorwachstum durch Suppression oder Stimulation des Immunsystems zu beeinflussen. BCC sind die häufigsten Tumoren der Haut und entstehen in besonders hoher Anzahl in genetisch prädisponierten Individuen und in organtransplantierten Patienten. Die Verfügbarkeit von nicht-invasiven, idealer Weise vorbeugenden Therapien würde die Lebensqualität und die Behandlung der Betroffenen wesentlich verbessern. Homogenität und geringgradige genetische Instabilitität sind charakteristisch für BCC, so dass diese Tumoren ein interessantes Modell zur Untersuchung von Strategien zur Tumorvakzinierung darstellen. Behaarte ptch1+/- Mäuse entwickeln nach Behandlung mit ultravioletter oder ionisierender Strahlung endogene BCC-ähnliche Tumoren. Unsere Untersuchungen zeigten, dass immunsuppressive Dosen von Medikamenten, die üblicherweise zur Vermeidung von Transplantatabstoßung eingesetzt werden, die Tumorlast in diesen Mäusen signifikante erhöhte. Dies weist darauf hin, dass ptch1+/- Mäuse ein geeignetes Modell zur Untersuchung der Entstehung von BCC und des Krankheitsverlaufes in Hochrisikopatienten sind. Mutationsbedingte Aktivierung des Hedgehog-Signaltransduktionsweges mit einer Überexpression von Zielgenen wie dem Hedgehog-Interagierendem Protein (Hip) ist der entscheidende pathogenetische Faktor für die Entstehung von BCC. Wir konnten nachweisen, dass Hip ein Tumor-assoziiertes Antigen ist und konstruierten zwei rekombinante Hip Polypeptide, um durch Immunisierung die Entstehung von BCC in ptch1+/- Mäusen zu inhibieren. Trotz seiner Eigenschaft als Selbstantigen wurden die Immunisierungen mit beiden Polypeptiden gut vertragen, induzierten B-Zell- und T-Zellantworten und führten zur einer signifikanten Reduktion der Anzahl von BCC. Immunisierung mit Proteinen,

die durch Hedgehog-Signalgebung hochreguliert werden, könnte demnach eine viel versprechende Option für die Behandlung von Hochrisikopatienten sein.

Diese Arbeit zur Tumorvakzinierung warf weitergehende Fragen bezüglich der Möglichkeiten auf, bestehende Impfstrategien zur optimieren. Dies könnte z. B. durch eine erleichterte Penetration der Impfstoffe an Orte, welche reich an APZ sind, geschehen. Berichte über die Beteiligung von epithelialen dendritischen Zellen an der Kreuzpräsentation von Antigenen und der Induktion von CD8 Zellen wiesen darauf hin, dass insbesondere transkutane (t.c.) Impfverfahren besonders relevant für die Induktion von zellulären Immunantworten sein könnten. Zudem zeigten Daten verschiedener Gruppen, dass die Adsorption von Antigen an Nanopartikel die Effektivität von Impfverfahren zusätzlich verbessern könnte. Ausgehend von diesen Ergebnissen untersuchten wir im Folgenden Möglichkeiten für das Heranbringen Partikel-basierter Impfstoffe an APZ der Haut. Wir etablierten zwei komplementäre experimentelle Modelle; exzidierte menschliche Haut zur Untersuchung der Penetration und Translokation von Nanopartikeln direkt in menschlicher Haut, und ein Mausmodell zur Darstellung und Verfolgung von Partikeln *in vivo* und für immunologische Untersuchungen. In beiden Modellen identifizierten wir den Haarfollikel als hauptsächlichen Penetrationsweg für topisch applizierte Partikel und fanden heraus, dass die Penetrationstiefe von Partikeln in Haarfollikel der Maus und in Vellushaarfollikel des Menschen abhängig von der Partikelgröße ist. Eine zentrale Beobachtung war, dass solide 40 nm Polystyrenpartikel nach Vorbehandlung der Haut mittels Cyanacrylatabriss (CSSS, exzidierte menschliche Haut) oder Tesafilmabriss (Mausmodell) nicht nur tief in Haarfollikelöffnungen, sondern auch durch das Haarfollikelepithel hindurch in das perifollikuläre Gewebe penetrierten. Mittels konfokaler Laserscanmikroskopie konnten wir auch topisch applizierte modifizierte Vakzinia Adenoviruspartikel identifzieren, die grün fluoreszierendes Protein exprimierten (MVA-eGFP) und diese *in vivo* sowohl in barrieregestörterter Maushaut und in drainierenden Lymphknoten nachweisen. Wir konnten mir dieser Art der t.c. Immunisierung Immunantworten gegen topisch applizierte DNA, Protein und Viruspartikel induzieren. In einer großen Serie von mikroskopischen Untersuchungen stellten wir Messdaten über die Größe und Proportionen von menschlichen Terminal- und Vellushaarfollikeln der Kopfhaut exemplarisch zusammen und zeigten, dass Haarfollikeltypen innerhalb bestimmter Körperregionen weitgehend homogene Gruppen bilden. Das von uns entwickelte Konzept des gezielten Einbringens von Wirkstoffen in bestimmte Haarfollikeltypen ist demnach auch auf größere Kollektiven von Individuen übertragbar. Da eine direkte Darstellung von einzelnen Nanopartikeln mit konventionellen Mikroskopietechniken nicht möglich war, entwickelten wir Protokolle für den Nachweis einzelner Partikel in menschlicher Haut mittels Röntgenmikroskopie. Diese Technologie könnte in Zukunft auch die Untersuchung von Partikel-

Gewebe Interaktionen ermöglichen. Anhand dieser Ergebnisse entwickelten wir das Grundkonzept der transkutanen Vakzinierung mittels Nanopartikeln als Impfstoffträger. Wir schlussfolgerten zudem, dass der Durchführung von CSSS, eine Methode, die Haarfollikel für die Penetration öffnet ohne die dazwischen liegende Epidermis zu zerstören, bei der t.c. Vakzinierung mit partikulären Impfstoffen wahrscheinlich eine zentrale Rolle zukommt.

Die Übersetzung unserer Ideen in klinische Anwendungen war ein wesentliches Ziel des Projektes. Ausgehend von den expreimentellen Protokollen entwickelten wir eine Standardprozedur zur t.c. Vakzinierung mittels CSSS, und führten eine Pilotstudie an gesunden Probanden durch, in der wir die Sicherheit und Effektivität dieses neu entwickelten Impfverfahrens mit Hilfe eines kommerziell erhältlichen Grippeimpfstoffs überprüften. CSSS wurde von allen Probanden gut vertragen. Die transkutane Impfung nach dem von uns entwickelten Protokoll induzierte Effektor CD4 und CD8 Zellantworten, während intramuskuläre Injektion deutlich Effektor CD4 T-Zellen, aber keine nachweisbaren CD8 T Zellen induzierte. Diese Untersuchungen erfolgten mittels intrazellulären Zytokinfärbungen, Durchflusszytometrie und Tetrameranalyse. Diese Daten weisen erstmals darauf hin, dass es in Abhängigkeit vom Weg der Impfstoffapplikation zu Unterschieden in der Qualität von zellulären Immunantworten kommen kann.

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ERKLÄRUNG

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

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